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EDITORIAL

History, ethics, advantages and limitations of experimental models for hepatic ablation

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Abstract

Numerous techniques developed in medicine require careful evaluation to determine their indications, limitations and potential side effects prior to their clinical use. At present this generally involves the use of animal models which is undesirable from an ethical standpoint, requires complex and time-consuming authorization, and is very expensive. This process is exemplified in the development of hepatic ablation techniques, starting experiments on explanted livers and progressing to safety and efficacy studies in living animals prior to clinical studies. The two main approaches used are *ex vivo* isolated non-perfused liver models and *in vivo* animal models. *Ex vivo* non perfused models are less expensive, easier to obtain but not suitable to study the heat sink effect or experiments requiring several hours. *In vivo* animal models closely resemble clinical subjects but often are expensive and have small sample sizes due to ethical guidelines. Isolated perfused *ex vivo* liver models have been used to study drug toxicity, liver failure, organ transplantation and hepatic ablation and combine advantages of both previous models.

Key words: Liver; Ablation; Experiment; *Ex vivo*; *In vivo*; Perfusion

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INTRODUCTION

Progress in science and medicine depends on new ideas being developed, tested and introduced into mainstream practice. Animal experimentation is used not only in medicine and surgery but also in science, the pharmaceutical industry, the military and educational establishments. In medicine it is essential to establish beyond doubt the safety of a drug, instrument or procedure before it even begins phase 1 of clinical trials. To establish the safety of this heterogeneous range of treatments the medical profession has frequently had no option but to the use of live animals. Although this practice is highly undesirable and many people are morally opposed to these experiments, unless and until viable alternatives are available legislation prevents the introduction of these new drugs and procedures without this prior testing. Indeed virtually all drugs and surgical treatments that are in use today have been tested on animals.

Although the best treatment for liver tumour remains surgical resection[1] the majority of patients are still affected by unresectable lesions. To these patients hepatic ablation provides an opportunity to increase survival[2]. Experimental research over the last twenty years has seen the development of a number of ablative modalities and

several of these have been applied clinically[3,4]. Ablation experiments are now focused to increase the diameter of the ablation zone to achieve better tumor margins[5-12], or to conduct safe ablations on lesions positioned close to major vessels[13-16]. Numerous experimental models are available, each with different advantages, disadvantages and ethical implications. It is therefore imperative that the researcher that wants to explore the field of experimental hepatic ablation is aware of their characteristics.

HISTORICAL BACKGROUND OF ANIMAL EXPERIMENTATION

The Greeks almost 2500 years ago described the earliest recorded animal experiments. Aristotle (384-322 BC), and Erasistratus (304-258 BC)[17,18] both performed studies on live animals and in 2nd century Rome Galen who is known as the father of vivisection dissected live goats and pigs[19]. These studies continued into the Roman era and were then passed on to medical schools in Arabia. The practice died out after this and was absent completely in the Dark Ages only being revived in Italy in the 16th century. Subsequently live animals have been used throughout history to study a wide range of problems and to assess new treatments particularly drugs and vaccines. In the 17th century, many pivotal discoveries came to light because of these studies including the understanding of lung function and the circulation of the blood. In the 1840's general anesthesia emerged (initially ether and chloroform) and it became possible to study unconscious animals. In 1881, 250 experiments were carried out in Britain, the first year that records were kept of the procedures carried out on animals. The following year (1822) the first animal protection law was passed in Britain and in 1876 the Cruelty to Animals Act came into being and was the first law specifically designed to regulate animal testing.

Many advances in our understanding did result from these animal studies including those in basic science and also medicine. These included Antoine Lavoisier demonstrating that respiration was a form of combustion using guinea pigs in calorimeters[20], Stephen Hales measuring blood pressure in the horses, and in the 1880's Louis Pasteur demonstrating the concept of "germs" by giving anthrax to sheep[21]. Also in the 1880's and 1890's Emil von Behring was able to isolate the diphtheria toxin and not only demonstrate its effects in guinea pigs but also, by 1898 produce immunity by the injection of a mixture of toxin and antitoxin, for which he was awarded the Nobel Prize in Physiology and Medicine in 1901. In 1921 Banting ligated the pancreatic duct of dogs and demonstrated that pancreatic isolates could be used to keep these diabetic animals alive, and by 1922 working with John Macloed he isolated insulin from bovine sources and famously treated Leonard Thompson, a 14-year-old diabetic boy with diabetes[22].

OBJECTIONS TO LIVE ANIMAL EXPERIMENTS

Despite these advances and those that took place subsequently many people remained understandably opposed to any form of live animal experimentation. Claude Bernard was known as the "prince of vivisectors" and the father of physiology[23] but ironically his wife Marie Francoise Martin founded the first anti-vivisection society in France in 1883 and famously wrote in 1865 that "the science of life is a superb and dazzlingly lighted hall which may be reached only by passing through a long and ghastly kitchen[23,24]. Opposition to animal experimentation continued and many eminent scientists voiced their disapproval. Charles Darwin in 1871 wrote to Ray Lancaster in reply and stated "You ask about my opinion on vivisection. I quite agree that it is justifiable for real investigations on physiology; but not for mere damnable and detestable curiosity. It is a subject which makes me sick with horror, so I will not say another word about it, else I shall not sleep tonight"[25].

Objections to live animal experiments emerged in the United States in the 1860's and Henry Bergh founded the American Society for the Prevention of Cruelty to Animals. The first movement which specifically opposed vivisection was the American Antivivisection Society, which was founded in 1883. The continued opposition to the use of animals together with the dramatic increase in the number of procedure that were being performed (in 2002 in the United Kingdom 2.7 million live animal experiments were authorized and in the United States, it is estimated that between 19 and 29 million experiments were performed although exact estimates were difficult because 90% of the animals were rats, mice and other species recently exempt from legislation) encouraged many researchers in science and medicine to seek other methods to test drugs and procedures and much of this ethos has been incorporated into legislation or guidelines. Although British law requires that any new drug be tested on at least two different species of animals (one must be a large non-rodent), United Kingdom regulations in respect of animal experimentation are very strict and the Animals Act of 1986 requires that no animal experiments be conducted if there is a realistic alternative. It also exhorts investigators to examine their research carefully to determine the smallest number of animals that may be used to answer the questions posed by the research.

There remains however a dilemma with both ethical and legislational dimensions. All scientists agree that animal experimentation is unacceptable if there is a viable alternative but legislation will not allow the introduction of new products without rigorous testing. In 1988, the American Medical Association published a white paper defending biomedical research in animals[26], and stated that "In fact, virtually every advance in medical science

in the 20th century, from antibiotics and vaccines to antidepressant drugs and organ transplants, has been achieved either directly or indirectly through the use of animals in laboratory experiments". In Europe European Centre for the Validation of Alternative Methods was established in October 1991 by a communication from the Commission to the Council and the Parliament. The aim was to try and organize research and exchange information to limit the need for animal experimentation. As defined in the communication it was felt that this could be achieved in four ways: (1) to coordinate the validation of alternative test methods at the European Union level; (2) to act as a focal point for the exchange of information on the development of alternative test methods; (3) to set up, maintain and manage a database on alternative procedures; and (4) to promote dialogue between legislators, industries, biomedical scientists, consumer organizations and animal welfare groups, with a view to the development, validation and international recognition of alternative test methods.

Although there has been considerable progress in these areas, notably with the use of fresh and cryo-preserved cells (particularly hepatocytes) problems remain when it is necessary to investigate a treatment prior to its use inpatients. The problem is particularly acute in the field of biotechnology in which many of the devices that are proposed and developed are potentially harmful and effects in living tissue are essentially unknown. Today it is certainly possible to computer model some of the effects (widely used in the pharmaceutical industry to predict the usefulness and safety of new drugs) but many results remain unpredictable. Fresh tissue can be used and will provide answers if the procedure takes a short time and its effects and results do not rely on changes (particularly at the cellular level) that require interaction with living cells or evolve over a significant time period (several hours or longer). Unfortunately many devices do produce changes due to interaction with normally vascularized tissue and frequently take prolonged periods to produce effects. With these the only way to date that the results could be examined in detail was in living animals. In addition although prototypes could be studied in small animals instruments that have been developed for human use because of their size can often only be studied in large animals.

LIVER ABLATION EXPERIMENTAL MODELS

The number of patients with liver lesions, which are potentially suitable for treatment, continues to rise with advances in critical care, anesthesia, surgery and oncology. At present, the gold standard remains surgical resection that produces long-term survivors and cure in a significant number of patients. The advent of laparoscopic resection has further widened the indications but by far the most attractive concept remains ablation of the liver (for primary or secondary tumors) containing the tumour. The ablation techniques currently in place for the treatment of liver tumors include radiofrequency ablation (RFA), microwave ablation (MWA), cryotherapy, percutaneous ethanol injection, high intensity focused ultrasound, interstitial laser photocoagulation, electrolysis and bimodal electric tissue ablation[4]. The additional availability of methods for real-time monitoring to ensure that a sufficient volume is ablated (to engulf the tumor and reduce the likelihood of local recurrence) has seen an upsurge in the number of procedures performed and the consequent emergence of survival data that are very encouraging. In addition there is evidence that ablative techniques produce immunologically active components that may confer some extra survival advantage but also systemic effects with potentially harmful consequences.

The development of any new hepatic ablative therapy is a long process that requires a detailed understanding of the basic physical and chemical properties of the ablative modality and their application in clinical settings. It includes designing equipment suitable for clinical application, identifying methods of assessing tissue response to ablation, optimizing treatment monitoring, exploring local and systemic effect of the ablation, and finally, demonstrating the safety and efficacy in humans. Understanding of the tissue temperature changes and radiological evolution of lesion generated by each ablative modality is crucial before it can be used in a clinical setting. Further problems occur with some of the techniques that rely on temperature changes for their effectiveness. Particularly with RFA and MWA the effect can be significantly attenuated in the vicinity of large blood vessels due to the "heat sink effect" where the large flow in the vessels conducts heat away sufficiently rapidly to interfere with the treatment and potentially leave viable tumor cells around these vessels. Flows within the hepatic vessels have been shown to reduce the ablative energy via heat sink effects resulting in a greater energy requirement when ablating in close proximity to larger vessels with an associated higher risk of vascular and bile duct complications[15,27]. For the same reasons, thermal ablation techniques particularly are influenced by the effects of the separate occlusion of the hepatic artery, portal vein or both (Pringle maneuver). This delicate balance between safety and efficacy of ablation has been the focus of many experimental studies[15,28,29].

One further attraction of ablative techniques is the fact that they can often be performed percutaneously or laparoscopically and considerable effort has been expended in the development of suitable probes. These probes can be complex and are generally modeled initially on computers to predict the likely outcome after the delivery of a set amount of energy. Unfortunately the alterations in tissue resistance and electrical and thermal conductivity consequent upon the use of the probes, together with the complex designs (and frequent use of multiple probes or probes that assume unpredictable

shapes after deployment) means that to date it has been essential to test the devices in animal models. These will also be necessary to "calibrate" all new devices and produce standard dose-response curves, so that in addition to real-time monitoring a prediction can be made about the amount of energy that needs to be delivered to ablate a predetermined volume.

The increasing popularity and deployment of these techniques means that to accomplish these aims would require the use of a large number of experimental animals, and because of this it is valuable to consider whether there is an alternative approach that can produce reliable data of sufficient quality to facilitate the incorporation of these methods into clinical practice without the need for subsequent large animal studies. *Ex vivo* perfused liver models have been developed recently for the study of the hepatic toxicity of new pharmacological agents[30-32], and in an attempt to improve the outcome from organ transplantation. Organ transplantation models, which use pulsatile perfusion after harvesting, would seem to offer an excellent alternative that could closely mimic the *in vivo* situation and allow data to be collected for up to 24 h. In a sufficiently well developed perfusion model in which parameters can be accurately controlled it seems likely that data generated would be genuinely useful and achieve the aim of avoiding the use of live animal models.

EX VIVO NON-PERFUSED MODELS

The most commonly used model for the study of hepatic ablation is liver explanted from animals. Bovine and porcine livers are most frequently used because of their similarity to human livers in terms of size and density, and because they are farm animals that are readily available and inexpensive. In addition long-term use has resulted in a familiarity with the animals' anatomy that is essential in studies on the liver where the lobulation and blood supply are an important determinants of outcome. Although the organ is not perfused the position of the ablation in respect of vessels and bile ducts remains important. Their size also allows a number of ablations to be carried out on each individual model, which minimizes the number of animals required.

These models can be used to examine the relationship between ablative energy and lesion size, to assess the effect of different antenna configurations on lesion size and to study the dielectric properties of liver tissues[33-36]. Crocetti *et al*[37] used *ex vivo* calf livers to assess the feasibility and validity of fusion imaging that combines ultrasound and computed tomography in the monitoring of RFA. Unfortunately the results of this study were not directly transferable to the clinical setting because of the absence of respiratory excursion and subject motions of this model. The absence of perfusion within *ex vivo* models also limits their ability to assess the effect of blood flow on the size of the lesion created by ablation with a set amount of energy. Furthermore detailed studies of the cellular changes in the ablated area cannot be conducted due to the ischemia produced by explantation and the rapid cessation of metabolic and physiochemical processes.

IN VIVO MODELS

In view of the limitation of *ex vivo* models particularly with respect to the absence of blood flow, *in vivo* animal models have been widely used for the study of hepatic ablation. Data generated from these studies are very reliable and the close similarity to results in humans allows the experience to be directly transferred to clinical practice. Small laboratory animal models such as murine models are easier to handle but have significant differences in organ size, function and anatomy compared to the human liver. The relatively small organ size also limits the number of ablations that can be carried out per liver. In contrast, *in vivo* porcine liver closely resembles human livers in size, vascularity and metabolic function and although the shape is different the organ is also lobulated in a similar way to the human liver. This allows comparisons to be drawn with respect of the positioning of probes, real dosage levels and consequences of treatment. The result is that the porcine model is by far the most commonly used at present for the final evaluation of all ablative techniques.

The two major approaches to date for the study of hepatic ablation in animals, *ex vivo* non-perfused liver models and *in vivo* animal studies, and their respective advantages and limitations are compared in Table 1. A number of studies have been carried out simultaneously on *in vivo* and *ex vivo* non-perfused models to try and assess the magnitude of the changes produced and also to evaluate the specific differences. The volume of the ablated regions is always significantly smaller in the *in vivo* models when similar energy of ablation is applied[5,38,39]. These findings can probably be attributed to the presence of vascular flow, which removes some of the energy (heat) during the treatment in the *in vivo* models and hence these models are used to examine the influence of vascular occlusion on the size of ablated area. *In vivo* models also allow histological study of different zones, and the evolution of these zones in lesions generated by thermal ablations.

Results generated from *in vivo* models are directly transferable to clinical practice and are at present the gold standard for pre-clinical experimental studies of hepatic ablation. However the use of living animals requires specific laboratory facilities and research teams with expertise in anesthetizing and handling different species. The size and temperament of larger animal models such as porcine and bovine models poses practical problems when animals need to be anesthetized, moved or examined post-procedure (particularly if blood sampling is required).

Table 1 Advantages and limitations of *ex vivo* and *in vivo* models for experimental study of hepatic ablation

Models	Applications	Advantages	Limitations
Ex vivo (non perfused) models	Compare the efficacy of different antenna configurations Trial of different energy setting	Allow histological examination of whole lesion to study zones of ablation Cheap Larger study sample size Easy to manipulate during experiment Does not require ethical approval/animal license	Non-physiological Homogenous parenchyma Absence of respiratory excursion and subject motions Lack of cooling effect secondary to tissue perfusion Unable to study heat sink effect
In vivo models	Study of lesion evolution over time Histological examination of lesion Study of heat sink effect and the effect of bile duct cooling Study of systemic responses to ablation Study of the effect of large volume ablation (in larger animals)	Small animals Easier to handle Cheaper Ability to have larger sample size	Small animals Small volume of liver Limit number of ablation on each liver Not suitable for the study of large volume ablation
		Large animals Closer resemblance to human liver in terms of size and physiology More ablations can be carried out in each liver	Large animals Size and temperament poses challenges during anesthesia Difficult vascular access in porcine models Also limited by strict ethic regulation Small study sample size
			Common limitations Expensive Expertise in animal handling and anesthesia is required Duration of study is limited to the lifespan of the model Absence of interacting organ systems which may have a role in generating systemic response Unable to assess the impact of ablation on end organs
Isolated perfused *ex vivo* liver models	Study of lesion evolution over time Study of heat sink effect and the effect of bile duct cooling Study of early inflammatory response	Cheaper than *in vivo* experiments Sophisticated and accurate manipulation of hepatic inflow (e.g., Pringle manoeuvre) Does not require ethical approval Greater control of perfusion characteristics (e.g., portal vein and hepatic arterial flows and pressures)	Perfusion circuit itself may activate some degree of systemic response

EX VIVO PERFUSED MODELS

Ex vivo non-perfused and *in vivo* models have significant advantages with respect to specific scientific questions but an alternative model, the isolated perfused *ex vivo* model, theoretically would have most of the advantages of both. A normothermic liver perfusion system using autologous blood maintains physiological and metabolic functions and the hemodynamic changes resemble those of *in vivo* models with good preservation of liver architecture[40-42]. The organ would be perfused over a number of hours, and within this period valuable data could be produced within a relatively inexpensive model with no ethical problems and no requirement for licensing (Table 1)[43-45]. This should permit the histological study of lesions generated by hepatic thermal ablation, real-time monitoring of the evolution of the lesions and experiments designed to study the "heat sink" effect with different modalities. In addition, with a sufficiently sophisticated and stable model it should also be possible (by venous sampling) to study the early inflammatory responses following hepatic ablation[44,46].

Liver perfusion studies date back as far as the 19th century when they were first performed for research on physiological function of the liver[47]. A number of studies have been conducted subsequently to establish valid models of liver perfusion using asanguineous perfusates or autologous blood[41,48-50]. These models have been used for physiological research, treatment of liver failure, studies of metabolism and toxicity of new pharmacological compounds and in transplant units for the recovery and preservation of organs, which are to be implanted[51-53]. Hildebrand *et al*[54] have advocated the use of a perfused *ex vivo* liver model for the training in laparoscopic RFA.

For the *ex vivo* perfused model the main limitation is the limited lifespan and the determination of intervals during this lifespan when valid data can be extracted. Nevertheless perfusion models, and particularly those using autologous blood and pulsatile perfusions, have become very sophisticated and physiological parameters can be very accurately maintained for many hours. The interaction of blood with non-biological surfaces of the perfusion circuit does activate a number of biological pathways producing some degree of systemic response and interpretation of responses to ablation will have to take these into account. However the response will be the same for each experiment and should not interfere with comparisons of different dose responses or different modalities[55]. The absence of other interacting

organs may also affect the extent of systemic responses following hepatic ablation but this model still potentially represents an exciting new method of studying these new devices while avoiding the need to use live animals. The true potential and weakness of an isolated perfused liver model for the study of hepatic ablation remain to be seen (and will be determined by future research) but recent advances have certainly developed the model to the stage that merits this research[56].

FUTURE DIRECTIONS AND ADVANCES

To overcome the limitations of disconnecting the liver from the remaining organs, Chung *et al*[56] recently created the first multiorgan *ex vivo* perfused model in which the liver was serially connected with the kidney. The viability of both organs and the inflammatory reaction elicited have been investigated in two separate studies[56,57]. The addition of the kidney to the circuit improved the biochemical milieu of the circuit and consented a more physiological environment for those experiments requiring strict conditions[57]. At the same time, the combination of two organs in the circuit did not increase the cytokine production compared to the classic "liver-alone" circuit[56] and therefore could be used to test the inflammatory reaction produced by ablative techniques[44] without significant interferences by the newly added organ.

CONCLUSION

The key advantages of isolated perfused liver models are the avoidance of live animal experiments, the ability to control accurately physiological parameters and to analyze morphological changes following hepatic ablation. In addition detailed study of the "heat sink" effect and changes consequent upon bile duct perfusion can be assessed. Other organs and tissues from the same animals from which the livers are procured can also be used in other research leading to an overall reduction in the number of animals required. Validation of the role of isolated perfused liver models for experimental studies of hepatic ablation is essential to establish their position in this field of research.

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FIELD OF VISION

Bowel preparation prior to colonoscopy: A continual search for excellence

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Abstract

Bowel preparation prior to colonoscopy is essential to maximize the benefits of colonoscopy. Numerous bowel preparations have been studied, ranging from 4 L polyethylene glycol (PEG) to split-dose regimens to 2 L PEG with an adjunct laxative (senna, bisacodyl, ascorbic acid). Due to the large volume of PEG required for adequate bowel preparation, many studies have focused on reducing this large volume to only 2 L PEG with the addition of an adjunct. Recently, a randomized controlled trial by Tajika *et al* showed that the addition of mosapride to only 1.5 L PEG was non-inferior to mosapride and 2 L PEG for bowel cleansing but did provide improvements in patient tolerance. This study offers yet another potential bowel preparation for patients undergoing colonoscopy and may trigger further studies with 1.5 L PEG with an adjunct. In this letter, we discuss the current state of bowel preparation prior to colonoscopy and offer information to guide clinicians on choosing the appropriate bowel preparation for their patients.

Key words: Colonoscopy; Polyethylene glycol; Mosapride; Bowel preparation

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INVITED COMMENTARY ON HOT ARTICLES

As clinical gastroenterologists, we read the recent article by Tajika *et al*[1] describing a new lower volume bowel preparation prior to colonoscopy with considerable interest. This article examined the use of mosapride and only 1.5 L of polyethylene glycol (PEG) for bowel preparation prior to colonoscopy. Given the continued need and search for more options for bowel preparation, we recommend reading and possibly implementing the information in this article.

Colorectal cancer is a common and devastating disease which affects many patients worldwide. Being in top three of cancers and cancer-related deaths in the United States, colorectal cancer has become a focus of gastroenterology, with much of our practice being significantly impacted by colorectal cancer screening and surveillance[2].

Over the past 10 years, colonoscopy has become the screening test of choice for colorectal cancer. Unlike other non-invasive colorectal cancer screening tools, such as fecal occult blood testing and computed tomographic (CT) colonography, colonoscopy is the only modality which encompasses both diagnostic and therapeutic potential. However, colonoscopy is dependent on adequacy of bowel preparation for complete visualization of the

Table 1 Common bowel preparations utilized prior to colonoscopy

First tier			
Split-dose PEG			
2 L the night prior and 2 L morning of colonoscopy			
Second tier			
Full-dose PEG 4 L the night prior	2 L PEG + Adjunct Ascorbic acid Bisacodyl Senna Magnesium Mosapride	Sodium phosphate (Tablet)	Sulfate prep (Na, K, Mg)
Third tier			
Miralax + Gatorade	Magnesium citrate	Enemas	1.5 L + Adjunct Mosapride

PEG: Polyethylene glycol.

colonic mucosa. If visualization is compromised, missed lesions, prolonged procedure time, and increased patient discomfort become increasingly possible, significantly impacting patients and healthcare costs[3,4]. Therefore, bowel preparation prior to colonoscopy is extremely important for an adequate colonoscopic examination.

The optimal bowel preparation prior to colonoscopy must adequately clear the fecal material to visualize the underlying mucosa but also must be tolerable to the patient. Even if the bowel preparation is exceptional at clearing the colon of feces, if it is not palatable, the patients will likely not complete the full preparation. Over the past few years, many studies have been performed with varying uses of PEG, such as split-dose PEG and PEG with adjuncts, in an effort to improve patient tolerability without decreasing the efficacy of the bowel preparation. A meta-analysis in *Gastrointestinal Endoscopy* in 2011 demonstrated that split-dose PEG (consisting of 2 L PEG the night prior and 2 L PEG the morning of the procedure), improved both the cleansing of the colon and patient tolerability as compared to full-dose PEG (4 L the night prior to colonoscopy)[5]. Subsequently, the split-dose PEG has become a very common bowel preparation and is the preferred bowel preparation of the American College of Gastroenterology. However, patients are still required to consume a large volume of PEG. Therefore, many studies have elected to reduce the volume of PEG to 2 L and add a laxative adjunct (ascorbic acid, bisacodyl, senna, magnesium, *etc*.) and monitor for bowel cleansing and patient tolerability[6-12]. However, these studies resulted in conflicting results.

In 2011, two meta-analyses were published as abstracts in the *American Journal of Gastroenterology* showing that 2 L PEG with ascorbic acid cleansed the colon as well as 4L PEG but was equally tolerated[13]; however, 2 L PEG with bisacodyl demonstrated equal bowel cleansing and improved patient tolerability[14]. Despite the results, limitations have been placed on using bisacodyl with PEG due to risk of ischemic colitis. Therefore, the search continues for an adequate low-dose PEG bowel preparation.

In the randomized controlled trial by Tajika *et al*[1] mosapride (15 mg) was utilized as an adjunct laxative to low-dose PEG (1.5 L *vs* 2 L). The study revealed that mosapride with 1.5 L PEG resulted in equal bowel cleansing and increased tolerability as compared to mosapride with 2 L PEG. Given these results, mosapride with 1.5 L PEG appears promising as an alternative bowel preparation prior to colonoscopy. However, given a few limitations within this study, the results need to be interpreted cautiously.

First, this study utilized a non-inferiority model for analysis. Most bowel preparation studies are performed using a superiority model due to limitations of the non-inferiority model. The non-inferiority model is dependent upon the margin at which non-inferiority is defined. If the margin is not accurate, the results may be significantly affected. In this study, mosapride with 1.5 L PEG was non-inferior to mosapride with 2 L PEG; however, it appears that 2 L PEG with mosapride group has many more excellent and good preparations as compared to 1.5 L PEG with mosapride group (right colon: 88 *vs* 60; left colon: 97 *vs* 82). Given this difference, especially in the right colon, it is difficult for us to believe that equality of cleansing exists for these two regimens. Therefore, this leads us to believe that the sample size estimation and the non-inferiority margin may not be as accurate as hoped. Second, although the Aronchick scale has been shown to be a valuable tool in assessing bowel preparation, it is designed for the entire colon[15]. This study used the Aronchick scale but divided it among right and left colon. Ideally, the Ottawa or Boston bowel preparation scales which utilize different segments of the colon with numerical scores may have been a better choice for bowel preparation assessment[16,17].

At this point, based upon the available literature, we believe the ideal bowel preparation prior to colonoscopy is the split-dose PEG given 2 L the night prior and 2 L the day of the colonoscopy (Table 1). However, given its large volume, it may not be suited for all patients. Furthermore, given the number of other potential options including the preparation described in this study, alternative bowel preparations are readily available and may be utilized in certain cases without adversely affecting bowel cleansing.

The choice of bowel preparation is extremely important for patients and practices. Adequate bowel preparation which is tolerable to patients will likely enhance satisfaction among patients, who would be more likely to return for another colonoscopy. It will also likely reduce the need for early repeat procedures due to poor prep quality and overall costs. Given the number of preparations available, bowel preparation prior to colonoscopy in the future may be tailored to the needs and desires of the patients. Although split-dose PEG seems to be the gold-standard at this time, other preparations may be utilized to enhance patient satisfaction without adversely affecting overall preparation quality. The choice is up to you and your patient.

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FIELD OF VISION

Internal frontier: The pathophysiology of the small intestine

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Abstract

Even though the small intestine occupies a major portion of the abdominal space and is essential for life, in most pathology textbooks any chapter on small intestinal diseases, especially in human beings, is typically shorter than those for other gastrointestinal organs. Clinical and experimental investigations of the small intestine in various clinical situations, such as nutrition management, obesity interventions, and emergency care, have elucidated several important biological problems associated with the small intestine, the last frontier of gastroenterology. In this issue, a review by Professor Basson and his team at Michigan State University sheds light on the changes in the human small intestine under various conditions based on their clinical and surgical experience. With the advent of recent innovations in enteroscopy, a form of endoscopy used to examine deep within the small intestine, the issue that they highlighted, i.e., mucosal adaptation and atrophy of the human small intestine, has emerged as a major and manageable challenge for gastroenterologists in general, including the readers of the *World Journal of Gastroenterology*.

Key words: Ileum; Jejunum; Small intestine; Atrophy; Adaptation; Enteroscopy

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INVITED COMMENTARY ON HOT ARTICLES

Juan Rosai's Surgical Pathology (9th edition) contains 45 pages on the small intestine, compared with 80 pages on the large intestine[1]. The actual length of the small intestine is much longer than that of the large intestine, and the vital importance of the small intestine is well known. Why then are fewer pages devoted to diseases of the small intestine? Obviously, the access of surgical pathologists and gastroenterologists to the small intestine is limited, compared with access to the large intestine and appendix. Actually, the old version of Morson and Dawson's textbook Gastrointestinal Pathology[2] is fairer: the same number of pages is devoted to the small intestine and to the large intestine, probably because this book is a product of the century during which autopsies made major contributions to our knowledge base. This issue of the *World Journal of Gastroenterology* has a wide-ranging review article on the pathophysiology of the small intestine by Professor Basson and his colleagues[3]. These authors are surgeons, and a tremendous amount of data based on their own clinical experiences and those of others, as well as on animal experiments, are comprehensively discussed in their review. This review article addresses conditions such as starvation and parenteral nutrition in patients with severe trauma or pancreatitis and in patients with postoperative short-gut syndrome for various reasons including bariatric surgery for obesity. Although these

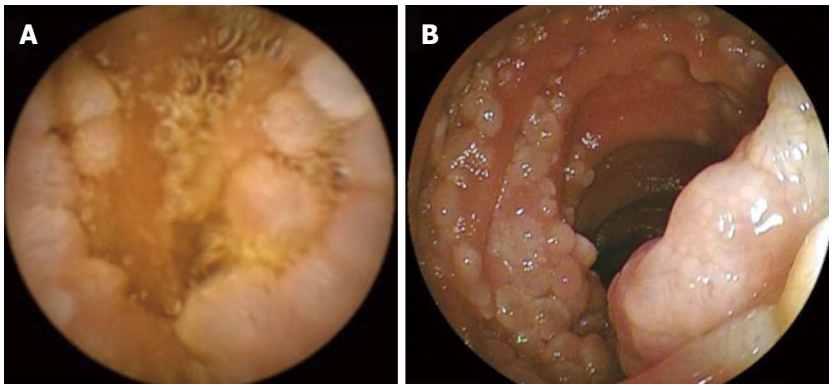

Figure 1 Endoscopy. Images obtained using capsule endoscopy (A) and double-balloon enteroscopy (B) in a 61-year-old man with follicular lymphoma who visited us because of gastrointestinal bleeding from an unknown cause and a hemoglobin concentration of 9.7 g/dL.

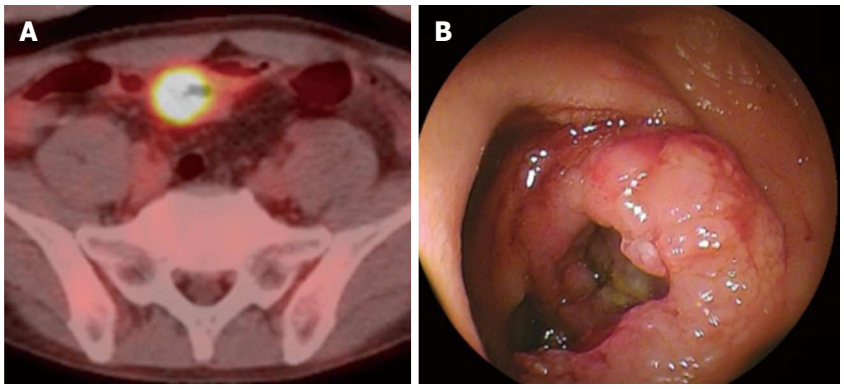

Figure 2 A mass positive by positron emission computed tomography actually was a tumor arising the mucosa of the small intestine of a 48-year-old woman who had suffered Crohn disease. A: Positron emission computed tomography/computed tomography showed an accumulation in site of wall thickening of ileum; B: Image obtained by double-balloon enteroscopy.

conditions are undoubtedly serious, recognition and explicit statements by medical professionals regarding the importance of the small intestine, and of its diseases as a "great burden of human distress", have only recently appeared in medical literature[4]. Most clinical conditions that highlight the importance of the small intestine are familiar only to experts in a particular area of medicine. These conditions are not typically a concern of non-surgical gastroenterologists, such as pathologists who are accustomed to diagnosing neoplasms of the large intestine, grading inflammatory bowel disease, and validating ischemic necrosis of the resected small intestine in cases with an acute abdomen. The small intestine is most often investigated because of its involvement in a disease originating from another organ, such as the mesothelium[5]. Changes in the mucosa of the small intestine itself are an exceptional category among the slides that pile up next to the microscope.

The situation is now changing. As avid readers of *World Journal of Gastroenterology* may have noticed[6-9], recent progress in enteroscopy has enabled areas deep within the small intestine to be reached, providing clinicians active in the wider branches of gastroenterology with the opportunity to evaluate changes in the small intestine, which have previously only been observed by surgeons. This also means that many non-surgeons must commit to the management of the small intestine, based on findings and evaluations associated with specimens of small intestine obtained using these newly developed modalities. For ordinary pathologists, for example, the concepts mainly discussed in this review, i.e., adaptation and atrophy of the small intestine, may be new and unfamiliar. These lesions were encountered only after specific "congenital or acquired disease or medical and surgical intervention" had occurred in the patients. When specimens obtained by enteroscopy become routine in the future, however, the concepts and knowledge of adaptation and atrophy of the small intestine will become an essential "must" for all practitioners including general pathologists.

One of the important conditions that the authors of this review addressed is the change in the small intestine in subjects receiving total parental nutrition. This lifesaving modality has many variations in terms of its nutritional regimen and the effects of these variations on pathophysiology, that is, on the grade of adaptation of the small intestine. These effects on the small intestinal mucosa and the consequent outcomes of individual patients have been thoroughly investigated and published in many scientific articles, but the scientific evidence in human beings remains insufficient according to the

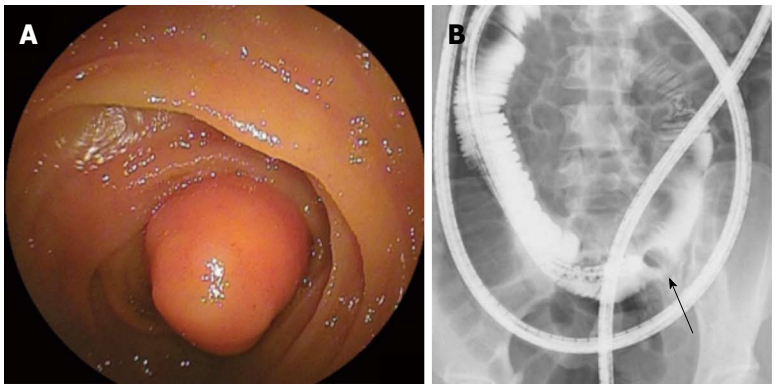

Figure 3 Identification of lipoma clarified the reason of intermittent abdominal discomfort in a 29-year-old female. Endoscopic finding (A) and selective small bowel series (B) obtained using double-balloon enteroscopy. This required surgical intervention. An arrow in the B indicates a defect by a lipoma.

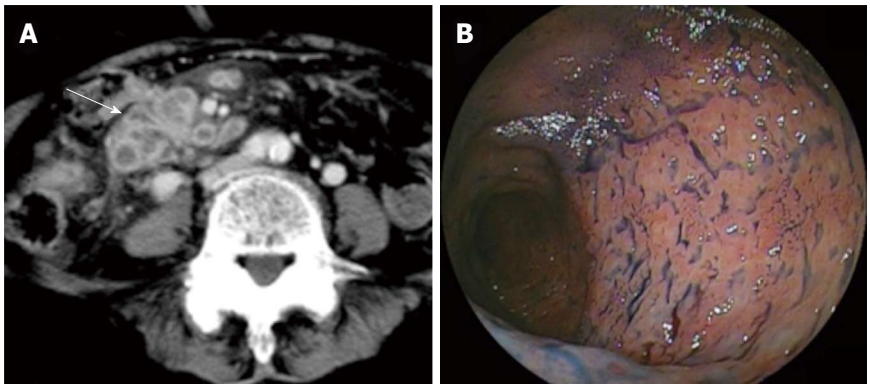

Figure 4 Intestinal tuberculosis is also revealed by double-ballon enteroscopy. A: Computed tomography showed wall thickening of the ileum with contrast enhancement (arrow); B: Double-balloon enteroscopy showed destruction of the small intestinal villi.

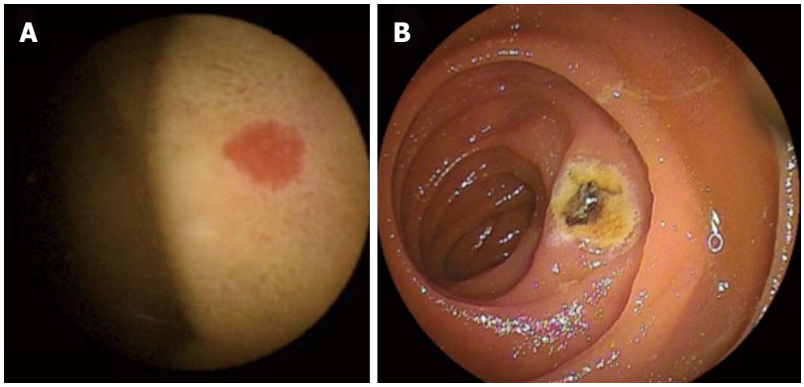

Figure 5 Angioectasia. A: Diagnosis of angioectasia was made by capsule endoscopy; B: Argon plasma coagulation successfully treated the lesion.

above-mentioned authors. The concept of mucosal adaptation[10] includes proliferation, functional augmentation, and cellular differentiation. Biochemical changes include alterations in molecules related to apoptosis, proliferation, signal transduction, and fatty acid metabolism. These meticulous frameworks in intestinal cells and tissues have so far been revealed by studies using *in vivo* and *in vitro* manipulative systems. From now on, in the era of enteroscopy, lingering questions such as "what is the reality and examples of mucosal adaptation in human clinical settings?" and "how can we validate the accumulated experimental findings in human beings and exploit them in clinical practice?" will be answered.

Small intestinal enteroscopy is now available in ordinary hospitals, thus facilitating the detection of previously unobserved pathological conditions. Capsular endoscopy, the latest wireless version of enteroscopy, has become a popular practical procedure since the publication of a seminal report on this modality a decade ago[11]. In the minds of laymen, this technique seems like a dream[12]. The use of capsular endoscopy and refined enteroscopy using a double-balloon method[13] in clinical practice have

revealed thousands of new anecdotal findings (Figure 1), and the rapid accumulation of this kind of basic knowledge of the small intestine will help to set up principles of surveillance for mucosal adaptation and atrophy of the small intestine (especially morphological changes) in various clinical conditions. For example, introduction of capsular endoscopy and a double-balloon method disclosed previously unrecognized lesions such as adenocarcinoma arising from Crohn disease in the small intestine (Figure 2)[14], submucosal lipoma (Figure 3), tuberculosis at the terminal ileum (Figure 4), and angioectasia (Figure 5). None of these lesions were accessible until the recent development of capsule endoscopy and the double balloon method. The morphology is new to pathologists and endoscopists, and these developments will critically influence the managements of the patients. The concepts that Professor Basson and colleagues have illuminated in their review will soon become an important guidepost for evaluating the histopathology of the small intestine in daily practice and for patient care by a broader range of gastroenterologists.

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FIELD OF VISION

Recurrent hepatitis C virus after transplant and the importance of plasma cells on biopsy

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Abstract

Hepatitis C virus (HCV) is the leading indication for liver transplantation in the United States. It recurs universally after transplant but the rate of fibrosis and the development of graft failure is variable. Different donor and recipient features have been demonstrated to impact fibrosis. Plasma cell hepatitis, a histologic finding, is one feature associated with poor graft and patient outcomes. The pathogenic mechanism resulting in plasma cell hepatitis is poorly understood, with evidence suggesting a role for both the HCV and the immune system.A recent publication described plasma cell hepatitis in a larger context of immune medicated graft dysfunction in transplant recipients receiving interferon based therapy. This manuscript will highlight the topic of plasma cell hepatitis and provide commentary on the lack of recognition, the data regarding pathophysiologic mechanisms and the potential management options.

Key words: Hepatitis C virus; Plasma cells; Biopsy; Sustained virological response

Kallwitz ER. Recurrent hepatitis C virus after transplant and the importance of plasma cells on biopsy. *World J Gastroenterol* 2013; 19(2): 158-160 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i2/158.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i2.158

INVITED COMMENTARY ON HOT ARTICLES

Hepatitis C virus (HCV) is the most common indication for liver transplantation in the United States. Recurrence of hepatitis C is nearly universal after transplantation and ensuing graft dysfunction occurs commonly. Ten percent of recipients progress to cirrhosis within 3 years of transplant[1] demonstrating that some patients develop aggressive recurrent HCV. Plasma cell hepatitis, diagnosed histologically, is one of a number of conditions associated with adverse outcomes and graft failure in patients with posttransplant HCV. Plasma cell hepatitis can develop in the context of interferon based therapy or in the absence of treatment with interferon. Levitsky *et al*[2] present a multicenter case-control study which included a large subset of patients found to have plasma cell hepatitis associated with interferon therapy for HCV.The manuscript should be read with interest by transplant hepatologists as it highlights important concepts regarding plasma cell hepatitis in patients with HCV after transplant. First, plasma cell hepatitis is under recognized. Second, the pathologic process resulting in plasma cell hepatitis is poorly understood. Finally, with a paucity of data, it is not possible to determine the best treatment for transplant recipients with this condition.

The case control series by Levitsky *et al*[2] reported that the incidence of any immune medicated graft dysfunction on interferon based therapy varied by center, ranging between 3.2%-16.3%. Persons found to have immune mediated graft dysfunction on HCV therapy had significantly worse survival, and more graft failure leading to higher rates of retransplantation. Plasma cell hepatitis was the most common manifestation of interferon induced immune mediated graft dysfunction. It is important to note that plasma cell hepatitis was also commonly identified prior to the use of interferon. The incidence of plasma cell hepatitis

Table 1 Generalized histologic and clinical features seen in post transplant patients with hepatitis C

Pathologic entity	Histologic features	Clinical and laboratory features
Plasma cell hepatitis	Plasma cells (often in sheets) Centrilobular necrosis	HCV PCR positive ANA or ASMA may be positive (often with low titers) Low level of immunosuppression Can be caused by interferon based therapy
De novo autoimmune hepatitis	Lymphoplasmacytic infiltrate Interface hepatitis	Positive ANA or ASMA Elevated immunoglobulins In persons on treatment HCV RNA often not detected (occurs in other settings in addition to HCV) Low level of immunosuppression Can be caused by interferon based therapy
Acute cellular rejection	Mixed inflammatory infiltrate Endotheliitis Nonsuppurative Cholangitis Centrilobular necrosis (variable)	Low level of immunosuppression Can be caused by interferon based therapy
Recurrent hepatitis C	Lymphocyte Aggregates Portal based fibrosis FCH is one variant (Cholestasis, Apoptosis, Fibrosis)	HCV PCR positive In FCH, markedly high viral load In FCH, high level of immunosuppression

HCV: Hepatitis C virus; FCH: Fibrosingcholestatic hepatitis; ANA: Antinuclear antibodies; ASMA: Anti-smooth muscle antibody; PCR: Polymerase chain reaction.

on pretreatment biopsies was much more common in persons who developed immune mediated graft dysfunction (36.5%) compared to the control group (7.7%, $P = 0.003$) on treatment[2]. Fourteen of the cases labeled as plasma cell hepatitis by the central pathologist were not recognized by the local pathologist who initially interpreted the liver biopsy[2]. The authors conclude that plasma cell hepatitis often predicts the development of immune mediated graft dysfunction occurring during interferon based treatment. In addition, the author's recommend that clinicians should not reduce immunosuppression doses and should not initiate interferon based therapy in those with immune features including plasma cell hepatitis on pretreatment biopsies.

The informative and interesting conclusions of this article deserve further comment. A main feature of the article from Levistky and colleagues is that plasma cell hepatitis is under recognized and is often mistaken for recurrent hepatitis C or other forms of rejection[2]. A histologic scoring system was developed in 2008[3]. Diagnostic features of plasma cell hepatitis include numerous plasma cells, often in sheets or clusters, accompanied by centrilobular necrosis. Despite the existence of standardized criteria, it is not surprising that plasma cell hepatitis is under recognized. With few publications describing plasma cell hepatitis, it is not topical to hepatologists and pathologists. Additionally, in post-transplant patients, other processes such as recurrent HCV, *de novo* autoimmune hepatitis and acute cellular rejection present alternative diagnoses. Table 1 highlights clinical and histologic features which might help distinguish the different diagnoses. It is important to realize significant overlap does exist between the pathologic process making a definite diagnosis impossible in some cases. The recent publication in a high profile journal will hopefully lead to better recognition of this disorder.

The pathogenesis of plasma cell hepatitis has yet to be defined. It has been described both as a manifestation of hepatitis C[4,5] and as a form of rejection[3]. Evidence exists for both possibilities. Prior series have shown that HCV plays a role. A two subject case series examined plasma cell hepatitis as a lymphoproliferative disorder[5]. Both patients in this series had serum or urine protein electrophoresis demonstrating a monoclonal protein and RNA probes for hepatitis C were positive within the plasma cell infiltrate[5]. An association between plasma cell hepatitis and mixed cryoglobulinemia has not been studied. In the current article, patient survival with plasma cell hepatitis was improved with a sustained virologic response to treatment for HCV[2]. Although graft failure and retransplantation occurred in some cases, five year survival was above 80% and similar to control subjects[2]. Additionally, there was a trend toward improved graft survival in cases of immune mediated graft dysfunction when hepatitis C was eradicated. In Kaplan-Meier analysis, the majority of graft loss occurred early after transplant with approximately 60% graft survival and no further graft loss occurring after two years in the group that achieved a sustained virological response (SVR)[2]. In contrast, the group that did not achieve an SVR continued to develop graft loss during the entire period of follow up and graft survival was less than 40% at five years[2]. It is additionally notable that SVR rates in series of patients with plasma cell hepatitis ranged between 40%-67%[2,3]. Given that poor outcomes are observed with plasma cell hepatitis in persons who never received interferon[3,6], prospective, randomized data are needed to compare outcome of interferon treatment versus no interferon treatment with respect to HCV eradication.

Other studies suggest the development of plasma cell hepatitis is an immune mediated event. Explanted livers of post transplant patients who later develop plasma cell hepatitis were more likely to have extensive plasma cell infiltrates[6] suggesting that immunologic predisposition exists even prior to transplant. However, not all persons with plasma cell infiltrates on explant will develop plasma cell hepatitis, and additional factors after transplant appear to play a role. There are data describing the development of plasma cell hepatitis in the setting of lowered immunosup-

pression[2,3]. In a series including 38 subjects with plasma cell hepatitis, 31 had either recently lowered immunosuppressant dosing or subtherapeutic drug levels[3]. In the series by Levitsky *et al*[2] significantly more patients with immune mediated graft dysfunction had a reduction in immunosuppression prior to interferon based therapy. Additionally, more subjects with immune mediated graft dysfunction had immunosuppression reduced during therapy[2]. One would expect interferon would have a role in the development of an immune mediated event and the contribution of interferon to the development of plasma cell hepatitis is not quite clear. In a retrospective series, interferon was not associated with the development of plasma cell hepatitis and its use did not impact outcome once plasma cell hepatitis developed[3]. In the series published by Levitsky *et al*[2] persons with existing plasma cell hepatitis had worsened immune mediated graft dysfunction after their immunosuppression was reduced and interferon was started. Increasing baseline immunosuppression prior to initiating interferon in patients with plasma cell hepatitis should be considered for future study, especially given data showing improved outcomes with augmenting immunosuppression alone[3].

With an immune predisposition, plasma cell hepatitis and *de novo* autoimmune hepatitis have overlapping features. They are nearly histologically indistinguishable, and some refer to them interchangeably[7]. A few subtle clues suggest that these processes have a different underlying pathophysiology. Case series have described *de novo* autoimmune hepatitis developing in conjunction with interferon based therapy with elevated autoimmune titers, undetected HCV RNA levels and pretreatment biopsies showing no plasma cells[8]. In addition, in transplant recipients for indications other than HCV, *de novo* autoimmune hepatitis has been shown to respond well to steroid therapy[9], whereas plasma cell hepatitis in HCV infected recipients typically does not[3].

Plasma cell hepatitis represents an important entity which is likely under reported as the result of poor recognition. Agreement on standardized nomenclature distinguishing plasma cell hepatitis from *de novo* autoimmune hepatitis in the post transplant setting may improve recognition. Plasma cell hepatitis best refers to plasma cell infiltration in the setting of post transplant hepatitis C. *De novo* autoimmune hepatitis best refers to plasma cell infiltration that occurs commonly with positive autoimmune titers, steroid responsiveness and, in the setting of interferon based therapy, may be best reserved when in a lymphoplasmacytic infiltrate develops without active viremia. As shown in Table 1, it must be recognized that overlap between the two conditions in both pathology and pathophysiologic mechanisms exist such that diagnostic certainty will not always occur.

Currently, the best management of plasma cell hepatitis that develops independent of HCV therapy is unclear. Limited data showed that augmentation of immunosuppression without the addition of prednisone may be of benefit[3]. Once on interferon based therapy, achieving an SVR was also shown to benefit patient survival[2]. The recommendation by Levitsky *et al*[2] that interferon should not be initiated in patients with plasma cell hepatitis may be overreaching based on the data presented. It also would suggest an alternate option with better outcomes existed. A practical approach may be augmenting baseline immunosuppression and a repeat liver biopsy. If the liver biopsy shows decreased immune features than interferon based therapy might be attempted. Ultimately, until there is better prospective data, responses to this entity will likely be reflective of single center experiences.

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TOPIC HIGHLIGHT

Mansour A Parsi, MD, *Series Editor*

Endoscopic management of difficult common bile duct stones

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Abstract

Endoscopy is widely accepted as the first treatment option in the management of bile duct stones. In this review we focus on the alternative endoscopic modalities for the management of difficult common bile duct stones. Most biliary stones can be removed with an extraction balloon, extraction basket or mechanical lithotripsy after endoscopic sphincterotomy. Endoscopic papillary balloon dilation with or without endoscopic sphincterotomy or mechanical lithotripsy has been shown to be effective for management of difficult to remove bile duct stones in selected patients. Ductal clearance can be safely achieved with peroral cholangioscopy guided laser or electrohydraulic lithotripsy in most cases where other endoscopic treatment modalities have failed. Biliary stenting may be an alternative treatment option for frail and elderly patients or those with serious co morbidities.

Key words: Choledocholithiasis; Mechanical lithotripsy; Laser lithotripsy; Electrohydra-ulic lithotripsy; Difficult to remove biliary stones; Endoscopic large balloon papillary dilation; Peroral cholangioscopy

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INTRODUCTION

Common bile duct (CBD) stones are seen in approximately 7%-12% of patients who undergo cholecystectomy for symptomatic cholelithiasis and are a common indication for referral to a biliary endoscopist[1]. They vary in size ranging from rather small (approximately 1-2 mm) to very large (> 3 cm). Endoscopic retrograde cholangiopancreatography (ERCP) with endoscopic sphincterotomy (ES) and basket or balloon extraction are well established therapeutic procedures for the management of CBD stones. It is estimated that nearly 85%-95% of all CBD stones can be managed effectively by these conventional endoscopic methods[2,3]. Failure to clear the bile duct renders the patient vulnerable to biliary obstruction, cholangitis and pancreatitis, thereby increasing the morbidity[4,5]. The occurrence of acute cholangitis is associated with significant mortality, especially in the elderly, underscoring the need for early intervention to clear the bile duct of stones and to relieve the obstruction to achieve adequate biliary drainage. Extraction of CBD stones is one of the most commonly performed procedures by therapeutic endoscopists. With novel advances in extraction techniques and instruments emerging routinely, it is vital to keep abreast of the new developments in order to improve the outcome. This review focuses on the alter-

native endoscopic management options for the treatment of difficult to remove CBD stones.

REVIEW CRITERIA

In July 2011, we searched MEDLINE from 1982 to the present using the Medical Subject Headings terms common bile duct stone, endoscopic retrograde cholangiopancreatography, difficult stone, endoscopy, and the key word "common bile duct stone". Full papers and abstracts in English language were considered. Important developments in research, reports from centers of excellence, and our own clinical experience in managing them, form the basis of this review article.

FACTORS ASSOCIATED WITH DIFFICULT TO TREAT BILE DUCT STONES

Multiple factors have been postulated to govern the success or failure of endoscopic extraction of CBD stones. In approximately 10%-15% of patients, managing biliary stones becomes formidable primarily due to difficulties in accessing the bile duct (periampullary diverticulum, sigmoid shaped CBD, post-gastrectomy Billroth type Ⅱ anatomy, Roux-en-Y-gastrojejunostomy), large number of stones (greater than 10), large size of stones (stones with a diameter > 15 mm which cannot be grasped with a basket), unusually shaped stones (barrel-shaped) or location of the stones (intra hepatic, cystic duct, proximal to strictures)[6]. In addition, endoscopic management becomes challenging in Mirizzi syndrome, in which stones in the cystic duct cause obstruction of the main bile duct[7]. Kim *et al*[8] prospectively evaluated the factors contributing to technical difficulty during endoscopic clearance of CBD stones. They reported that older age (> 65 years), previous gastrojejunostomy, larger stone size (≥ 15mm), impaction of stones, shorter length of the distal CBD arm (≤ 36mm), and more acute distal CBD angulation (≤ 135 degrees) are all contributors to technical difficulty for endoscopic removal of bile duct stones[8].

MANAGEMENT OF DIFFICULT STONES

In high risk patients, the risks and benefits of alternative techniques for removal of bile duct stones not amenable to conventional endoscopic techniques must be carefully balanced against each other and with surgery. The individual decision concerning the appropriate therapy is also influenced by the local expertise and the availability of the technical equipment.

CBD stones up to 1.5 cm in diameter can be extracted intact after endoscopic sphincterotomy. The rate of successful retrieval progressively declines with increasing size of the stone[9]. Larger stones especially those with a diameter ≥ 2cm may need fragmentation before removal to reduce the risk of stone impaction.

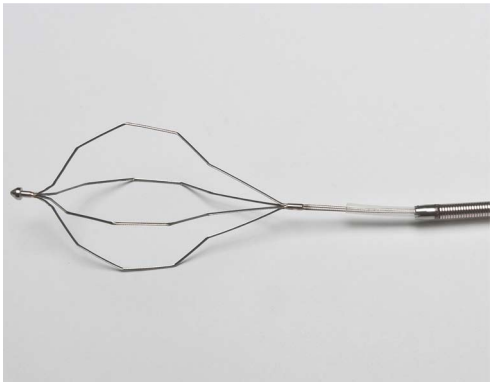

Figure 1 Example of a three-layered mechanical lithotripsy device with the basket, inner plastic sheath and an outer metal sheath. The stone is captured by the basket and crushed against the outer metal sheath.

Mechanical lithotripsy

In 1982, Riemann *et al*[10], first introduced mechanical lithotripsy (ML). ML is currently the most widely used technique for fragmentation of stones. Contemporary lithotripter baskets have a high breaking strengths and have improved the success rate of ML for extraction of large CBD stones (> 2 cm) to well over 90% without serious complications[11]. Broadly speaking, there are two types of baskets for ML. The type of basket used depends on whether lithotripsy is done on an elective ("through the scope") basis or on an emergent basis (salvage device) for basket impaction[12]. The 'through the scope' model is typically a three-layer system with the basket, inner plastic sheath, and an outer metal sheath (Figure 1). The stone is captured with the basket and the outer metal sheath is advanced to the stone which will be crushed against it. Sometimes, unexpectedly, stone and basket impaction can occur even during routine extraction of smaller stones. Under such circumstances, stone fragmentation can be done after removing the handle from the basket and the duodenoscope from the patient. An endotriptor (a spiral metal sheath) is introduced under fluoroscopic guidance, over the basket wires, and the stone is crushed after connecting the bare basket wires to the crank handle (Figure 2). The broken basket and the stone are then removed. The shaft of the endotriptor is generally shorter and thicker than that employed in standard ML[13]. Although basket impaction can occur with through the scope lithotripsy baskets; it is more commonly encountered with extraction baskets, which have thinner wires and weaker handles not suitable for fragmentation of stones.

In patients with multiple large stones, lithotripters with a sleeve system can be employed multiple times without withdrawal of the endoscope, facilitating stone fragmentation[14,15].

ML has been widely used as it is a readily available, cost effective, and simple procedure. Unfortunately the failure rate is high especially in patients with stones greater than 2.8 cm in diameter[15]. In a retrospective study the size of the stone was the only factor that significantly af-

Figure 2 Image of a mechanical lithotripter which can be used as a "salvage device" for removal of impacted baskets.

fected the success or failure of bile duct clearance. In this study of 162 patients, the cumulative probability of bile duct clearance ranged from > 90% for stones with a diameter less than 10 mm to 68% for those greater than 28 mm in diameter ($P < 0.02$)[15]. A subsequent prospective study by Garg *et al*[16] however reported that stone size alone may not be important unless considered together with the diameter of the bile duct. They concluded that the only important predictive factor that compromised the success of mechanical lithotripsy was stone impaction in the bile duct, with either an inability to pass the basket proximal to the stone or a failure of the basket to open fully around the stone to allow it to be grasped properly[16]. Although stone composition was not included in the study by Leung *et al*[12], some endoscopists believe that stones that are hard and densely calcified (visualized on a plain radiograph) resist mechanical fragmentation, resulting in an extraction failure with standard baskets. Although the stones which are molded to the shape of the bile duct may be softer, they are more difficult to crush because they may not be easily engaged by the lithotripter basket[12].

A multi-center study reported the rate of complications associated with ML to be around 3.6%[13]. Among the spectrum of complications, basket impaction or fracture of the basket wire are uniquely associated with ML. Non-surgical interventions that have been utilized in this setting include extension of sphincterotomy, awaiting spontaneous passage of the impacted basket and stone after successful stent placement, use of a second lithotripter, extracorporeal shock wave lithotripsy (ESWL), laser lithotripsy, electrohydraulic lithotripsy, and transhepatic lithotripsy and stone dislodgement[13-18]. Other complications include broken handle and perforation or injury to the bile duct[13-18].

In about 10% of the patients ML proves to be cumbersome, protracted and ineffective[6] wherein one has to resort to other methods such as electrohydraulic, or laser lithotripsy for stone fragmentation and subsequent removal.

Electrohydraulic lithotripsy

Initially used as an industrial tool for fragmenting rocks in mines, its application was extrapolated to medical use when Koch attempted fragmentation of biliary stones using this technology[19]. Electrohydraulic Lithotripsy (EHL) consists of a bipolar lithotripsy probe which discharges sparks with the aid of a charge generator in an aqueous medium. The sparks generated under water generate high-frequency hydraulic pressure waves, the energy of which is absorbed by nearby stones and results in their fragmentation[20]. The shock waves can cause inadvertent injury or perforation of the bile duct wall if the probe is not deployed close to the stone and away from the ductal wall. EHL can be performed under fluoroscopic guidance by using centering balloons or direct cholangioscopic vision[20]. The disadvantage of using only fluoroscopic guidance is related to the two dimensional imaging and the inability to confirm correct positioning of the probe. Therefore direct visualization is frequently preferred to avoid damage to the ductal wall[21]. A cholangioscope is inserted through the instrument channel of the mother scope. One or two dedicated biliary endoscopists are needed for this procedure. Continuous irrigation with water during the procedure generates a fluid medium for propagation of the shock waves and in addition offers a clear view of the stones by flushing away the debris[20].

The overall complication rate ranges from 7% to 9%[22,23], with most common complications being hemobilia, cholangitis, and less commonly, ductal perforation. Binmoeller *et al*[6], in one of the earlier large studies reported that EHL was successful in 63 of the 64 patients who had failed previous attempts of ML. Smaller published studies report stone fragmentation rates between 77%-100% for peroral EHL with minimal complications[22,24-28]. Arya *et al*[23], reported a stone fragmentation rate of 96% and final stone clearance of 90%. In a retrospective study of 94 patients who had failed stone extraction by conventional techniques, Hui *et al*[29], compared the outcomes of EHL with further endoscopy to stenting alone in a subset of elderly and infirm patients. They demonstrated that EHL and further ERCPs had a higher success rate (80%) with a low complication rate (7.7%) and recommended that elderly and frail patients should be referred to tertiary centers for EHL in order to prolong survival and decrease biliary complications[29].

The EHL equipment is compact, requires no special electricity, and is relatively inexpensive[30]. Other advantages are that the EHL procedure does not require special training or protective gear. In the United States, use of EHL for fragmentation and removal of biliary stones is quite common in centers with special interest in biliary disorders.

Laser lithotripsy

In laser lithotripsy (LL), laser light at a particular wavelength is focused on the surface of the stone to induce a wave-mediated fragmentation. The pulsed laser energy utilized in stone fragmentation is in contrast to the continuous laser energy used in tumor ablation[7]. The first successful use of pulsed laser for shock-wave lithotripsy of bile duct stones was reported in 1986[31]. Since then

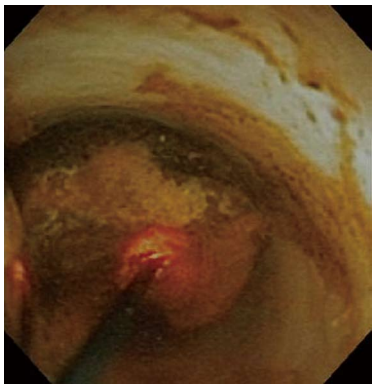

Figure 3 Laser lithotripsy of a bile duct stone under cholangioscopic guidance.

the technology has evolved and other laser types such as neodymium: yttrium-aluminum-garnet (Nd: YAG), flash lamp-pulsed dye (coumarin), the flash lamp-pulsed dye (rhodamine) with an automatic stone recognition system and the new Frequency Doubled Double Pulse Nd:YAG (FREDDY) system have been introduced[32-35]. LL is typically performed perorally under cholangioscopic or fluoroscopic guidance or by the transhepatic approach. As with EHL, LL under direct visualization using a cholangioscope is often preferred to avoid damage to the ductal wall (Figure 3).

Based on some reports, ductal clearance can be accomplished in 64% to 97% of patients by using[7,36] LL. In the majority of patients ductal clearance could be achieved in one session, although more sessions were required occasionally. LL has been demonstrated to be more effective than ESWL in terms of stone clearance rate and more rapid stone fragmentation with a shorter duration of treatment leading to a significant reduction in cost[37,38]. In some centers where the laser equipment is available, laser lithotripsy has gained popularity and has managed to replace EHL as the primary modality for fragmentation of difficult to remove stones.

A recent innovation worth mentioning is the introduction of a double-lumen basket which allows passage of a laser probe for effective laser lithotripsy after the stone is captured by the basket[39]. For a selected group of patients, this technique was shown to be feasible and effective, and the authors hope that continuous improvements in designs and construction materials would further enhance the success rate of this device.

Extracorporeal shockwave lithotripsy

In ESWL, high-pressure shock waves are generated outside the body (extracorporeal) by underwater spark gap (electrohydraulic) generated by piezoelectric crystals or electromagnetic membrane technology[30]. The shock waves are focused by elliptical transducers to the designated target through a liquid or tissue medium which prevents the energy attenuation. ESWL is performed under ultrasound or fluoroscopic guidance. Since most biliary stones are radiolucent and are not adequately visualized by fluoroscopy alone, placement of a nasobiliary tube for contrast instillation is required if ESWL is performed under fluoroscopy. In case of first generation lithotripters, the patients needed to be immersed in a water bath. Subsequent ESWL lithotripters employ water-filled compressible bags and a gel is applied to the skin surface for interface with the patient. Comparison between the lithotripters at a single center showed no significant difference in fragmentation of CBD stones[40]. General anesthesia is usually needed as the discomfort produced may not be adequately controlled by conscious sedation. The critical determining factor for success of single ESWL session is stone size and microcrystalline structure and architecture of the stone[41,42]. The presence or absence of bile duct stenosis can also influence the success of ESWL[43]. Sauerbruch *et al*[44], reported the efficacy of ESWL in achieving CBD stone fragmentation in over 90% of patients with minimal side effects.

In most institutions that already have access to ESWL for treatment of renal calculi, no other purchases of equipment needs to be made. For ESWL direct contact with the calculi is not needed, and multiple stones can be managed simultaneously[41]. ESWL can be of particular help in patients with abnormal anatomy such as those who have undergone Billroth-Ⅱ or Roux-en-Y surgeries in whom endoscopic access to the major papilla is difficult.

Although in general ESWL is tolerated well, it can be associated with adverse events such as transient biliary colic, subcutaneous ecchymosis, cardiac arrhythmia, self limited hemobilia, cholangitis, ileus and pancreatitis[7,45]. Perinephric hematoma, biliary obstruction, bowel perforation, lung injury and splenic rupture are among the rarely reported complications[42]. Multiple ESWL sessions may be required in a subset of patients to achieve ductal clearance, and endoscopic procedures between the ESWL sessions may become necessary to clear the bile duct of debris to assure drainage. The recurrence rate of CBD stones after ESWL clearance during a 1 to 2 year follow up was considerable and was around 14%[45,46]. In a randomized trial comparing fluoroscopic guided ESWL and LL, LL was preferable not only for successful stone free rate (73% *vs* 97%), but also in terms of the number of sessions needed to clear the duct (3 in ESWL *vs* 1.2 in LL) and the duration of treatment[30]. Another randomized trial comparing ultrasound guided ESWL and laser lithotripsy in the treatment of CBD stones refractory to conventional treatment clearly showed superior stone clearance rate and cost effectiveness in laser lithotripsy (52.4% *vs* 82%)[40]. However, a prospective trial comparing EHL *vs* ESWL showed no difference in success rates for clearing the CBD, duration and cost of hospitalization between the two modalities[24].

In the United States, ESWL is rarely performed for management of biliary stones and most centers prefer cholangioscopy-guided LL or EHL for this purpose.

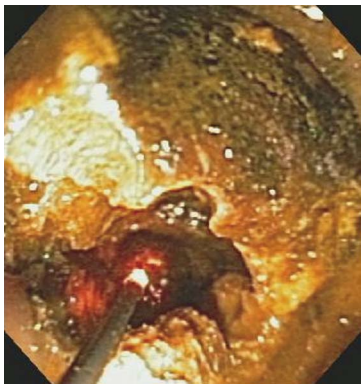

Figure 4 Direct peroral cholangioscopy guided laser lithotripsy of a bile duct stone. The red laser light makes targeting of the stone easier).

Cholangioscopy-guided lithotripsy

EHL or LL is ideally performed under direct visual control using a cholangioscope. The traditional cholangioscopy systems consist of a duodenoscope (also called "mother scope") and a dedicated cholangioscope (also called "baby scope" or "daughter scope") which is introduced into the bile duct through the accessory channel of the mother scope. The baby scope itself has an instrument channel through which the EHL or laser probe is introduced. Peroral cholangioscopy guided EHL or LL is cumbersome and can be labor intensive requiring an additional endoscopic unit and often participation of two skilled endoscopists, one to handle the duodenoscope and the other to maneuver the cholangioscope. The traditional cholangioscopes are capable of only 2-way steering, which may limit the field of view. These cholangioscopes are also extremely vulnerable to damage, requiring frequent expensive repairs. Furthermore, sharp angulations in the biliary tree may limit access into intrahepatic ducts or the cystic duct[47].

To negate these limitations, the Spyglass Direct Visualization System (Boston Scientific Corp, Natick, Mass) was designed for single operator examination of the bile ducts, 4-way steering, and dedicated irrigation channels[48,49]. In a large international multicenter study conducted at 10 centers in the United States and 5 centers in Europe, ductal clearance was successfully achieved in 71% of the patients using the Spyglass cholangioscopy system[50]. Despite its effectiveness, the spyglass system has been underutilized mainly due to its fiber optic image quality which is inferior to the video image quality offered by the new videocholangioscopes[51].

Direct peroral cholangioscopy

In direct peroral cholangioscopy (DPOC), an ultraslim upper endoscope is maneuvered across the biliary sphincter and into the bile duct for direct observation. With the introduction of high-definition ultraslim endoscopes with narrow band imaging capability, direct peroral cholangioscopy has gained popularity. This is mainly due to the many advantages of this technique. Compared to ductoscopy using a dedicated cholangioscope, direct cholangioscopy has several advantages. It offers a single operator platform, digital image quality and simultaneous irrigation and therapeutic capabilities. The most profound disadvantage of DPOC, however, is the difficulty associated with traversing the biliary sphincter to gain access to the bile duct. This is mainly due to looping of the ultraslim upper endoscope in the stomach or in the duodenum. To enhance the success rate of DPOC, specialized accessories or techniques are needed to advance the ultra slim endoscope into the proximal biliary system.

Larghi and Waxman[52] reported their experience in which the ultraslim upper endoscope was inserted with the aid of a guidewire placed during ERCP to maintain access. Additional use of manual pressure applied on the patient's abdomen has been shown to ease the passage of the ultraslim endoscope into the hilar area in some patients[53]. The main drawback encountered with passage of an ultraslim upper endoscope over a guidewire for gaining access to the bile duct is the dislodgement of the guidewire from the bile duct and also large loop formation hindering the entrance of the endoscope into the biliary system. Moon *et al*[54] demonstrated the ropeway technique using an intraductal balloon that can be anchored within an intrahepatic bile duct to advance an ultraslim upper endoscope into the biliary tree for performance of DPOC. However, withdrawal of the balloon may cause technical difficulties in maintaining access which underscores the need for other accessories to maintain the scope's position within the bile duct. The same group also reported use of an overtube balloon originally designed for double balloon enteroscopy to facilitate the introduction of an ultraslim upper endoscope into the biliary tree[55]. However the large inner diameter of the overtube (10.8 mm) compared to the outer diameter of the ultraslim upper endoscopes (5.2-6 mm), makes it difficult to manipulate both instruments and results in discomfort to the patient, looping of the endoscope in the duodenum, and difficulty in reaching the proximal bile duct[55,56].

In a recent study, we assessed utility of a novel anchoring balloon for performance of DPOC. Use of the anchoring balloon allowed consistent access to the biliary tree for performance of diagnostic and therapeutic DPOC distal to the confluence of the right and left hepatic ducts. More proximal access, however, was challenging owing to looping of the ultraslim endoscope after balloon removal.

Efforts are underway to develop the combined use of an intraductal anchoring balloon and an overtube especially designed for DPOC. Very recently, air embolism was reported following DPOC, resulting in a left sided hemiparesis[57]. Endoscopists must be conscious of the fact that air embolism could remain asymptomatic, as in regional embolism (portal venous gas) or manifest as hypoxia, shock, cardiac arrest or cerebral ischemia as noted in this case[57].

Once the hurdle of introducing an ultraslim upper endoscope into the bile duct has been overcome, a laser

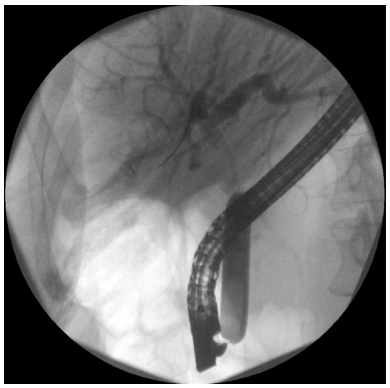

Figure 5 Large diameter papillary balloon dilatation to remove bile duct stone.

or an EHL probe can easily be passed through the working channel of the ultraslim endoscope. Stones can be directly visualized before and during lithotripsy by DPOC (Figure 4). Lithotripsy is performed using this technique until the stones are satisfactorily fragmented to allow removal through the biliary sphincter. In a small study with 18 patients who had failed conventional endoscopic therapy including ML, DPOC guided EHL or LL was successful in approximately 90% of the patients with an average of 1.6 treatment sessions per patient[58].

Specialized ultraslim upper endoscopes are being designed to facilitate access to the biliary tree for DPOC[59]. Ultraslim upper endoscopes can be passed through the nasal cavity for performance of DPOC. Direct transnasal cholangioscopy has been reported for successful extraction of CBD stones[60].

Endoscopic papillary balloon dilatation

Endoscopic papillary balloon dilation (EPBD) was introduced as an alternative to endoscopic sphincterotomy for removal of bile duct stones in 1980's[61]. In an initial report involving 10 patients with CBD stones, biliary sphincteroplasty to 15 mm allowed removal of CBD stones in 6 patients[61]. In the other 4 patients, ductal clearance required a combination of biliary sphincterotomy and mechanical lithotripsy. The use of large diameter papillary balloon dilatation (up to 20 mm in diameter) for management of difficult to remove biliary stones was reported by Ersoz *et al*[62] in 2003. They reported a high success rate for stone removal. However, their complication rate was also high. Several reports have suggested that EPBD is associated with risk of severe pancreatitis which raises safety concern of this procedure[61,63]. In our institution, we use EPBD selectively and try to avoid its use in those with high risk of post ERCP pancreatitis (Figure 5).

Since those initial reports, multiple studies have shown that EPBD alone or in combination with other techniques can be of use for management of difficult to remove biliary stones[62-68]. EPBD is especially attractive in patients who are at risk for bleeding after endoscopic sphincterotomy or in those with altered anatomy in whom a full sphincterotomy cannot be successfully achieved.

Some authors have suggested that the stone recurrence rate may also be higher with EPBD than with endoscopic sphincterotomy and mechanical lithotripsy[69]. However the results of a Japanese multicentric trial with a mean follow up of 6.7 years demonstrated that there is lesser risk of stone recurrence following EPBD when compared with sphincterotomy[70]. Further, a recent meta-analysis which included 15 randomized trials comparing EPBD and endoscopic sphincterotomy showed reduced risk of bleeding and infections and is especially indicated in older patients, those who are at risk for infection and coagulopathy[71]. Despite its effectiveness, EPBD has been associated with serious complications[63]. A higher risk of post ERCP pancreatitis has been observed which has been attributed to the inadequately loosened sphincter of Oddi and the intra mucosal hemorrhage and inflammation/edema around the papilla. This may cause compression of the pancreatic duct and may accentuate the risk of pancreatitis[72]. In this regard, a randomized controlled trial demonstrated that a 5-min dilation time as opposed to the conventional 1-min time resulted in an adequately loosened sphincter of Oddi and consequently reduced the risk of post ERCP pancreatitis and improved its efficacy[73]. The rate of these complications can be reduced by strict patient selection, avoidance of forced procedures, optimal dilation duration and immediate conversion to an alternative procedure if any difficulty is encountered during EPBD.

Endoscopic biliary stenting

In very old patients and those with serious co-morbidities where other endoscopic or surgical procedures may confer unacceptably high risks, endoscopic biliary stenting is a useful alternative[69]. Biliary drainage by stenting is mandatory if ductal clearance cannot be achieved during ERCP or in between procedures in patients who require more than one session for ductal clearance. CBD stones have been reported to reduce in size in 60% of patients within one to two years after biliary stenting[69].

Mechanical irritation of the stent on the stone is postulated to be one of the mechanisms.

In a study involving 28 geriatric patients with CBD stones refractory to conventional endoscopic removal, endoscopic biliary stent placement combined with oral ursodeoxycholic acid and terpene therapy for a mean of six months led to significant reduction in the size of CBD stone[74]. Subsequently, endoscopic stone removal was successfully performed in 26 of 28 patients with a mean of 1.7 ERCP procedures. This combination therapy may be of use for treatment of difficult to remove CBD stones in a subset of patients with significant co-morbidities and intolerance to prolonged endoscopic treatment modalities.

In conclusion, the past several years have witnessed the emergence of new technologies and techniques for management of difficult to remove biliary stones. Treat-

ment of such stones is generally accomplished using a multimodal approach combining conventional techniques such as endoscopic sphincterotomy, use of extraction balloons and baskets and mechanical lithotripsy, with newer techniques such as cholangioscopy guided laser or electrohydraulic lithotripsy. Recent advances in the development of videocholangioscopes, single operator catheter-based cholangioscopes and specially-designed ultrathin upper endoscopes for DPOC have made lithotripsy under direct visual guidance safer, more reliable, and more routine. Future studies will certainly shed more light on the safety of different modalities for stone extraction and will help determine the best management approach for different subgroup of patients with difficult to remove bile duct stones.

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Prognostic value of innate and adaptive immunity in colorectal cancer

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Abstract

Colorectal cancer (CRC) remains one of the major public health problems throughout the world. Originally depicted as a multi-step dynamical disease, CRC develops slowly over several years and progresses through cytologically distinct benign and malignant states, from single crypt lesions through adenoma, to malignant carcinoma with the potential for invasion and metastasis. Moving from histological observations since a long time, it has been recognized that inflammation and immunity actively participate in the pathogenesis, surveillance and progression of CRC. The advent of immunohistochemical techniques and of animal models has improved our understanding of the immune dynamical system in CRC. It is well known that immune cells have variable behavior controlled by complex interactions in the tumor microenvironment. Advances in immunology and molecular biology have shown that CRC is immunogenic and that host immune responses influence survival. Several lines of evidence support the concept that tumor stromal cells, are not merely a scaffold, but rather they influence growth, survival, and invasiveness of cancer cells, dynamically contributing to the tumor microenvironment, together with immune cells. Different types of immune cells infiltrate CRC, comprising cells of both the innate and adaptive immune system. A relevant issue is to unravel the discrepancy between the inhibitory effects on cancer growth exerted by the local immune response and the promoting effects on cancer proliferation, invasion, and dissemination induced by some types of inflammatory cells. Here, we sought to discuss the role played by innate and adaptive immune system in the local progression and metastasis of CRC, and the prognostic information that we can currently understand and exploit.

Key words: Colorectal cancer; Immunity; Inflammation; Prognosis; Metastasis

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INTRODUCTION

Despite progresses in our biological and clinical knowledge, colorectal cancer (CRC) remains one of the major public health problems throughout the world[1]. By its frequency, CRC ranks third in men and women worldwide[1]. In addition, CRC continues to be one of the most common fatal types of cancer. Originally depicted as a multi-step dynamical disease, CRC develops slowly over several years and progresses through cytologically distinct benign and malignant states, from single crypt lesions through

adenoma, to malignant carcinoma with the potential for invasion and metastasis[2,3]. According to the theory of multi-step carcinogenesis, colorectal epithelial cells accumulate a number of molecular changes to eventually become fully malignant[4,5]. In spite of unifying theories, genetic and epigenetic events during the carcinogenesis process differ considerably from tumor to tumor. Thus, CRC is not a single disease; rather it encompasses different molecular and pathological entities with a wide range of clinical behaviors[6]. At the molecular level, CRC is the tip of an iceberg the basis of which encloses a complex array of gene alterations, affecting supra-molecular processes. Essentially, like individual fingerprints, each tumor arises and behaves in a unique fashion that is unlikely to be exactly recapitulated by any other tumor. Nevertheless, molecular changes allows for a categorization of CRC, which is largely accepted, although likely to oversimplistic. It has been demonstrated that main genetic and epigenetic features, such as microsatellite instability (MSI), chromosomal instability, CpG island methylator phenotype (CIMP) or global DNA hypomethylation, lead to alterations of gene function on a genome-wide scale. It is known that activation of oncogenes, including *KRAS*, *BRAF*, *PIK3CA* and *TP53*, affects intracellular signaling pathways[6,7]. The suppressor pathway is disrupted in CRC with chromosomal instability occurring in the majority of CRCs (approximately 85%), which have a molecular profile characterized by specific chromosomal amplifications and transformations, aneuploidy, and loss of heterozygosity[6-8]. Differently, CRCs of the mutator pathway (approximately 15%) have a defective DNA mismatch repair system, which leads to accumulation of thousands of unrepaired mutations[8]. This inability to repair DNA slippage errors and mismatches can easily be demonstrated because it results in variability in the length of DNA microsatellites, formed by repetitive sequences, that is MSI. It is accepted that MSI CRCs have a heterogeneous histological appearance, better prognosis due to a reduced metastatic potential, and a different response to chemotherapy[9-14].

Histopathological examination reveals that likely other solid tumors, CRC are associated with diverse immune cell infiltrates[15-20], and that in the cancer context, epithelial cells coexist with extracellular matrix components and non-neoplastic cell types, including fibroblasts, myofibroblasts, adipocytes, endothelial cells, pericytes, which collectively form the tumor stroma. Several lines of evidence support the concept that tumor stromal cells, are not merely a scaffold, but rather they influence growth, survival, and invasiveness of cancer cells, dynamically contributing to the tumor microenvironment, together with immune cells[20-25]. The types of immune system cells that are found infiltrating CRC consist of cells of the innate immune system i.e., macrophages, neutrophils, mast cells and natural killer cells, as well as cells associated with an adaptive immune response i.e., T and B lymphocytes. Although it is commonly thought that an immune response localized to the tumor inhibit cancer growth, it is clear that some types of tumor-associated inflammation may also exert an opposite action, at least at some point of CRC natural history[26] (Figure 1). Here we sought to briefly review these two contradictory aspects of the immune response to CRC.

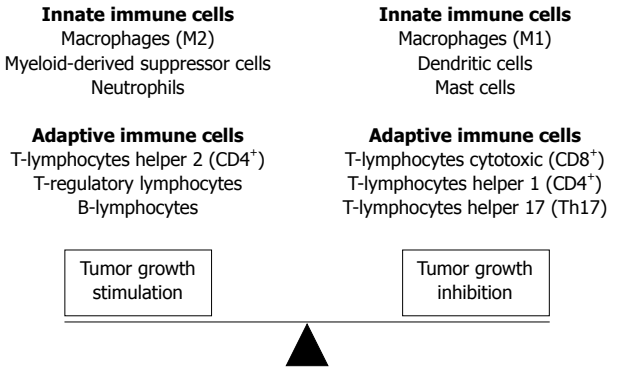

Figure 1 Immune cells have variable behavior controlled by complex interactions in the tumor microenvironment. Although it is commonly thought that an immune response localized to the tumor inhibit cancer growth, it is now clear that some types of tumor-associated inflammation can exert an opposite action.

INNATE IMMUNITY AND COLORECTAL CANCER

It is well known that innate immunity represents the body's first defense or "gut reaction" to an abnormal situation, such as cancer, and does not involve specific recognition of immunogenic peptides, or antigens[20]. Aside T and B lymphocytes , innate immune cells orchestrate an inflammatory environment that may function to either stimulate or inhibit cancer growth[20]. Various innate immune cells have been implicated in CRC development and progression[15,27,28]. Among these, macrophages, are a primary source of secreted pro-inflammatory cytokines, and are generally distinguished as type 1 (M1) or type 2 (M2)[29-31]. M1s generally have an interleukin (IL) 12^{low}IL-10^{high} phenotype, show impaired expression of reactive nitrogen intermediates, poor antigen presentation and have tumoricidal capacity, while show high expression of angiogenic factors [including Vascular-endothelial growth factor (VEGF) epidermal-growth factor (EGF) and semaphorin 4D], metalloproteases (MMPs) and cathepsins as well as of the growth arrest-specific protein 6 (GAS6)[29-32]. Additionally, M1s can support T-helper 1 (Th1) adaptive immunity[33]. Conversely, M2s secrete immunosuppressive cytokines and promote tumor growth[22,34]. It has been shown that cancer cells shape their interaction with macrophages by escaping phagocytosis and by promoting a M2-like polarization throughout chemokines and polarizing cytokines including chemokine (C-C motif) ligand 2 (CCL2), colony stimulating factor 1 (CSF1), macrophage slowing factor (MSF), tumor necrosis factor-alpha (TNF-α, IL-10 and transforming growth factor-β (TGF-β).

Among the cells with M2 phenotype, the tumor-associated macrophages (TAM) have been shown as capable of secreting proteases that enhance invasion and metastases, together with a range of cytokines inhibiting an adaptive tumor-specific immune response, and angiogenic factors that increase neovascularity. In patients with CRC, macrophages are usually found located around necrotic areas of tumor and the advancing tumor margin[28]. Their role in CRC still remains controversial. While, it was originally thought that the main function of TAMs was a direct cytotoxic effects on tumor cells, phagocytosis apoptotic/necrotic cell debris, and present tumor-associated antigens to T lymphocytes, current evidences suggest that inflammation and TAMs can also promote tumor growth and metastasis. Kang *et al*[35] have recently highlighted an association between intra-tumoral TAM densities with CRC malignant aggressiveness. Additionally, increased frequencies of intra-tumoral TAM have been associated with high levels of MMP type 2 and 9 expression in CRC cells[36,37]. These findings are in accord with a previous cell-line study showing that co-culturing of tumor cells with macrophages enhances cancer cell migration, invasiveness, and MMP-2 and MMP-9 secretion[35].

Kaler *et al*[38] have recently established that macrophages promote Wnt signaling pathway in CRC cells and thus enhance their proliferation, and demonstrated that macrophages exert their pro-tumorigenic activity mainly through the release of IL-1β. The same authors demonstrated that tumor necrosis factor related apoptosis inducing ligand (TRAIL) induced apoptosis of CRC cells is inhibited by macrophage derived IL-1β, and showed that macrophages and recombinant IL-1β counteract TRAIL-induced apoptosis through activation of Wnt signaling and stabilization of the nuclear transcription factor Snail in tumor cells.

A number of studies have also shown that macrophages can release a vast diversity of cytokines, proteolytic enzymes, growth factors, and inflammatory mediators that may directly influence and stimulate the growth and migration of tumor cells[29-33]. Li *et al*[39] first reported that IL-6 released by macrophage directly promotes CRC cell progression. They showed that monocyte/macrophage-derived IL-6 enhances migration of HT-29 CRC cells *in vitro*[39]. Although, how IL-6 enhances HT-29 cell migration still remains unknown, it has been suggested that TGF-β may be involved in this process. Using monoclonal antibodies to neutralize IL-10 in macrophage supernatant, Li *et al*[39] found that the IL-6-mediated effects on HT-29 CRC cells were all enhanced. Therefore, the interaction between IL-6 and IL-10 released from macrophage is indeed involved in CRC progression and prognosis. The above findings support the fact that TAMs play a regulatory role in the tumor microenvironment by modulating secretion of cytokines such as IL-6 and IL-10, thereby causing cancer cells to manipulate their microenvironment to facilitate cancer growth[40,41].

TAM activation and maturation is under the influence of the tumor microenvironment. TAMs retain a relatively immature phenotype, characterized by a low expression of differentiation-associated antigens in hypoxic microenvironments[35,42,43]. Conversely to an M2 phenotype with pro-tumoral functions in CRC[35], it has been shown that the macrophages, especially those secreting IL-12 and IL-23, infiltrating the tumor front are positively correlated with a favorable outcome, which implicates TAMs as possessing anti-tumor functions. Forssell *et al*[44] stained with the pan-monocyte/macrophage marker CD68 a series of CRC specimens and showed that the higher macrophage infiltration along the tumor invasive front correlated with improved survival in colon cancer compared to rectal cancer. They concluded that a dense macrophage infiltration at the tumor front positively influences prognosis in colon cancer and that the degree of cell-to-cell contact may influence the balance between pro-tumorigenic and anti-tumorigenic properties of macrophages. High levels of tissue macrophages have been also associated with earlier disease stage, absence of nodal and lymphovascular metastases and an overall better prognosis. Zhou *et al*[45] by analyzing the relationship between the density of TAMs and the potential of hepatic metastasis and survival have shown that a higher density of macrophages along the invasive front of CRC was associated with a higher 5-year survival rate. In addition, according to Forssell's scoring system that defines CD68 hot-spots as small areas among which the infiltration of macrophages was considerably above the average level of CD68-positive cells, the highest CD68 hot-spot was associated with both the incidence of hepatic metastasis and the interval between colon resection and the occurrence of hepatic metastasis[45].

The mechanisms behind the anti-tumor effects of TAMs have still not been fully elucidated, and seem potentially be ascribed to the M1 phenotype, which is in part controlled by the $CD4^+$T lymphocytes and the death of cancer cells[45-47]. It has been ascertained that recruitment of TAMs contributes to the development of an adaptive immune response against cancer, and the balance between antigen availability and clearance through phagocytosis and subsequent degradation of senescent or apoptotic cells.

It is undeniable that the discrepancies in results between different studies may be due to a number of factors related to the location of the TAMs and the assessment methods employed. Recent studies have reported that different macrophage phenotypes localized to different regions of the carcinoma have variable effects on tumor cells[18,19]. Furthermore, evidences have shown that the relationship between TAMs and tumor progression is tumor type-dependent.

Other innate immune system cells can be detected in CRC microenvironment, including mast cells (MCs), neutrophils, natural killer (NK) cells, and eosinophils.

Nagtegaal *et al*[48] have shown that peritumoral MCs prevent local and distant recurrence, with improved survival as a consequence. The significant benefit of MCs on

tumor progression in CRC was also highlighted by Gounaris *et al*[49] who reported that depletion of MCs, either by drugs either in MC-deficient mice, led to remission of existing polyps. Similar to other immune cell types, high numbers of MCs are associated with earlier CRC disease stage and have been proposed as an independent prognostic marker for improved survival[50]. It has also been reported that the number of MCs progressively decreases from normal mucosa through premalignant conditions and the lowest numbers are seen in cancers. Because of their location, MCs may prevent the metastasis of carcinomas that are restricted to the sub-mucosa. *In vivo* studies have observed that destruction of lymphatic vessels in the peritumoral infiltrate is always accompanied by MCs. This led to the hypothesis that MCs degranulate in the sub-mucosa when they come into contact with the inflammatory infiltrate or CRC cells, thus leading to the destruction of lymphatic vessels and thereby preventing further metastasis. Gulubova and Vlaykova[51] proposed the MCs density along the invasive front of the primary CRC as a helpful tool for prognosis of patients after surgical therapy. In their study, it has been shown that patients with low MCs density had significantly better prognosis compared to those with high MCs density. In addition, Blatner *et al*[52] reported that in CRC, MCs contribute to systemic regulatory T-cell dysfunction. MCs have an intricate interaction with T-regulatory cells that controls the functions of both cell types in a reciprocal manner. MCs play also an important role in allograft acceptance, where they are required to sustain the peripheral tolerance mediated by T-regulatory cells. These latter can inhibit MCs differentiation and hinder degranulation by contact-dependent mechanisms and production of soluble factors, such as IL-10. Conversely, the activation and subsequent degranulation of MCs breaks peripheral tolerance. MCs degranulation or direct cell contact and secretion of IL-6 promote Th-17 conversion of T-regulatory cells with loss of both forkhead box P3 transcription factor (Foxp3) expression and T-cell-suppressive properties.

Neutrophils may form up to 15% of the inflammatory infiltrate associated with CRCs and this proportion increases within areas of tumor necrosis[28]. In patients with rectal cancer, high concentrations of neutrophils have been shown as independent predictors of improved prognosis especially when microscopic abscesses form[28]. However, an elevated neutrophil/lymphocyte ratio was, however, found by Halazun *et al*[53] led to a poorer survival time and higher rate of recurrence in CRC patients undergoing surgery for liver metastasis.

NK cells are granular lymphocytes that form part of the innate cellular immune response[28]. In CRC, high numbers of NK cells in the inflammatory infiltrate has been associated with better prognosis[28]. The number of NK cells decreases with increasing cancer stage. Additionally, it has been shown that the ratio of NK cells in the peripheral blood is an important prognostic indicator in CRC patients and it is of interest to note that 5-fluorouracil-based chemotherapy increases the number of NK cells[54,55].

Aside the above innate immune cell types, dendritic cells (DCs), antigen-presenting cells that are critical to the stimulation of effective anti-tumor adaptive immune responses, can become defective in the tumor microenvironment and aid in tumor immune evasion by failing to stimulate T lymphocytes. It has been suggested that the presence of DCs may be of significant benefit in patients with CRC. Xie *et al*[56] also demonstrated that the presence of DCs was found predominantly in early compared to later disease stages and mostly located in tumor surrounding tissue. Suzuki *et al*[57] showed the presence of mature $CD83^+$ DCs at the cancer invasive front and by light and electron microscopy demonstrated their aggregation into clusters with lymphocytes, the majority of which were $CD45RO^+$ T lymphocytes. They concluded that mature $CD83^+$ DCs at the invasive margin promote T-cell activation for the generation of tumor specific immunity.

Although, there is growing evidence that the host innate immune system has a critical role in regulating carcinogenesis, the specific receptors involved and the importance of their interaction with commensal bacteria remain to be elucidated. Two major classes of innate immune receptors, the Toll-like receptors and Nod-like receptors, many of which are upstream of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), have been investigated[58,59]. Particularly, the toll-like receptors have been implicated in promoting colon tumorigenesis. Fukata *et al*[60] have shown that toll-like receptor-4 (TLR4) is over-expressed in human colitis-associated cancer and, that mice deficient in TLR4 are markedly protected against colitis-associated neoplasia. A high TLR4 expression in the tumor microenvironment has recently been reported as a possible marker of disease progression in CRC[60]. Conversely, the role of nod-like receptors in regulating colorectal tumorigenesis remains unclear. Chen *et al*[61] reported an increased intestinal permeability associated with enhanced inflammatory cytokine production and epithelial cell proliferation in Nod1-deficient mice. As the depletion of the gut microbiota suppressed tumor development in Nod1-deficient mice, a link should exist between commensal bacteria and host innate Nod1 signaling pathway involved in the development of inflammation-mediated CRC.

ADAPTIVE IMMUNITY AND COLORECTAL CANCER

It is today well recognized that cells of the adaptive immune system are recruited in CRC and colitis-associated tumours (CAC), where they have either pro- and anti-tumorigenic roles. T lymphocytes participate in inflammation, cancer development and progression, as well as in anticancer immunity[20-22]. In CAC the adaptive immune system seems to have mainly a pro-tumorigenic role, while in CRC it may play a double-faced role, being the

balance between immune-surveillance (carried out by $CD8^+$ and $CD4^+$ T lymphocytes) and tumour-promoting inflammation (by various sub-types of T lymphocytes) to change over time, and eventually dictating disease progression. It has been suggested that immune-surveillance might mediate the early recognition and elimination of transformed cells and aberrant crypt foci, keeping small tumours in a dormant state[62]. Immune-surveillance is retained important during the metastatic process, when small numbers or isolated tumoral cells travel and can be attacked by antitumor immune cells not inhibited by factors in the tumour microenvironment. Galon *et al*[18] suggested that once human CRC become clinically detectable, the adaptive immune response plays a role in preventing tumour recurrence and metastasis. Independently, Chiba *et al*[63] and Banerjea *et al*[64] reported that intra-tumoral T cells modify the tumour stroma or CRC cells in a way that attenuate the metastatic potential.

Cytotoxic T lymphocytes ($CD8^+$ T cells, or CTL) constitute one of the leading effector of antitumor immunity. In order for $CD8^+$ T cells to recognize antigens, these need to be exposed on the tumour cells in association with the human leukocyte antigen (HLA) class I proteins[65]. Upon encounter of a tumour cell antigen/HLA I complex for which their T cell receptor (TCR) is specific, $CD8^+$ T lymphocytes clonally expand and differentiate[65]. Once activated, cytotoxic T lymphocytes can mediate specific destruction of tumour cells through the release of lytic components *via* cell-cell interaction[65,66]. Perforin, a cytolytic protein found in the granules of CD8 T-cells and NK cells, and enzymatic proteases, including granzyme B, are secreted determining cell death by disruption of the cell membrane and activation of the apoptotic pathway respectively. $CD4^+$ T cells, which only respond to antigens presented by the HLA class Ⅱ proteins expressed by DCs, are important for antitumor immunity. On the peculiar cytokine profile induced, $CD4^+$ T lymphocytes are mainly subdivided in Th1 or Th2 lymphocytes[67]. Th1 cells secrete cytokines such as interferon-gamma (IFN-γ) and TNF-α, and support cytotoxic T lymphocytes by producing IL-2, required for $CD8^+$ T cells proliferation. Conversely, Th2 cells principally secrete IL-10, IL-4, and IL-5, and limit cytotoxic T lymphocytes proliferation.

Regulatory T cells (Treg cells) have been defined as a T-cell population that functionally suppresses an immune response by influencing the activity of another cell type[68]. Treg cells have been categorized into two main classes based on their ontogeny: naturally occurring Treg (nTreg), which develop in the thymus and are present in mice and healthy humans from an early postnatal period, and Treg which can arise in the periphery (or *in vitro*)[69]. nTreg are characterized by their high expression of CD25 ($CD4^+CD25^+$) and co-expression of the FOXP3[69]. Needham *et al*[70] reported that depletion of intra-tumoral Tregs enhances antitumor immunity and tumour rejection in mouse models. Clarke *et al*[71] have shown that depletion of Tregs in the peripheral blood of patients with CRC was recently shown to unmask $CD4^+$ T-cell responses to tumour antigens. It is known that self-tumour antigens may also induce the preferential proliferation of $CD4^+CD25^+FOXP3^+$ Treg cells. Tregs function to maintain immune homeostasis and limit acute inflammation. These cells interfere with T cell priming and can affect the antitumor function of effector cells *via* secretion of TGF-β and IL-10. A number of investigations suggest that Tregs infiltrating the tumour may adversely affect prognosis[72-75]. Increased levels of Treg cells have been, however, associated with a favourable prognosis in CRC[76-79]. The mechanisms of this dual effect, which seems dependent on the tissue type, are not well understood, although it is hypothesized that Tregs might suppress inflammation induced by growth promoting innate immune system cells.

Although the role of B lymphocytes in cancer has been overshadowed by the interest in developing T-cell-mediated cellular responses, it is now apparent that B lymphocytes can play a complementary role in the host response against tumour. B lymphocytes represent a cell population that express clonally diverse cell surface Ig receptors recognizing specific antigenic epitopes. In addition to the role of B lymphocytes in antibody production, these cells mediate/regulate several other functions fundamental for immune homeostasis. Of significant importance is the antigen-presenting role of B lymphocytes in the initiation of T-cell immune responses. Moreover, B lymphocytes can play a significant role in infection and autoimmunity as regulatory cells (indicated as Bregs) via the elaboration of suppressive cytokines, such as IL-10, TGF-β, or IL-4. The role played by B cells in cancer immunology remains still complex and somewhat controversial. Depending upon their state of activation, B lymphocytes have had divergent roles on T-cell differentiation and effector function. Oversimplifying, resting B lymphocytes have been reported to suppress T-cell-mediated antitumor immunity, by acting on both $CD4^+$ and $CD8^+$ T lymphocytes. In contrast, a number of reports suggest the efficacy of activated B lymphocytes in cellular immunotherapy of malignancies. In particular, activated B lymphocytes have been reported to enhance the ability to generate tumour-infiltrating lymphocytes in vitro involving anti-CD3 and IL-2.

The therapeutic targeting of tumours or components of the immune system with molecule-specific monoclonal antibodies (mAb) is now considered a viable treatment option for cancer patients. One of the currently applied antibodies in clinics is represented by rituximab (Rituxan) that targets B cells for elimination by binding the B cell-associated marker CD20. Interestingly, it has been recently developed a C57BL/6 TRAIL-sensitive tumour model with the aim of being able to use gene-targeted mice to better evaluate the innate and adaptive immune cells contributing to the tumoricidal activity of the MD5-1 mAb in more clinically relevant established tumours. C57BL/6 gene-targeted or immune cell-depleted mice were used to examine the antitumor activity of

MD5-1 against the TRAIL-sensitive mouse MC38 colon adenocarcinoma. It has been shown that an intact B cell compartment is critical for the therapeutic activity of MD5-1 against established tumours. B cells were confirmed to trigger tumour cell apoptosis by FcR-mediated cross-linking of the MD5-1 mAb *in vitro* and *in vivo* B lymphocytes were critical for directly triggering MD5-1-mediated tumour cell apoptosis.

Although the role of B-cells in human CRCs is still not completely characterized, B-cell-deficient mice exhibit spontaneous regression of MC38 colon carcinoma cells. Studies involving BCR transgenic mice indicated that B lymphocytes might inhibit antitumor T lymphocytes responses by antigen-nonspecific mechanisms. Shah *et al* investigated the role of B lymphocytes in tumour immunity by studying immune responses of mice genetically lacking B lymphocytes to primary tumours. They highlight that although the effects of B lymphocytes on anti-tumour response warrant further study, adoptive transfer of CD40(-/-) B lymphocytes into B lymphocytes-deficient mice resulted in restored growth of MC38 colon carcinoma cells suggesting additional factors other than CD40 are involved in dampening anti-tumour responses.

PROGNOSTIC INFORMATION OF IMMUNE CELL INFILTRATE

In contrast to infiltration of cells responsible for chronic inflammation, the presence of high numbers of T lymphocytes has been reported to be a positive prognostic factor in several cancers. The first reports on the beneficial effect of lymphocytic infiltration in CRC appeared already in the Eighties. They were later confirmed until recent most studies highlighting a prominent function for memory T lymphocytes and $CD8^+$ T lymphocytes in predicting disease-free survival and overall survival. In general terms, it has been suggested that prognosis in patients with cancer is positively affected by (1) the presence of a tumour gene signature consistent with a type I adaptive immune response (i.e., increased antigen presentation, IFN-γ signalling, and TCR signalling); and (2) the presence of T cells that penetrate through tumour stroma and deeply infiltrate the parenchyma to become intra-tumoral T cells[20]. Thus, besides a Th-1 response signature, the other key feature of an effective immune response is the ability of T cells to reach the site of the tumour and to infiltrate it. Because T-cell infiltration is not spatially homogeneous in CRC, attention has been focused on the predictive values of T lymphocytes located in the center of the tumor (CT), along the invasive margin (IM) and in lymphoid aggregate mainly detectable in proximity of the tumor (these aggregates are called tertiary lymphoid structures)[80,81].

In a large series of CRCs, Pagès *et al*[82] assessed the immune component of the tumoral microenvironment by a combination of high-throughput gene expression and immunophenotypic analyses, and evaluated its possible influence on tumour dissemination. They found an association between evidence of an immune reaction within the tumour and the absence of tumour local invasion of vascular, lymphatic, and neural structures (collectively referred to as VELIPI). CRCs without VELIPI were associated with an enhanced immune cell infiltration and an increase of mRNA expression of adaptive Th1 effector T-cell markers [CD8, T-box transcription factor 21 (T-bet), IFN regulatory factor-1 (IRF-1), IFN-γ, granulysin, and granzyme B]. The immunohistochemical analysis of adaptive immune markers i.e., CD3, CD8, granzyme B, and CD45RO, by tissue microarrays prepared from the CT and from its IM revealed a statistically significant correlation between the density of these immune cells and outcome for all patients but those with metastatic disease at diagnosis. Further, the combined analysis of both tumour regions improved the accuracy of survival prediction compared with single-region analysis[18].

Independently, Deschoolmeester *et al*[83] showed that the presence of a pronounced lymphocytic infiltration within the tumour is associated with improved survival. They found that $CD3^+$ and $CD8^+$ T lymphocytes within tumour nests and of $CD3^+$ in the stroma had a major impact on the patients' overall survival. The improved survival associated with infiltration of T lymphocytes has been suggested to result from the effective suppression of micrometastases. Therefore, the densities of $CD8^+$ T cells within the primary tumour might be a potential marker of the presence of a systemic immunosurveillance mechanism. In addition, tumour cells secrete substances in the stromal compartment, which might be recognized by the immune system that subsequently attack the tumour. A weak adaptive immune reaction correlated with a very poor prognosis even in patients with early tumour invasion. Conversely, a high density of adaptive immune cells correlated with a highly favourable prognosis whatever the local extent of the tumour and the regional lymph node invasion.

In mice, targeted disruptions of genes that encode critical components of the immune system (i.e., mice lacking: IFN-γ or its receptor, signal transducer and activator of transcription-1 mediating IFN-γ receptor signalling, perforin, recombination activating gene-2, or IL-12) induce an increased susceptibility of the host to tumours[84]. These findings make it possible to hypothesize an immune-mediated control of tumour development by the adaptive compartment.

A number of studies have reported that MSI, CIMP, BRAF mutation, PIK3CA mutation, and tumour LINE-1 hypomethylation are associated with CRC prognosis and that lymphocytic infiltration is associated with many of these molecular variables. The associations of a prognostic biomarker with a given disease, strongly suggests its stage-dependency as outcome predictor. This is best exemplified by MSI CRC, whose overall prognostic advantage is associated with a low frequency of stage Ⅲ and Ⅳ cases at diagnosis as compared to microsatellite stable counterpart. Most MSI CRCs show a pronounced

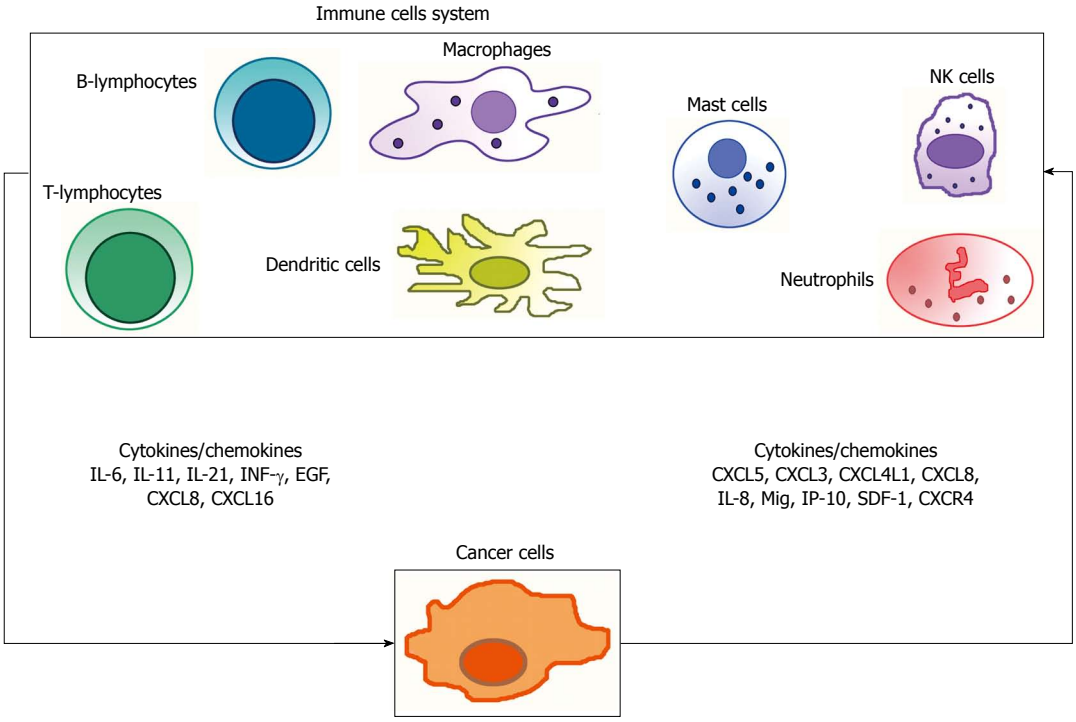

Figure 2 Anti- and pro-tumor immune response involves the interaction of several cell types of the adaptive as well as of the innate immune system, and an intricate network of products (i.e., cytokines and chemokines). IL: Interleukin; INF-γ: Intracellular interferon-γ; EGF: Epidermal-growth factor; NK cells: Natural killer cells.

intra-tumoral inflammatory reaction (in fact a criterion for MSI testing), the mechanistic explanation of which, however, is still incompletely understood. Within these tumours, infiltrating lymphocytes have been identified as predominantly activated $CD8^+$ T cells. The presence of these cytotoxic T lymphocytes has been attributed to the inherently greater production of abnormal peptides as a result of unreliable DNA repair in MSI-positive tumours. It is known that truncated peptides produced by frameshift mutations due to MSI may be immunogenic and contribute to the host immune response. However, little is still known about the interrelationship between tumour-infiltrating T lymphocytes, MSI status, and other tumour molecular features. It is indubitable that to define the prognostic effect of tumour-infiltrating T-cells independently of those potential confounders, large studies of CRC with extensive molecular characterization are needed. Additionally, caution is needed before incorporating tumour-infiltrating T cells into tumour staging. To minimise the risk of inappropriate tumour down-staging at diagnosis, survival data need to be confirmed in independent series of patients studied in the past decade. Moreover, the association has to be conclusively proven between low densities of tumour-infiltrating T cells and the clinical detection of metachronous metastases, which remains the most appropriate outcome measure for recognising a role of the local immune response in micrometastasis suppression. Recently, Laghi *et al*[85] investigated the relationship between the density of $CD3^+$ T infiltrating lymphocytes along the tumour invasive margin, and the occurrence of metachronous distant-organ metastases after potentially curative resection, in a large, consecutive series of patients with deeply invading (pT3 or pT4) MSI-typed CRC, and no evidence of distant organ metastasis at diagnosis. They found that large areas of $CD3^+$ cells at the invasive front of pT3 or pT4 CRCs are associated with a low risk of metachronous metastasis and consequently a survival advantage, only in patients with node-negative cancers, but not in patients whose cancers involved lymphnodes. Of interest, the prognostic advantage conferred by a high density of $CD3^+$ cells was independent of tumour microsatellite status in patients with stage Ⅱ CRC. CD3-immunostaining of CRC tissue might therefore be useful for selecting stage Ⅱ patients who, because they are at very low risk for cancer progression, could be spared adjuvant treatments.

Nosho *et al*[86] examined the prognostic role of tumour-infiltrating T-cell subsets in a database of 768 CRCs from two prospective cohort studies. They concurrently assessed the densities of $CD3^+$, $CD8^+$, $CD45RO^+$, and $FOXP3^+$ lymphocytes as well as other relevant molecular and pathological features, therefore making possible to evaluate the independent effect of each T-cell subset density on patient survival. They found that the density of $CD45RO^+$ cells, but not that of $CD3^+$, $CD8^+$, or $FOXP3^+$ cells, was an independent prognostic biomarker of longer survival in CRC patients. In contrast, Salama *et al*[76] by analysing T-cell infiltrates in 967 CRCs including 593 stage Ⅱ and 374 stage Ⅲ cases, reported that $FOXP3^+$ lymphocytes density had stronger prognostic significance than $CD8^+$ and $CD45RO^+$ cells, and predicted a better outcome. $FOXP3^+$ lymphocytes were

found not associated with any histopathologic features. At multivariate analysis, stage, vascular invasion, and $FOXP3^+$ cell density in tumoral tissue were independent prognostic indicators. These results led Salama *et al*[76] to conclude that the inclusion of $FOXP3^+$ cell density may help to improve the prognostication of early-stage CRC, although these authors do not explored this parameter with tumor stage.

CONCLUSION

It is accepted that human cancer is a complex dynamical disease[87,88]. It is also now well recognized that cancers are not just composed of malignant cells, but that they are microenvironment consisting of many cell types, including a range of immune cells. It is indubitable that the antitumor immune response involves the interaction of several cell types and products (Figure 2), of the adaptive as well as of the innate immune system. It is also clear that CRC can escape immune surveillance using several strategies. The molecular profiles of the function and interaction of innate and adaptive immune cells, and the definition of tumor antigens have all led to build the basis of the knowledge of how the immune system modulates tumor growth and inhibition. The challenge remains to determine not only how the "rejection" pathway initiates in human malignancy, but also how that rejection is maintained. It is indubitable that the analysis of the type, quantity, location and the functions of the immune infiltrate becomes a primary step in understanding CRC natural history, and, in a clinical perspective, its prognostic determinants. A comprehensive analysis of all components of the lymphocytic infiltrates in the context of their localization, organization and impact at various steps of tumor progression remains largely, if not entirely, to be reported to prospective studies. In parallel, understanding the mechanisms of efficient immune reactions, the place where they are initiated, the cells and key cytokines and chemokines involved (Figure 2), and their impact at different stages of the disease should provide new tools and goals for more effective and less toxic targeted therapies.

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REVIEW

Gastrointestinal radiation injury: Symptoms, risk factors and mechanisms

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Abstract

Ionising radiation therapy is a common treatment modality for different types of cancer and its use is expected to increase with advances in screening and early detection of cancer. Radiation injury to the gastrointestinal tract is important factor working against better utility of this important therapeutic modality. Cancer survivors can suffer a wide variety of acute and chronic symptoms following radiotherapy, which significantly reduces their quality of life as well as adding an extra burden to the cost of health care. The accurate diagnosis and treatment of intestinal radiation injury often represents a clinical challenge to practicing physicians in both gastroenterology and oncology. Despite the growing recognition of the problem and some advances in understanding the cellular and molecular mechanisms of radiation injury, relatively little is known about the pathophysiology of gastrointestinal radiation injury or any possible susceptibility factors that could aggravate its severity. The aims of this review are to examine the various clinical manifestations of post-radiation gastrointestinal symptoms, to discuss possible patient and treatment factors implicated in normal gastrointestinal tissue radiosensitivity and to outline different mechanisms of intestinal tissue injury.

Key words: Radiation enteritis; Radiation proctitis; Symptoms; Pathophysiology; Risk factors

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INTRODUCTION

Radiation delivery methods

Radiation therapy can be delivered by three main methods. External beam radiotherapy is a method in which radiation beam is delivered from outside the body to the target tumour through two or three-dimensional beam arrays using linear accelerators. Advances in planning and delivery techniques such as 3-dimensional simulation and intensity modulated radiation therapy are associated with a reduced risk of normal tissue toxicity and allow a higher radiation dose to be used compared to conventional two dimensional methods[1-4]. Enhanced target definition of both tumours and surrounding normal tissues and combining beams of varying intensity in intensity modulated radiation therapy allow for better dose delivery and improved tumour control with less toxicity[5,6]. Quantitative dose tracking to both normal tissues and tumors in the form of dose-volume histograms[7] provide a graphic display of a simulated radiation treatment plan and generate valuable information on the dose distribution within the volume of interest[8]. These are now common planning

tools in modern external beam radiation delivery. Brachytherapy is an internal form of radiation therapy where the radiation sources are implanted within or in close proximity to the target tumour to deliver a high dose of localized radiation. This procedure is a highly effective dose delivery method for certain tumours such as prostate and gynaecological cancers. The third method of the radiation therapy is the systemic administration of radioactive particles which is termed radioisotope therapy. In this method radioactive particles (radionuclides) are injected into the blood stream to be adsorbed specifically by the targeted tissue such as thyroid gland[9]. Gastrointestinal radiation injury most commonly occurs following external beam therapy.

A highly precise radiation delivery can be achieved through newer techniques such as image guided radiotherapy techniques which allow verification of the target position on a daily basis to account for internal target motion[10]. Stereotactic radiation therapy[11] can focus a narrow radiation beams on a small target such as early cancer or metastatic lesions[12].

TREATMENT RELATED RISK FACTORS FOR GASTROINTESTINAL INJURY

Radiation dose, fractionation and field size

Radiation dose is a major determinant of the severity of acute and late normal tissue toxicity[13-20], the desired optimal radiation dose is defined as the dose that maximizes the difference between "tumor" and "normal tissue" damage within the sigmoid shape dose-effect relationship curve[11]. With respect to the gastrointestinal tract, the severity of toxicity is reported as Grades of severity to different symptoms or clinical manifestations ranging from minor symptomatic changes to severe life threatening complications. Multiple toxicity grading systems have been developed to assess adverse events of cancer treatment[21]. Generally, Grade 1 and 2 radiation injury are frequent and they are often requiring no treatment although they can cause a considerable effect on patient quality of life. Examples of commonly used toxicity grading system to assess radiation injury severity are the Radiation Therapy Oncology Group[22] and the Common Terminology Criteria for Adverse Events grading system (Table 1)[21]. Radiation dose per fraction and altered fractionation schedules are important factors linked to increased risk of intestinal radiation toxicity[23]. The radiosensitivity of the cell depends on two factors, the intrinsic radiosensitivity which is linearly related to the radiation dose and it is represents the initial slope of the cell survival curve (alpha). The second factor is (beta) which represents the curvature of the cell survival curve and it is a factor of dose-per fraction and dose-rate variations in radiobiology[24].

The alpha/beta ratio represents the dose at which the linear and quadratic components of the Linear-Quadratic model contribute equally to cell killing and has been shown to have a connection to early and late radiation response[25]. In radiotherapy of tumors with long turnover time such as prostate cancer, the alpha/beta ratio is smaller than that of early reacting normal tissues. In this case, hypo-fractionation will be a better strategy for radiotherapy than the many small fractions used for other tumors[24,26,27].

A data analysis of 918 head and neck cancer patients reported a variable prevalence of mucositis between patients treated with continuous hyperfractionated accelerated radiotherapy (CHART) and patients received conventional fractionation radiotherapy. The incidence of Grade 3 confluent mucositis reported after CHART was 75% compared to 44% following conventional fractionation radiotherapy[28].

Modification of the radiation delivery regimes through

Table 1 Example of some gastrointestinal symptoms grades following radiation injury

Grade	Gastrointestinal symptoms
	Nausea
1	Loss of appetite without alteration in eating habits
2	Oral intake decreased without significant weight loss, dehydration or malnutrition; IV fluids indicated < 24 h
3	Inadequate oral caloric or fluid intake; IV fluids, tube feedings, or TPN indicated ⩾ 24 h
4	Life-threatening consequences
5	Death
	Anorexia
1	Loss of appetite without alteration in eating habits
2	Oral intake altered without significant weight loss or malnutrition; oral nutritional supplements indicated
3	Associated with significant weight loss or malnutrition (e.g., inadequate oral caloric and/or fluid intake); IV fluids, tube feedings or TPN indicated
4	Life-threatening consequences
5	Death
	Haemorrhage-GI
1	Mild, intervention (other than iron supplements) not indicated
2	Symptomatic and medical intervention or minor cauterization indicated
3	Transfusion, interventional radiology, endoscopic, or operative intervention indicated; radiation therapy (i.e., hemostasis of bleeding site)
4	Life-threatening consequences; major urgent intervention indicated
5	Death
	Ulceration-GI
1	Asymptomatic, radiographic or endoscopic findings only
2	Symptomatic; altered GI function (e.g., altered dietary habits, oral supplements); IV fluids indicated < 24 h
3	Symptomatic and severely altered GI function (e.g., inadequate oral caloric or fluid intake); IV fluids, tube feedings, or TPN indicated ⩾ 24 h
4	Life-threatening consequences
5	Death
	Incontinence anal
1	Occasional use of pads required
2	Daily use of pads required
3	Interfering with ADL; operative intervention indicated
4	Permanent bowel diversion indicated
5	Death

According to the Common Terminology Criteria for Adverse Events system v 3.0. IV: Intravenous; GI: Gastrointestinal; ADL: Activities of daily living; TPN: Total parenteral nutrition.

Table 2 Summary of risk factors for gastrointestinal radiation injury

Risk factors	
Radiation techniques	Treatment volume, total dose, fractionation dose and schedules
Combined modality therapies	Surgery Chemotherapy: Particularly concurrent
Medical co-morbidities	Vascular disease, connective tissue disease, inflammatory bowel disease, HIV
Genetic susceptibility	Single nucleotide polymorphism, ataxia telangiectasia

HIV: Human immunodeficiency virus.

hypofractionation was also suggested to be safer than conventionally fractionated conformal radiotherapy in a randomized study of prostate cancer radiotherapy[29].

Treatment field size and intestinal volume irradiated are important factor and a key determinant of radiation toxicity. Bowel toxicity was found to be directly related to the volume of small intestine irradiated[13]. Moreover, irradiation to a larger volume of small intestine was reported to increase the operative mortality in rectal cancer patients treated with anterior and posterior field irradiation technique[30,31]. Furthermore, the impact of the field volume has been demonstrated in a randomized study of prostate cancer radiotherapy. Patients who were treated with conformal shielding with 48% less volume irradiation had less rectal toxicity at 5 years than patients treated with conventional radiotherapy, despite identical radiation dose[32,33] (Table 2).

Combined modality approaches

Combined modality therapy increases the risk of radiation toxicity. Surgery or concurrent chemotherapy is linked to an increased incidence of radiation toxicity. Previous abdominal surgery increases the risk of radiation toxicity[34]. Anatomical changes that increases intestinal exposure to radiation such as postoperative small intestine prolapse into the pelvic cavity[13,35] or surgical adhesions that fix intestinal segments within the radiation field can all predispose part of the intestine to receive higher doses of radiation[36]. Combining prostatectomy with radiotherapy can increase rectal toxicity during prostate cancer treatment[37]. An analysis of acute toxicity was performed in 405 prostate patients in The European Organization for Research and Treatment of Cancer randomized trial 22863. In those patients it was reported that among other factors, previous genitourinary surgery was found to be predictive of lower gastrointestinal radiation toxicity[38].

Combining chemotherapy with radiation has been reported to increase the rate of acute intestinal toxicity, while the long term effect of this combination is not clear[20,39]. Oral mucositis was reported in more than 90% of patients treated with a combined chemo-radiotherapy regime for head and neck cancer[40,41] in comparison to another study which reported an incidence of oral mucositis in 62% of patients treated with radiotherapy alone[42]. Concurrent chemotherapy with radiation has also been reported to increase the risk of oesophageal radiation injury by 12-fold[43].

In a study of cervical cancer patients treated with chemoradiotherapy, the incidence of Grade 3 late intestinal toxicity increased from 10% to 26% in patients treated with both mitomycin and fluorouracil compared to a fluorouracil alone group, suggesting a possible role for the type of chemotherapeutic agent used as determinant of severity of gastrointestinal toxicity[44].

Different mechanisms have been suggested to explain the sensitizing effect of adding chemotherapy in increasing the risk of intestinal radiation injury. Examples of possible mechanisms are alterations in cell cycle kinetics, or synchronization of replicating cell populations. Halopyrimidines such as fluorouracil, fluorodeoxyuridine and iododeoxyuridine may sensitize tumors both by inhibiting effective DNA repair and by increasing the amount of radiation induced DNA damage[45-47].

SYMPTOMS RESULTING FROM RADIATION INJURY TO THE GASTROINTESTINAL TRACT

Acute and chronic gastrointestinal radiation injury

During external beam radiotherapy, ionising radiation enters and exits the body and therefore affects normal tissues surrounding the target tumour. The gastrointestinal tract which extends over a large surface area and any part of the gastrointestinal tract that falls within the radiation field can be affected, resulting in acute and chronic symptoms of gastrointestinal radiation injury (Table 3).

Clinical manifestations of gastrointestinal radiation injury can present during or soon following radiotherapy. These symptoms are related to acute mucosal injury and inflammation. Delayed symptoms occur a few months or years after radiotherapy and are attributed to a chronic process of transmural fibrosis and vascular sclerosis. Typically symptoms are considered "acute" if they occur within the course of treatment or up to 90 d following treatment. These are generally reversible. Chronic side effects are much less common and occur more than 90 d post radiation; they are less likely to reverse. The onset of delayed symptoms has been reported as much as after 30 years following radiotherapy[48].

Mouth, pharynx, and oesophagus

During radiotherapy for head and neck or thoracic cancer, the upper gastrointestinal tract falls within the radiation field. Irradiation to this area results in acute mucosal injury causing mucositis and ulceration which manifests within the first two weeks in 30%-60% of patients, causing dysphagia and odynophagia[49]. In a study of 254 non-small-cell lung cancer patients, acute toxicity of Grade 2 or worse has been reported in 78% of patients[50]. Mucositis is debilitating, can be a dose limiting side effect and

Table 3 Acute and chronic manifestations of gastrointestinal radiation injury

Clinical manifestations	Radiation tolerance dose TD5/5, TD50/5 (Gy)	Gastrointestinal organ
Oral mucositis occurs in > 90% of patients with concurrent chemotherapy[40]	Parotid gland: TD5/5 (32) TD50/5 (46)[60,179,180]	Mouth, salivary glands, hypopharynx, parotid
Xerostomia and altered saliva composition		
Acute Grade 3-4 oesophageal injuries occur in 46%with concurrent chemotherapy[181]	TD5/5 (55-60)	
Dose > 58 Gy predicts Grade 3-5 acute oesophagitis[54]	TD50/5 (68-72)[60,179]	Oesophagus
60 Gy resulted in Grade 3 toxicity in 42%[182]		
Radiation can lead to late stricture and/or perforation of the oesophagus[53,60]		
40 Gy: Severe late toxicity in 7% including ulceration, gastritis and small-bowel obstruction/perforation[183]	TD5/5; (50-60) TD50/5 (65-70)[60,179]	Stomach
Elevated liver enzymes in 5%[184]	TD5/5; (30-50)	Liver Small intestine
(31.3-37 Gy resulted in RILD in 9.4%[66,185]	TD50/5; (40-55)[60,179]	
45 Gy cause 5% Grade 3-4 toxicity and 14% with concurrent chemotherapy[71]	TD5/5; (40-50)	
Diarrhoea, abdominal pain in 20-70%[72]	TD50/5; (55-60)[60,179,180]	
Transmural fibrosis leading to obstruction in 5%-10%[68,77-78]		
Intestinal fistulation occurs at a rate of 0.6% to 4.8%[68,79]		Colon and rectum
50 Gy 5 year estimate of small bowel obstruction is 11%[186]		
Colitis in 25%-50% of patients[186]	Colon	
Grade 2-3 acute proctitis 40%[91]	TD5/5; (45-55)	
Chronic rectal symptoms in 6.7%-31%[91]	TD50/5 (55-65)[60,179,180]	
Acute symptoms of anus and rectal injury occur in up to 75% of patients during radiotherapy[187]	Rectum TD5/5; (60-61.38) TD50/5 (80-81.38)[60,179,180]	

TD5/5: Radiation dose associated with 5% of patients' risk of delayed toxicity in 5 years; TD50/5: The radiation dose associated with 50% of patients' risk of delayed toxicity in 5 years. RILD: Radiation-induced liver disease.

is difficult to treat. Severe symptoms may require therapy interruption, or the provision of an alternative nutritional route to avoid dehydration and malnutrition. Therefore, elective percutaneous endoscopic gastrostomy tube insertion before radiotherapy is a recognized practice and it is associated with improved quality of life and a lower rate of hospital admissions[51,52]. In severe cases, acute radiation oesophagitis can lead to more serious complications such as significant bleeding or oesophageal perforation[53].

Clinical predictors for acute oesophageal toxicity include maximum radiation dose. A maximum dose of > 58 Gy was reported to predict the risk of Grade 3-5 oesophageal toxicity in 207 non-small-cell lung cancer patients treated with 3-dimensional conformal radiotherapy[54] while a dose of 50 Gy was significantly associated with Grade 2 or worse oesophagitis in another cohort 36 non-small-cell lung cancer patients[55]. In both studies patients received concurrent chemotherapy which is also a risk factor for oesophageal toxicity. The volume of the irradiated oesophagus has also been suggested to predict acute oesophageal toxicity[55]. In another series of 208 non-small-cell lung cancer patients treated with three dimensional conformal radiotherapy, concurrent chemotherapy and maximal point dose to the oesophagus > 60 Gy were found to be significantly associated with the risk of Grade 3-5 oesophageal injury[56]. The Quantitative Analyses of Normal Tissue Effects in the Clinic paper by Werner-Wasik *et al*[57] published in 2010 reviewed the published data on the dose-volume effect and concluded that it was not possible to identify the best threshold volumetric parameter for oesophageal irradiation given the variety of the volumetric metrics in the published data.

Delayed symptoms of oesophageal injury can manifest after several months following radiotherapy and include chronic ulceration, fistulisation or chronic dysphagia. Dysphagia can be secondary to tissue fibrosis and stricture formation or due to motility disorder induced by muscular or nerve injuries. Delayed oesophageal toxicity has been reported in 17% of non-small-cell lung cancer patients[50] and the median and maximal time to the onset of late toxicity was 5 and 40 mo respectively after radiotherapy.

Stomach and duodenum

Gastric injury during radiotherapy occurs when the stomach falls within the radiation field of an adjacent tumour. Nausea, vomiting, dyspepsia and abdominal pain has been reported to occur early after radiotherapy to the upper abdomen in 50% of patients[58]. These symptoms result from acute mucosal injury causing erosions and ulceration of gastric and duodenal mucosa.

Later symptoms include chronic dyspepsia and abdominal pain due to chronic ulceration secondary to mucosal injury[59]. Rarely, gastric wall fibrosis can lead to gastric outlet obstruction. The radiation dose associated with 5% and 50% of patients risk of delayed gastric toxicity in 5 years (TD5/5 and TD50/5) have been estimated at 50 Gy and 65 Gy respectively for gastric ulceration or perforation. An accurate data on dose-volume constrain for partial gastric irradiation is not available. However, the threshold dose of 45 Gy to the whole stomach has been associated with ulceration in 5%-7% of patients[60,61].

Liver injury

Following irradiation to the liver, radiation induced liver disease can occur in patients with normal pre-radiotherapy liver function, causing anicteric hepatomegaly and mild alkaline phosphatase serum level elevation. A more

severe derangement of liver function occurs in patients with pre-existing liver disease[62]. Radiation induced liver disease can progress to fibrosis, cirrhosis, and liver failure[63]. Abdominal imaging with computed tomography scan or magnetic resonance imaging can be helpful to show low-attenuation injury areas or areas of atrophy in the irradiated liver segment[64].

Risk factors for radiation induced liver disease include baseline liver dysfunction, Hepatitis B virus carrier status[65], mean dose > 30 Gy for partial liver radiotherapy, concurrent chemotherapy and volume of liver irradiated[66,67].

Small intestine

The small intestine receives irradiation during radiotherapy of pelvic or abdominal malignancies. The degree of injury depends on the radiation dose and the volume of intestinal segment that falls within the radiation field[68,69]. Significant correlation has been suggested between the volume of irradiated small bowel and the likelihood of acute toxicity, regardless of the radiation dose delivered[70]. Other predictors of acute small intestine toxicity include the use of concurrent chemotherapy. This effect has been reported in 186 cervical cancer patients who received 45 Gy preoperative pelvic radiotherapy alone where 5% of patients experienced Grade 3-4 toxicity in comparison to 14% of 183 patients who received radiotherapy and weekly cisplatin[71]. The relatively fixed portions of the small intestine such as the duodenum and the terminal ileum are at increased risk of radiation toxicity as they are more susceptible to receive higher doses of radiation than the mobile parts of small intestine.

The radiation dose associated with delayed small bowel toxicity have been estimated by Emami *et al*[60]. The TD5/5 and TD50/5 doses for one third of small bowel irradiation were estimated at 50 Gy and 60 Gy respectively. The TD5/5 and TD50/5 for the whole-organ irradiation were 40 Gy and 55 Gy respectively. These doses estimates remained as a guide for two decades and more recent data were consistent with these ranges[60,61].

Clinically, nausea, vomiting and abdominal pain are early symptoms that can occur during the first two weeks following abdominal radiotherapy, and may be mediated by the release of inflammatory cytokines following radiotherapy. Diarrhoea and abdominal pain occur during the first two weeks of radiotherapy to abdominal or pelvic malignancies in 20% to 70% of patients[72]. This may be a result of direct radiation injury to the small intestinal mucosa causing epithelial atrophy, and reduced mucosal blood flow[73].

The acute symptoms usually settle within three weeks after completion of radiotherapy and the intestinal epithelium regenerates from stem cells at the base of the crypts.

Delayed symptoms of radiation small intestinal injury manifest months to years after radiotherapy with symptoms of diarrhoea, recurrent abdominal pains and malabsorption.

Chronic diarrhoea following radiotherapy can result from different pathophysiological processes such as bile salt malabsorption, bacterial overgrowth, fat malabsorption, rapid intestinal transit or lactose intolerance[74]. More chronic symptoms occur as a result of pathological abnormalities to the intestinal vascular compartment resulting in intestinal ischemia as well as progressive intestinal fibrosis leading to structural abnormalities such as strictures and fistulation. Bacterial overgrowth contributes to malabsorption and diarrhoea, particularly in patients with intestinal strictures[73,75].

Intestinal obstruction can complicate 5% to 10% of severe small intestinal radiation injury[76]. The rate of severe small intestinal complications following radiotherapy for rectal cancer can vary considerably depending on the tumor and treatment characteristics, Reports indicate rates of 0.8% to 13% for small intestinal obstruction[68,77,78] and 0.6% to 4.8% for intestinal fistulation[68,79]. Patients with severe small intestinal injury have a poor prognosis since surgery to manage strictures is complex and has been associated with poor outcomes[73,80].

Colon and rectum

During abdominal and pelvic radiotherapy, the colon and rectum are commonly affected as their anatomical locations fall within the radiation field of a variety of cancers. The fixed portions of the colon, the caecum and the rectum are at greater risk of receiving higher doses of radiation than the remainder of the colon.

Acute radiation injury to the rectum and anal canal can result in a diversity of symptoms such as abdominal pain, diarrhoea, tenesmus, rectal pain, urgency, rectal discharge, incontinence, and fresh rectal bleeding. These symptoms occur primarily as a consequence of direct mucosal damage[81-83]. Acute radiation injury to the colon can be severe and in 5%-15% can lead to therapy interruption or treatment plan alteration[84].

A recent study showed that 47% of women who received radiotherapy for cervical or endometrial cancer reported symptoms of radiation intestinal injury affecting quality of life within 3 mo following therapy completion[85]. These results are consistent with a previous structured questionnaire study[86] which showed that 53% of patients had reported bowel symptoms significantly affecting their quality of life, whilst 81% of patients in the study described new-onset gastrointestinal problems after receiving radiotherapy.

The recent data on dose-volume effect in radiation induced rectal injury was reviewed by Michalski *et al*[87]. The incidence of Grade > 2 injury from different studies was variable according to dose, treatment parameters and scale used in each study. Among the recent studies reported, an incidence of 13.5% and 16% of Grade > 2 rectal injury. Identified predictors for Grade > 2 rectal injury or rectal bleeding include the volume of the rectum irradiated and a total radiation dose > 60 Gy during 3-dimensional conformal radiotherapy. The effect of concurrent chemotherapy has been observed in the

European Organization for Research and Treatment of Cancer study where patients received 45 Gy preoperative radiotherapy or radiotherapy and 5-flurouracil (5-FU). Grade > 2 diarrhoea occurred in 17% of radiotherapy alone group compared to 38% of radiotherapy and 5-FU group[88].

Delayed symptoms of radiation colonic injury are insidious and usually follow a progressive course. They can manifest after a latent period of few months or years. One study has reported a 15% incidence of bowel toxicity 20 years after receiving radiotherapy in a cervical cancer cohort[89]. Severe life threatening complications occur after radiotherapy such as fistulation, sepsis, perforation or bleeding at a rate between 4%-8% within 5-10 year time after radiotherapy[90]. Patients suffer from a variety of symptoms, such as abdominal pain, changing bowel habits with intermittent diarrhoea. Constipation can result from altered colonic motility due to fibrosis and stricture formation. An abnormal bowel transit can manifest as recurrent pain and increased risk of obstruction or pseudo-obstruction secondary to fecal loading. Excessive fibrosis can cause loss of ano-rectal compliance and manifests as urgency and frequency[73]. Faecal incontinence has been reported in up to 20% of patients and significantly reduces patients' quality of life[86,91].

Unlike radiation injury to the small bowel, radiation injury to the colon does not compromise nutrient absorption and malabsorption is uncommon[73].

Adverse effects of radiation to the pelvis primarily affect the colon and the rectum. However, other organs can be irradiated causing increased morbidity for example injury to the urinary tract or the genital system resulting in symptoms affecting quality of life were reported in 30% of patients[92-95]. A rare but serious complication of prostate brachytherapy is recto-vesical fistula which occurs with a low frequency of 1 in 250 to 1 in 1000 patients implanted[96-98].

Radiation exposure increases the risk of a secondary malignancy. Patients who received radiotherapy were shown to have significantly higher risk of developing second cancers both overall and in the areas that were exposed to the radiation field[99]. In an analysis of testicular cancer survivors which included 28 843 men, the risk of a second cancer was estimated. The patterns of second cancer suggested that many factors may be involved, including previous treatment received, but the precise roles of different factors is still to be clarified. It has been reported that secondary leukaemia was associated with both radiotherapy and chemotherapy, whereas excess cancers of the stomach, bladder, and possibly pancreas were associated mainly with radiotherapy[100]. This risk also includes colorectal cancer, which can occur more than 10 to 20 years after radiation exposure[99,101].

PATIENT RELATED RISK FACTORS

Patient factors and individual variations

Individual patient phenotypic factors have been suggested to influence the susceptibility to intestinal radiation injury. It was reported that older patient age is associated with an increased risk of developing reduced organ function after radiotherapy[102-104]. Body habitus has been reported as another susceptibility factor, where thin patients with narrow antero-posterior diameter can suffer an increased risk of intestinal radiation toxicity compared to normal individuals[36]. Smoking status has been associated with risk of chronic intestinal toxicity[20,105] as well as previous history of surgery[13,35,36].

Medical co-morbidities

Vascular disease: Co-morbid vascular disease such as hypertension, diabetes mellitus and atherosclerosis were suggested to predispose patients to an increased vascular injury following radiation and subsequent intestinal wall ischemia and impaired tissue repair[74]. The microocclusive vascular disease in addition to increased blood viscosity in diabetes mellitus were suggested to predispose to intestinal tissue ischemia[106,107]. One study investigated the possible effect of diabetes mellitus during prostate cancer radiotherapy. The study reported higher rates of late genitourinary/gastrointestinal toxicities in diabetic patients than in non-diabetics (34% and 23% respectively). It was also noticed that diabetics developed complications earlier than the non-diabetics (10 mo and 24 mo respectively)[108].

Inflammatory bowel disease: Co-morbid inflammatory bowel disease (IBD) is considered in some institutions as a relative contraindication to radiotherapy for concerns of greater risk of acute and late side effects[109-111]. Intolerance to radiotherapy in IBD patients has been demonstrated in case reports[112,113] and in a larger retrospective analysis where the incidence of severe acute and late events was 21% and 29% respectively[114,115]. However, in a large retrospective analysis in patients with colorectal cancer, the data on treatment modality received for 170 colorectal cancer patients with history of IBD found no significant difference in cancer treatment modalities between patients with or without history of IBD. This observation points out that a history of IBD was not a barrier to receive radiotherapy treatment in this patient group[116].

It has been postulated that co-morbid IBD and intestinal inflammation may alter the acute tissue response to radiotherapy through inflammatory mediators, growth factors and cytokine cascades produced at the site of intestinal injury. Some mediators and cytokines were suggested to decrease the sensitivity to radiation injury e.g., fibroblast growth factor 2, prostaglandin-E2, tumor necrosis factor-α and interleukin (IL)-1 and IL-11. However, others were suggested to have mixed effects e.g., IL-12 protecting bone marrow-derived cells but sensitizing intestinal epithelial cells to radiation injury[117-123].

Collagen vascular diseases: Collagen vascular diseases (CVD) increases the risk of both acute and chronic radia-

tion toxicity, as has been reported by Chon *et al*[105] in 4 different studies in patients with and without CVD. On the other hand, radiation may cause an acute exacerbation of systemic symptoms in patients with CVD[124], possibility through release of fibroblast-triggering mediators by the inflammatory cells[105].

Human immunodeficiency virus infection: It has been reported that human immunodeficiency virus (HIV) infection induces a state of radiosensitivity because severe mucositis was observed in HIV patients who received radiotherapy for the treatment of Kaposi sarcoma[125,126]. Support for this hypothesis was found by an increased radiosensitivity of skin fibroblasts of HIV patients with Kaposi sarcoma compared to healthy control[127]. It was also noted in a study involving 59 HIV positive patients that T-lymphocytes of HIV infected individuals were considerably more sensitive to X-rays compared to that of HIV negative donors[128]. Housri *et al*[129] reviewed the recent evidence and suggested recommendations for radiotherapy in HIV patients, based on the strength of the best available evidence, and classified according to Strength of Recommendation Taxonomy. There was no conclusive evidence to support the need for special precautions for HIV patients during radiotherapy[130].

Genotypic variations

It has been suggested that patient's genotype may impact their individual susceptibility to radiation toxicity. This can occur through inherited germ-line mutations in genes involved in DNA damage detection, DNA repair or cell cycle regulation[131-133]. Recently the term Radiogenomics has been introduced to refer to the science that aims to predict clinical radiosensitivity and to optimize radiotherapy treatment from individual genetic profiles[134].

Genetic variations are thought to be a key determinant of normal tissue radiosensitivity and may account for up to 80% of the inter-individual variations in normal tissue reaction to radiotherapy[135,136]. Support for this hypothesis was provided in a study of breast cancer radiotherapy, which reported the incidence and time to development of radiation-induced telangiectasia[137]. The results of the study revealed a wide range of variation suggesting that patient-related factors can explain 81%-90% of the patient-to-patient variation in telangiectasia level seen after radiotherapy despite similar radiation treatment given. The results further supported reports of other studies[138,139].

The state of extreme tissue radiosensitivity which has been identified in patients with germ-line mutations in genes involved in DNA damage detection or DNA repair e.g., Nijmegen breakage syndrome, Fanconi's anemia and Ataxia telangiectasia has supported the potential role of genetic variations as an important determinant of individual's radiation response. Nevertheless, this risk is probably confined to patients and carriers of those mutant genes and is not known to be relevant to other patients receiving radiotherapy[31,45,140,141].

Candidate gene studies, [single nucleotide polymorphism (SNP) association studies] have investigated the role of many genes which have been linked to different elements of the mechanisms related to the pathogenesis of radiation toxicity. Genes investigated include those involved in DNA repair such as *ATM*, *BRCA1*, *BRCA2*[142,143], apoptosis such as *TP53, BCL2*[144,145], antioxidant enzymes such as *SOD1*[146], and growth factors *FGF2*[147,148] and *VEGF*[147,148]. In this regard, an association has been suggested between candidate SNPs in the genes *TGFB1*, *SOD2*, *XRCC3*, *XRCC1* and late radiation toxicity in breast cancer patients[133,149]. Similarly, SNP association studies in pelvic tissue have suggested correlations between some risk genes such as *XRCC1*, *XRCC3*, *TGFB1*, *OGG1* and an increased risk of developing late gastrointestinal and genitourinary radiation toxicity following radiotherapy[150-152].

MECHANISMS OF RADIATION INJURY

Ionising radiation induces double strand breaks in DNA. This triggers activation of a signalling pathway that leads to activation of tumour suppressor p53. Depending on the extent of the DNA damage, which depends on the radiation dose, and on other factors in the cellular milieu, p53 activation leads to cell cycle arrest and DNA double strand break repair, or apoptosis. In cancer radiotherapy, apoptosis of tumour cells is the desired outcome. However, intestinal crypt epithelial cells are quite sensitive to radiation and the killing of these cells leads to mucosal injury[153,154]. Specifically, when the dose of radiation is sufficient to kill all of the epithelial stem cells in a crypt, then as the epithelial cells migrate up the crypt and are eventually shed into the intestinal lumen; the crypt cannot be repopulated with epithelial cells, and consequently involutes. When this happens to a large proportion of crypts in a region of intestine, normal barrier function is lost which leads to the exposure of the normally sterile lamina propria to luminal microbes. This triggers an acute inflammatory response associated with immune cellular infiltrates; T lymphocytes, macrophages and neutrophils causing loss of epithelial cells as well as degradation of the extracellular matrix in the lamina propria due to enzymes and mediators released by the immune cells[155]. A further damage to the mucosal and submucosal tissues are caused by reactive oxygen metabolites which are produced in large amounts by activated leukocytes in the inflamed mucosa and this can induce significant damage to various cellular components, including structural and regulatory proteins, carbohydrates, lipids, DNA, and RNA[31].

During radiotherapy, ionising radiation kills crypt epithelial stem cells. As a result, crypts involute and epithelial barrier integrity is lost. This provides access of luminal microbes and their products to innate immune cells in the lamina propria, with activation of immune cells. An impaired recognition of bacterial translocation can further exacerbate the inflammatory process and promote

stricture formation by two possible mechanisms. The bacterial wall antigens could causes a secondary excessive up regulation of pro-inflammatory transcription factors, such as nuclear factor kappaB[156]. This might be followed by prolonged macrophage activation and induction of NADPH oxidase expression[157] leading to a further increased in oxygen radical secretion to eradicate bacteria leading to further tissue destruction[155]. Meanwhile translocated bacteria could directly stimulate neighboring mesenchymal cells via pattern recognition receptors leading to increased activation of immune cells[156,158].

The acute inflammatory process continues but eventually, after the cessation of radiation, through poorly understood mechanisms, crypts start to regenerate and this restores normal epithelial barrier function, which is followed by resolution of inflammation[159]. In some patients this inflammatory process becomes exaggerated for unknown reasons with severe ulceration and inflammatory process runs a chronic course characterised by extensive fibrosis and intestinal ischemia[160,161]. Recent observations in animal models of radiation injury indicate that repair after radiation may depend on the recruitment of mesenchymal stem cells from the bone marrow to the site of radiation injury. Mesenchymal stem cell mobilization and engraftment is thought to be induced by cytokines and potentially specific homing induced by chemokines, all of these are released by inflammation[162,163].

The final pathological outcome of the radiation injury in the intestinal tissue will depend on a complex crosstalk between various cellular components of the tissue within the extracellular matrix which eventually determine the process of tissue recovery or long term complications[73,164].

Radiation injury to the vascular compartment is thought to be a key feature in the pathological processes of intestinal radiation injury as well as an important determinant of both acute and chronic effects after radiotherapy[165,166]. It has been regarded as a major component in the initiation, progression and maintenance of delayed intestinal tissue damage and enhanced fibrosis which lead to loss of mucosal function and stricture formation[167,168].

Endothelial cell apoptosis has been implicated as the primary lesion leading to epithelial stem cell dysfunction and subsequent intestinal tissue damage following radiotherapy. Support for this hypothesis was found by identifying a state of radioresisitance following inhibition of endothelial apoptosis in experimental mice. Radiation-induced crypt damage, organ failure, and death from radiation injury were all prevented when the endothelial apoptosis was inhibited pharmacologically, by the administration of fibroblast growth factor or genetically by deletion of the acid sphingomyelinase gene[169]. However, subsequent studies challenged this concept by demonstrating an epithelial cell apoptosis at lower radiation doses which is insufficient to cause endothelial cell death. This result has been enforced further by experiments using high dose Boron therapy radiation, specifically targeted to the endothelium. The results showed no effect on epithelial stem cell survival[168,170].

Formation of new blood vessels (angiogenesis) is a crucial requirement for tumour growth and survival[171]. The tumour vasculature is prone to hypoxia which results in further production of proangiogenic factors by tumour cells. Angiogenesis inhibitors target tumour endothelial cells and cause inhibition of new vessel formation and a transient tumour hypoxia[172,173]. Although tumour hypoxia has been linked to increasing tumour radio-resistance, studies have shown that the administration of angiogenesis inhibitors improves tumour oxygenation and response to radiotherapy[174-177]. Different mechanism has been suggested for the radio-sensitising effect of combining angiogenesis inhibitors with radiotherapy[173]. Mazeron *et al*[178] has recently reviewed the clinical trials on angiogenesis inhibitors, but despite the promising value of these new agents, the biological basis for their synergistic effect and the safety and efficacy of these agents are still to be determined.

CONCLUSION

Intestinal radiation injury is a significant clinical issue which is expected to increase in prevalence due to improved survival of cancer patients as well as to increased availability of radiotherapy as an affordable treatment option. Radiotherapy treatment can cause a wide variety of gastrointestinal side effects. Following radiation injury to the gastrointestinal tract, symptoms can present acutely or after a long period of time. Although severe intestinal injury is less common with the development of advanced radiotherapy planning and delivery techniques, a less severe degree of injury is common and continues to affect a considerable proportion of patients, significantly reduces their quality of life, ands an extra burden to the cost of health care. The accurate diagnosis and management of intestinal radiation injury represents a clinical challenge to practicing physicians in both gastroenterology and oncology. Despite the growing recognition of the problem and some advances in understanding the cellular and molecular mechanisms of radiation injury, relatively little is known about the pathophysiology of intestinal radiation injury or the exact factors that aggravate it. Patient and treatment related risk factors have been suggested although the exact influence posed by these factors is still to be better characterized. Combined modality therapies for cancer are commonly used and they increase the risk of radiation toxicity and further add to the problem. Medical co-morbid diseases such as vascular disease, inflammatory bowel disease and collagen vascular disease can pose a significant risk that can affect patient suitability to receive radiotherapy treatment. Genotypic variations can influence the risk of gastrointestinal radiation injury but future research findings on this area are needed to assess their clinical importance. A better understanding of the pathophysiology of radiation injury may provide the opportunity to develop more effective preventive and therapeutic strategies.

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REVIEW

Gastrointestinal radiation injury: Prevention and treatment

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Abstract

With the recent advances in detection and treatment of cancer, there is an increasing emphasis on the efficacy and safety aspects of cancer therapy. Radiation therapy is a common treatment for a wide variety of cancers, either alone or in combination with other treatments. Ionising radiation injury to the gastrointestinal tract is a frequent side effect of radiation therapy and a considerable proportion of patients suffer acute or chronic gastrointestinal symptoms as a result. These side effects often cause morbidity and may in some cases lower the efficacy of radiotherapy treatment. Radiation injury to the gastrointestinal tract can be minimised by either of two strategies: technical strategies which aim to physically shift radiation dose away from the normal intestinal tissues, and biological strategies which aim to modulate the normal tissue response to ionising radiation or to increase its resistance to it. Although considerable improvement in the safety of radiotherapy treatment has been achieved through the use of modern optimised planning and delivery techniques, biological techniques may offer additional further promise. Different agents have been used to prevent or minimize the severity of gastrointestinal injury induced by ionising radiation exposure, including biological, chemical and pharmacological agents. In this review we aim to discuss various technical strategies to prevent gastrointestinal injury during cancer radiotherapy, examine the different therapeutic options for acute and chronic gastrointestinal radiation injury and outline some examples of research directions and considerations for prevention at a pre-clinical level.

Key words: Radiation enteritis; Radiation proctitis; Prevention; Treatment; Gastrointestinal radiation injury

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INTRODUCTION

External beam radiotherapy is a common treatment modality for wide varieties of cancers. Approximately 70% of all cancer patients receive radiotherapy during the course of their disease, while radiotherapy plays a central role in 25% of all cancer cures[1-2]. Radiotherapy is a cost effective treatment, accounting for only 5% of the total cancer care expenditure[3]. This estimate has increased over the last decade with the wide use of modern technological innovations in simulation, delineation, dose calculation and radiation treatment delivery[4].

Gastrointestinal radiation injury is an important problem for two main reasons. First, it causes morbidity associated with a significant economic burden[5] and a significant reduction in patient's quality of life[6,7]. Second, it limits the dose of radiation that can be used to control cancer, as radiotherapy-related side effects often limit the tolerability for patients. Clinical manifestations of gastrointestinal

radiation injury can present acutely during or soon following radiotherapy due to acute mucosal injury and inflammation or they can present insidiously within few months or years after radiotherapy due to a chronic process of transmural fibrosis and vascular sclerosis.

Radiation toxicity to the gastrointestinal tract can be reduced by either of two strategies: technical strategies, which aim to physically shift the radiation dose away from the normal tissues or through biological strategies which aim to modulate the cellular and tissue response to ionising radiation.

TECHNICAL STRATEGIES FOR PREVENTION

Attention to detail regarding both radiotherapy planning and radiation delivery methods can minimise the risk of intestinal toxicity during pelvic radiotherapy. Multiple field planning and delivery can reduce the radiation dose to normal intestine as well as minimise the volume of small intestine exposed to radiation in the pelvis[8].

Physical manoeuvres and tissue expander techniques

The impact of different physical measures such as patient position during pelvic radiotherapy have been evaluated in a randomized trial of supine and prone positioning in patients undergoing conformal radiotherapy for prostate cancer. There were significant improvements for small bowel, rectal wall and bladder wall doses in the supine position[9].

Another study utilised combined manoeuvres to displace the small intestine from the pelvis during radiotherapy by bladder distension, lower abdominal wall compression with the patient in prone position using two-field and four-field planning radiographs of contrast-enhanced small bowel[10]. The results demonstrated in 50% of patients the four-field volume of pelvic small bowel was significantly less in the prone position than in the supine position. Similarly, other techniques such as the use of a "belly board" while the patient is in the prone position where the opening in the table allows the abdomen to fall below the level of the table, displaces the small bowel by the effect of the gravity during radiotherapy. These technique were found to reduce the volume of the small intestine within the pelvis by a mean of 66%[11].

Pre-treatment small bowel contrast studies can assess the location and mobility of the small bowel and hence, determine the optimum treatment position that could minimise the volume of small intestine within the pelvis[12]. Tissue expander techniques have been used to minimise the volume of exposed small bowel within the irradiation field during abdominal and pelvic external beam radiotherapy. Studies have shown a reduction of 50% in the risk of chronic intestinal complications with the use of intra-pelvic tissue expander[13]. Other techniques used to minimise the radiation dose to the rectum include transperineal injection of human collagen to increase the distance between the prostate gland and anterior rectal wall. The mean reduction in dose to the anterior rectal wall was 50% with no rectal toxicity reported[14].

Prophylactic surgical techniques

Various prophylactic surgical therapies were also employed to reduce the small intestinal exposure during pelvic radiotherapy such as insertion of biodegradable mesh slings, intra-pelvic breast prostheses or omentoplasty during operative resection whenever postoperative radiotherapy may be indicated[15-20]. Mesh slings were found to reduce the volume of small intestine exposed by 50%[21,22] while other techniques such as pelvic reconstruction, omentoplasty and transposition of the large bowel were found to reduce the volume of bowel at risk by 60%[22,23]. Prostate gland immobilization and rectal wall sparing has been suggested by the use of endorectal balloons during prostate cancer radiotherapy. Endorectal balloons are reported to be well tolerated by patients and showed a significant rectal wall sparing effect during three-dimensional conformal radiotherapy and intensity-modulated radiotherapy for prostate cancer[24,25].

Optimised planning and delivery techniques

A significant improvement in the safety of radiotherapy treatment has been achieved through the use of optimised planning and delivery techniques which aim to reduce the field size, focus the radiation beam into the lesion and minimise the volume of exposed surrounding tissues in the radiation field[10]. Multiple field arrangements may also reduce normal tissue toxicity during pelvic radiotherapy.

The dose-volume histogram is a plot of a cumulative dose-volume frequency distribution, that graphically summarizes the simulated radiation distribution within a volume of interest of a patient which would result from a proposed radiation treatment plan[26]. It provides a graphic display of a simulated radiation treatment plan and generates valuable information on the dose distribution within the volume of interest[27]. The dose-volume histogram is used to predict the development of radiation toxicity and to identify low and high risk patient groups[28]. Careful review of the dose-volume histogram of both the intended targets as well as the structures of avoidance constitutes a standard part of the assessment of the radiation treatment plans in modern radiation therapy.

Three-dimensional conformal techniques which comprise computerised tomography scanning simulation techniques and intensity modulated radiation therapy are associated with a reduced risk of normal tissue toxicity and allow a higher radiation doses to be used compared to conventional two dimensional methods[29-32]. Computerised tomography scan simulation techniques were found to reduce the volume of the bowel unintentionally irradiated by 5% compared to conventional radiotherapy[33].

Intensity modulated radiation therapy allows clear definition of both target lesions and surrounding normal tissues and hence the ability to apply two different inten-

sities of radiation (high and low) within the same treatment field[34,35]. It represents the ultimate combination of treatment planning and delivery which provides the most flexible delivery of radiation to complex targets while minimising radiation doses to surrounding critical structures. It does so by utilizing sophisticated planning algorithms such as inverse planning and multiple small beams of varying intensity, to optimally deliver the treatment. Intensity modulated radiation therapy was associated with a significant reduction in toxicity following pelvic radiotherapy in prostate cancer patients[36]. It was also associated with a reduction of 15%-18% in intestinal volume unintentionally irradiated compared to conventional two dimensional radiotherapy, and a 10%-13% reduction in intestinal volume unintentionally irradiated compared to three-dimensional computerised tomography simulation techniques[33]. Furthermore, intensity modulated radiation therapy has been found to reduce acute and late toxicity and maintains good long term quality of life even when high radiation doses are administered to prostate cancer patients[37-39]. In 208 head and neck cancer patients treated with intensity modulated radiation therapy it was found that intensity modulated radiation therapy was associated with reduced late toxicities without jeopardising local cancer control and overall survival[40]. The long-term quality of life has been compared among survivors of head and neck cancer patients whom were treated with either intensity modulated radiation therapy or three-dimensional conformal radiotherapy. The study results have shown that the early improvement in quality of life associated with intensity modulated radiation therapy was maintained and become more magnified over time[41].

Brachytherapy is another advanced technique in which the source of radiation is implanted within the malignant tissues (interstitial brachytherapy) or within a cavity in its immediate vicinity (intra-cavitary brachytherapy). The sources can be permanently inserted and emits their radiation over a prolonged period of time (low dose rate) or temporary and emit over a short period (high dose rate). In this fashion, a high radiation dose is limited to the target lesion sparing the surrounding normal tissues. Brachytherapy alone or in combination with external beam radiotherapy can reduce normal tissue toxicity without compromising treatment efficacy in prostate cancer[42,43]. Brachytherapy can be an option for patients with history of inflammatory bowel disease and it is well tolerated in low risk prostate carcinoma[44].

Combining target definition on a daily basis and adjusting the treatments to account for target motion is referred to as image guided radiotherapy techniques[45]. Stereotactic radiation therapy can focus an extremely narrow ionizing radiation beam on a small target from different directions using an immobilisation system. The high accuracy of this technique has enabled targeting a small lesions such as brain metastatic lesions[46]. Stereotactic radiation therapy uses a small number of high doses of radiation to target small lesions. It has been used in the treatment of early-stage non-small-cell lung cancer, prostate cancer, renal-cell carcinoma, and liver cancer, and in the treatment of oligometastases in the lung, liver, and spine[47]. Stereotactic radiation therapy is associated with high local control rates of cancer[48] and it was evaluated in the treatment of prostate cancer using an accelerated form of hypofractitionation through fewer but larger treatment fractions. The early results of this new technique suggest that it may induce an initial prostate specific antigen response similar to that seen with conventional fractionation radiotherapy but with fewer acute side effects. Disease control and chronic toxicity have not yet been fully evaluated[49]. The use of higher (often > 10 Gy) daily radiation doses in stereotactic radiation therapy needs to be carefully considered in view of the potential for more severe side effects[50]. The impact of the image guided radiotherapy techniques has been shown in a large study with 331 patients treated with high-dose intensity modulated radiation therapy with fiducial marker-based position verification for prostate cancer which allowed daily correction of the prostate position using the fiducial markers. The results showed that high dose was well tolerated with lower rate of acute toxicity, which provide possibilities for further dose escalation[37].

High-energy proton beams stop abruptly in the tissue at the end of their range and deposit most of their energy there, in this fashion, they provide dose distributions that are superior to X-rays when used in comparable beam configurations[51] In addition, they are more densely ionising along their treatment path, and therefore a higher quantum of energy to kill the cancer cell and high linear energy transfer. With proton beam radiation therapy a smaller volume of normal tissues is irradiated at high dose levels for most anatomic sites than is feasible with any photon technique[52]. Clinical studies have shown that proton beam therapy in patients with hepatocellular carcinoma has been shown to be effective, safe and well tolerable[53]. Proton beam therapy allows large tumour volumes to be irradiated to high doses without significant dose exposure to surrounding normal tissue which make it a promising modality for the treatment of large-volume tumours[54]. However, they have yet to show significant advantages in comparison with the conventional radiation therapy.

MEDICAL THERAPY FOR GASTROINTESTINAL RADIATION INJURY

Although technical strategies have achieved a significant degree of normal tissue protection during radiotherapy delivery, biological strategies may offer additional future promise. A wide variety of pharmacological agents, nutritional supplements, biological response modifiers, and dietary measures have been investigated for potential benefit to prevent or minimize the severity of intestinal tissue injury induced by ionising radiation exposure.

SUPPORTIVE TREATMENT

Patient selection for radiotherapy

Patient risk profile for developing radiation toxicity merits a careful consideration while assessing individual patient for radiotherapy treatment. Co-morbid diseases such as vascular disease, hypertension, diabetes mellitus and atherosclerosis[55], inflammatory bowel disease[56-58], collagen vascular disease[59] and human immunodeficiency virus (HIV) infection[60,61] have been linked to an increased risk of toxicity following external beam radiotherapy.

Nutritional status and effect of diet during radiotherapy

Patient general health and nutritional status during radiotherapy may affect outcomes. Measures to improve patient nutrition such as dietary counselling, oral supplements and intensive nutrition intervention have been associated with an improved outcome in terms of body weight, morbidity, and quality of life during and after radiotherapy[62,63]. McGough *et al*[64] reviewed the efficacy of nutritional intervention on bowel symptoms during pelvic radiotherapy in data from 2646 patients. They found no evidence base for nutritional interventions identified to mitigate bowel symptoms following radiotherapy. However, the role of probiotic supplements, low fat diet and elemental diet was recommended for further evaluation. Subsequent studies on elemental diet identified the difficulty of patients tolerance to large volumes of elemental diet regimen during radiotherapy period[65,66]. The effect of high-potency probiotic preparation VSL3 has been investigated in double-blind, placebo-controlled trial of 490 pelvic radiotherapy patients. The results showed more diarrhoea and daily bowel movements in the placebo group compared with VSL3 recipients[67].

Biological, chemical and pharmacological therapy

Biological, chemical or pharmacological interventions to ameliorate the effect of radiation injury on gastrointestinal tract can be categorized according to the time of administration with respect to radiation exposure. Agents administered prior to radiotherapy for normal tissue prophylaxis are described as radioprotectors. Agents administered during the course of radiotherapy to minimize the injury are called mitigators, while agents administered to ameliorate an established injury are called treatment[68].

The complexity of the pathophysiology of intestinal radiation injury along with the increasing prevalence of the problem has attracted significant research interest in exploring the effectiveness of many agents. Treatment of delayed radiation injury is challenging as it is often refractory to different therapeutic modalities[69].

At the pre-clinical level, there has been a significant interest to test the efficacy of different agents to modulate the biological process implicated in radiation induced tissue injury and normal tissue damage (Table 1).

At the clinical level, there are no formal trials to reliably assess the effectiveness of most of the suggested therapies; hence most of the evidence is obtained from small studies which are often from a single centre. In addition, the results reported in many studies are often directly related to the radiation toxicity scale in use and its sensitivity, specificity as well as to methods of interpretation and grading of symptoms reported by patients.

Topical therapy

Topical therapy for radiation proctitis has been tried with different agents. Local instillation of formalin has been reported to be effective in the treatment of severe haemorrhagic radiation proctitis[70]. Sodium butyrate enemas were reported to improve the acute symptoms of radiation proctitis with no impact on the incidence and severity of late proctitis[71]. Steroid enemas and other topical steroid preparations, commonly prescribed for other anorectal inflammatory conditions have also been in use for symptom relief in radiation proctitis, but without conclusive evidence of efficacy.

Hyperbaric oxygen was suggested to exert its therapeutic role in chronic radiation proctitis through induction of neovascularization that reverses tissue hypoxia[72,73] with trophic effect on vasculogenic stem cells[74]. Treatment of chronic radiation proctitis with hyperbaric oxygen has shown promising results in a large randomized double-blind trial with 120 patients with long term follow up. Hyperbaric oxygen therapy significantly improved the healing responses in patients with refractory radiation proctitis with enhanced bowel-specific quality of life[75].

A recent systematic review demonstrated the therapeutic benefit of hyperbaric oxygen based on the evidence and expert consensus opinion. The review demonstrated its efficacy in patients with radiation damage to the anus and rectum and refractory chronic radiation injury[73].

Anti-inflammatory agents

Anti-inflammatory agents were suggested to reduce the severity of acute intestinal inflammation following irradiation. 5 aminosalicylic acid (5-ASA) has been studied, but most have reported no benefit or even worse symptoms with the use of 5-ASA compared to placebo[76-79]. Sulphasalazine showed clinical improvement in symptoms in a case series of four patients[80]. Balsalazide has been suggested to improve proctitis and toxicity grade in patients undergoing prostate cancer radiotherapy, in a study of 27 patients[81]. Taken together, the clinical evidence on therapeutic role of 5-ASA are not sufficient to recommend routine use in radiation injury.

Antioxidants

Antioxidants were suggested to have cytoprotective effects by reducing cellular oxidative stress following radiation injury to intestinal tissue. A sustained therapeutic benefit after 4 wk therapy with vitamin E and C has been reported in a study involving twenty patients with chronic radiation proctitis[82]. More studies will be required to assess the therapeutic role of different antioxidant agents.

Table 1 Examples of putative intestinal radioprotectants tested in pre-clinical studies

Biological agent	Suggested effect/mechanism	Suggested radioprotection
Glutamine, arginine enriched diet[103]	Enhanced mucosal healing	Protective effect on rat intestine compared to control
Vitamin- E[104]	Reduction of oxidative stress	Protective effect on rat intestine compared to control
Captopril[105]	Inhibition of pro-inflammatory enzyme angiotensin-1-converting enzyme	Protective effect on mouse intestine compared to control
Rofecoxib[106]	Selective Inhibition of cyclooxygenases -2 enzyme	Protective effect on rat intestine compared to control
Clopidogrel[107]	Inhibition of platelets aggregation with reduced vascular sclerosis	Protective effect on rat intestine
Thalidomide[108]	Protection to microvascular bed	Attenuated injury to rat endothelial cells of micro vascular bed compared to control
Simvastatin[109]	Attenuate endothelial cell injury	Attenuated delayed intestinal injury in rats
Glucagon-like peptide-2 (GLP-2)[110]	Increased mucosal mass	Intestinal trophic and protective effect to rat intestine
Octreotide[111,112]	Modulation the inflammatory effects mediated by over expression of NFκB	Ameliorated inflammation and injury in rat intestine
Prostaglandin E-2[113]	Pro-proliferative and anti-apoptotic effect on intestinal epithelium	Increased crypts survival
Anti-Transforming growth factors beta receptor[114]	Biological inhibition of extracellular remodeling	Reduced intestinal injury and fibrosis compared to IgG treated control mice
Toll like receptor 5 agonist derived from Salmonella flagellin[115]	Activation of intestinal immune response *via* NFκB signalling pathway	Protective effect to mice intestine
Intestinal sterilisation[116] Germ-free raised mice	Reduced number of translocated bacteria, sepsis and inflammation	Reduced intestinal cell apoptosis
Probiotic bacteria[117] (*Lactobacillus species*)	Restored intestinal microbiota imbalance	Reduces intestinal damage, sepsis and death in rodents

Amifostine

Amifostine is a scavenger of reactive oxygen species. Its protective effects have been related to its ability to minimise the injurious effects of free radicals on intestinal cells[83]. Amifostine has been investigated for both systemic and topical routes of administration during radiotherapy. Intravenous amifostine administered daily before radiotherapy has been shown to reduce the incidence of radiation proctitis during pelvic radiotherapy[84,85]. Similarly, rectal suspension of amifostine in two doses (1 g in 18 patients and 2 g in 12 patients) administrated daily before radiation therapy for prostate cancer has resulted in significant improvement in acute and late bowel quality of life toxicity parameters, more noticeable with higher doses[86]. Despite the results of these studies and that of many others, the updated clinical practice guidelines for the prevention and treatment of mucositis published in 2007[87] concluded that data on amifostine are mostly obtained from small, single-centre studies with conflicting results. The group concluded that intravenous amifostine daily administration prior to radiotherapy may prevent radiation proctitis in patients who are receiving standard-dose radiotherapy for rectal cancer (Level Ⅲ evidence, grade B recommendation)[87].

In previous studies, amifostine has been shown to reduce the incidence of xerostomia in patients during head and neck radiotherapy[88]. The American Society for Clinical Oncology recommended its use in 2002 published guidelines[89]. However, the recommendation has been subsequently withdrawn in the guidelines update in 2008 due to an insufficient data[90].

Sucralfate

Protection of mucosal cells has been suggested with sucralfate (aluminium sucrose octasulfate). A beneficial effect of sucralfate has been previously reported in two double-blind placebo-controlled studies in patients who received pelvic radiotherapy. It reported that the frequency of defecation and stool consistency were improved by sucralfate[91,92]. Sucralfate enemas have been evaluated in 26 patients with radiation-induced proctitis with persistent rectal bleeding and the results showed a reduction in the severity of rectal bleeding in all patients with sucralfate enemas after four weeks treatment[93]. However, in another study the therapeutic effect of sucralfate rectal enemas were evaluated compared to placebo, both administered during the course of prostate cancer radiotherapy. The results showed no difference in the rate of rectal bleeding between sucralfate and placebo treated group after a median follow-up of five years[94]. The therapeutic benefits of oral sucralfate have also been assessed in a prospective randomised placebo-controlled study on fifty one patients receiving pelvic radiotherapy. Results from 44 study subjects showed significantly increased diarrhoea in the sucralfate group which led to cessation of the trial. The study concluded that sucralfate cannot be recommended for prophylaxis of acute radiation to the rectum and may even worsen the symptoms[95]. Similarly, the lack of effect of micronized sucralfate mouthwash has been reported in a randomized, controlled trial in radiation-induced oral mucositis patients[96]. Despite extensive investigation, clinical data on sucralfate have shown variable results, which do not support its protective role

for routine clinical use.

ENDOSCOPIC MANAGEMENT, BIOPSY AND LASER CAUTERY

A variety of endoscopic therapies have been tried for the treatment of chronic radiation injury such as the heater probe, bipolar electrocoagulation and argon plasma coagulation with newer methods of endoscopic ablation such as radiofrequency ablation and cryotherapy[97]. Endoscopic treatment has been used primarily aimed to treat bleeding rectal telengectasia. Argon plasma coagulation therapy has been shown to be effective and safe treatment for treatment of rectal bleeding resulting from chronic radiation injury[98,99]. Caution should be applied however when considering the use of these invasive techniques following high dose treatment delivery. For instance the use of rectal wall biopsy and laser cautery has been associated with the development of recto-urinary fistulae[100-102] in men treated with brachytherapy for prostate cancer.

CONCLUSION

Prevention of gastrointestinal toxicity starts with a thorough assessment of patient risk profile before radiotherapy. There is no prophylactic or therapeutic agent available to radiotherapy patients proven to mitigate the acute and chronic symptoms of gastrointestinal radiation injury or to allow safe radiation dose escalation for better control of cancer. Many therapeutic agents are in use, but often with little evidence base. The recent advances in radiotherapy planning and delivery techniques provide a considerable degree of protection to the gastrointestinal during external beam radiotherapy. Newer techniques such as image guided radiotherapy techniques, stereotactic therapy and proton beam therapy may confer additional protection. On the other hand, a better understanding of the pathophysiology of intestinal radiation injury will allow the development of more effective biological strategies that could increase the end organ resistance to radiation toxicity. In this regard, modulation of various cellular and cytokine pathways that have been implicated in the development of the acute pathological process of radiation injury can give future promise. In addition to targeting different mechanisms mediating chronic inflammatory and fibrotic process that underlie the delayed pathological changes in the gastrointestinal tract, such approaches may provide new therapeutics insights to this problem.

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ORIGINAL ARTICLE

Intestinal injury can be reduced by intra-arterial postischemic perfusion with hypertonic saline

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Abstract

AIM: To investigate the effect of local intestinal perfusion with hypertonic saline (HTS) on intestinal ischemia-reperfusion injury (IRI) in both *ex vivo* and *in vivo* rat models.

METHODS: All experiments were performed on male Wistar rats anesthetized with pentobarbital sodium given intraperitoneally at a dose of 60 mg/kg. *Ex vivo* vascularly perfused rat intestine was subjected to 60-min ischemia and either 30-min reperfusion with isotonic buffer (controls), or 5 min with HTS of 365 or 415 mOsm/L osmolarity ($HTS^{365mOsm}$ or $HTS^{415mOsm}$, respectively) followed by 25-min reperfusion with isotonic buffer. The vascular intestinal perfusate flow (IPF) rate was determined by collection of the effluent from the portal vein in a calibrated tube. Spontaneous intestinal contraction rate was monitored throughout. Irreversible intestinal injury or area of necrosis (AN) was evaluated histochemically using 2.3.5-triphenyltetrazolium chloride staining. *In vivo*, 30-min ischemia was followed by either 30-min blood perfusion or 5-min reperfusion with $HTS^{365mOsm}$ through the superior mesenteric artery (SMA) followed by 25-min blood perfusion. Arterial blood pressure (BP) was measured in the common carotid artery using a miniature pressure transducer. Histological injury was evaluated in both preparations using the Chui score.

RESULTS: *Ex vivo*, intestinal IRI resulted in a reduction in the IPF rate during reperfusion ($P < 0.05$ *vs* sham). The postischemic recovery of the IPF rate did not differ between the controls and the $HTS^{365mOsm}$ group. In the $HTS^{415mOsm}$ group, postischemic IPF rates were lower than in the controls and the $HTS^{365mOsm}$ group ($P < 0.05$). The intestinal contraction rate was similar at baseline in all groups. An increase in this parameter was observed during the first 10 min of reperfusion in the control group as compared to the sham-treated group, but no such increase was seen in the $HTS^{365mOsm}$ group. In controls, AN averaged 14.8% ± 5.07% of the total tissue volume. Administration of $HTS^{365mOsm}$ for 5 min after 60-min ischemia resulted in decrease in AN (5.1% ± 1.20% *vs* controls, $P < 0.01$). However, perfusion of the intestine with the HTS of greater osmolarity ($HTS^{415mOsm}$) failed to protect the intestine from irreversible injury. The Chiu score was lower in the $HTS^{365mOsm}$ group in comparison with controls (2.4 ± 0.54 *vs* 3.2 ± 0.44, $P = 0.042$), while intestinal perfusion with $HTS^{415mOsm}$ failed to improve the Chiu score. Intestinal reperfusion with $HTS^{365mOsm}$ in

the *in vivo* series secured rapid recovery of BP after its transient fall, whereas in the controls no recovery was seen. The Chiu score was lower in the $HTS^{365mOsm}$ group *vs* controls (3.1 ± 0.26 and 3.8 ± 0.22, $P = 0.0079$ respectively,), although the magnitude of the effect was lower than in the *ex vivo* series.

CONCLUSION: Brief intestinal postischemic perfusion with $HTS^{365mOsm}$ through the SMA followed by blood flow restoration is a protective procedure that could be used for the prevention of intestinal IRI.

Key words: Intestinal ischemia-reperfusion injury; Superior mesenteric artery; Perfusion; Hypertonic saline; Acute mesenteric ischemia; Intestinal perfusate flow rate

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INTRODUCTION

Acute mesenteric ischemia (AMI) is a life-threatening vascular emergency with a mortality rate as high as 60%-80%[1]. It can be caused by local arterial obstruction, impairment of venous outflow, or a systemic hemodynamic disorder resulting in severe intestinal hypoxic injury. The rather non-specific clinical presentation of AMI can be an obstacle to its early recognition, thus delaying the proper treatment, namely revascularization[2]. However, if AMI can be diagnosed unambiguously prior to the development of intestinal infarction, either embolectomy or the intra-arterial infusion of thrombolytic agents can materially improve the outcome[3]. Unfortunately, even after timely revascularization, the positive effect can be compromised, at least in part, by the development of reperfusion injury, including edema, the no-reflow phenomenon, and the progressive death of mucosal cells[4]. In experimental models, intestinal ischemia-reperfusion injury (IRI) has been shown to be alleviated by a variety of pre- and postconditioning procedures[5,6], while various antioxidants, anesthetics, vasodilators, anti-inflammatory agents, and cytokine receptor blockers can also be helpful[7,8]. Furthermore, the systemic administration of hypertonic saline (HTS) has given clearly positive results[9,10]. The protective effect of HTS in these experiments is believed to be based on the following mechanisms: arteriolar vasodilation, osmotic stress-induced inhibition of inflammatory cells, and prevention of intestinal edema[10-12]. It is to be noted that the application of either HTS or some of the above-mentioned drugs may have certain limitations, such as an increased risk of electrolyte imbalance[12] and the development of adverse drug reactions, in either case as a result of systemic administration. It has been hypothesized, therefore, that perfusion of the intestine with HTS through the superior mesenteric artery (SMA) might prove to be a more advantageous strategy, given its greater efficacy in delivering the hypertonic solution to the target.

The present study was an experimental investigation of the effect of local intra-arterial HTS administration during the early reperfusion phase on the functional and structural manifestations of intestinal injury. To the best of our knowledge, this approach has not yet been attempted.

MATERIALS AND METHODS

Chemicals and materials

2.3.5-triphenyltetrazolium chloride (TTC) was obtained from Sigma-Aldrich (St. Louis, MO, United States). Pentobarbital sodium was purchased from Apoteket (Umea, Sweden). Bovine serum albumin was obtained from Biowest (Nuaille, France). Dextran 60 000 was obtained from Biochemist (Saransk, Russian Federation). Other chemicals used for the preparation of both Krebs-Henseleit buffer and HTS were purchased from Acros Organics (Geel, Belgium).

Animals

All experiments were performed on male Wistar rats weighting 250-300 g. The animals were fed regular chow, and water was available *ad libitum*. The procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the local Ethics Committee. The animals were anesthetized with pentobarbital sodium given intraperitoneally at a dose of 60 mg/kg. Core body temperature was maintained at 37.0 ± 0.5 ℃ by a feedback-controlled heating pad (TCAT-2LV controller, PHYSITEMP Instruments Inc., United States).

Ex vivo experiments

Isolated perfused small intestine preparation: The intestine was isolated and perfused according to a previously described technique[13]. Briefly, the abdominal cavity was opened by a midline incision. To prevent blood coagulation, 50 units of heparin sodium were injected into the inferior vena cava. The abdominal aorta was exposed from the level of the celiac trunk (CT) to the aortic bifurcation. The intestinal segment between the pyloric sphincter and the distal portion of the ileum was cut using an electrocautery unit. The branches of the SMA that supply the cecum and proximal part of the colon were exposed, ligated, and cut, followed by the removal of the proximal colon. The intestinal lumen was rinsed with warm (37 ℃) saline. The aorta was cannulated through its infrarenal segment with subsequent advancement of the cannula tip to the SMA level. The aorta was then ligated immediately above the CT, and perfusion of the small

intestine with oxygenated (95% O_2 and 5% CO_2) Krebs-Henseleit buffer (KHB) was initiated through the SMA and CT at a pressure of 80 mmHg. KHB solution was modified to contain high molecular weight compounds (1.0% bovine serum albumin and 3.0% dextran 60 000) plus (all in mmol/L) 118.5 NaCl, 25.0 $NaHCO_3$, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.5 $CaCl_2$, 5.0 glucose, 2.0 lactate, 0.2 pyruvate. The portal vein was cannulated to ensure outflow of the perfusion fluid. After ligation of the splenic and gastric vessels, the exposed part of the intestine was placed into the water-jacketed bath filled with normal saline to maintain the temperature at 37 ℃ during the experiment. All surgical procedures described were performed within 50-60 min and were followed by a 20-min stabilization period.

Experimental protocol: After stabilization, ischemia of the intestine was induced by cessation of the perfusate flow for 60 min, followed by reperfusion. During the initial 5-min reperfusion, the perfusion was resumed with the modified KHB of either normal (physiological) or increased osmolarity (315 *vs* 365 or 415 mOsm/L, designated as $HTS^{365mOsm}$ or $HTS^{415mOsm}$, respectively), in each case delivered under a pressure of 80 mmHg, followed by perfusion with normotonic KHB for 25 min. The experiments were randomized and allocated to the following 4 groups**:** (1) Sham (n = 6): the intestine was continuously perfused for 110 min; (2) Controls (n = 6): 60-min intestinal ischemia, followed by 30-min reperfusion with normotonic KHB (osmolarity 315 mOsm/L); (3) $HTS^{365mOsm}$ (n = 5): 60-min intestinal ischemia, followed by 5-min reperfusion with HTS having an increased osmolarity of 365 mOsm/L (KHB supplemented with 50 mmol NaCl), followed by 25-min reperfusion with normotonic KHB; and (4) $HTS^{415mOsm}$ (n = 6): the same sequence of procedures as in the previous group, but using HTS with osmolarity increased to 415 mOsm/L.

Assessment of vascular intestinal perfusate flow rate: The vascular intestinal perfusate flow (IPF) rate was determined each 5 min during both the stabilization and reperfusion periods by collection of the effluent from the portal vein in a calibrated tube.

Determination of luminal intestinal flow: The outflow from the intestinal lumen was determined in all experiments during the stabilization period. Luminal flow exceeding 0.5 mL/min is indicative of pathological intestinal secretion, which is usually due to ischemic injury of the intestine during surgical manipulations. Thus, the experiments with luminal flow exceeding 0.5 mL/min were excluded from the final analysis.

Histochemical analysis: We used the technique of visualizing the area of necrosis (AN) with 2.3.5-triphenyltetrazolium chloride (TTC), a method generally considered to be a "gold standard" for AN delineation in the heart in other studies, including our own[14,15]. Briefly, immediately after the end of reperfusion into the SMA, 10 mL of 0.5% TTC solution were administered for 30 s. Fifteen minutes after that, 3 equally spaced segments of the intestine with a total length of 3 cm were excised and fixed in 4% buffered formaldehyde solution for 24 h at room temperature. The samples were cut into 7-8 transverse slices, each 1.5 mm thick, which were photographed for further analysis using Adobe Photoshop CS. TTC-positive tissue, brick red in color, was considered to be viable, in contrast to the necrotic tissue, which had a pale appearance. The AN volume in each sample was calculated by multiplying the TTC-negative area by the slice thickness. The AN volume of the whole intestinal segment was calculated by summing the AN volumes of the slices and was expressed as the percentage of AN in relation to the total volume of the intestinal tissue being analyzed.

Evaluation of spontaneous intestinal motility: According to the original technique described by Hoffman *et al*[16], this parameter is assessed by recording the movement of fecal pellets down the intestine. We used a different approach to register the spontaneous intestinal contraction rate. In brief, a pair of soft rubber rings, hand-crafted from a black tube with a diameter close to that of the intestine, were cut at one point and carefully placed around the intestine to signify equally spaced anatomical locations throughout the experiments. Sixty-second video recordings (5 frames per second) were taken every 10 min during the stabilization and reperfusion periods and were digitized with VirtualDub software (Avery Lee, United States). Based on the distances between the rings, the values were plotted against time, followed by a Fourier transform and probability plotting of the frequency distribution. The data were presented as the rates of low-frequency intestinal contractions (Hz).

In vivo experiments

For the investigation of the effect of HTS perfusion on the intestinal IRI, we developed a technique for perfusion through the SMA, which, in our opinion, offers some advantages over the systemic administration of HTS used before. The infrarenal aorta was occluded with a microclip and cannulated with an 18G polyethylene cannula (Figure 1A). The micro clip was then removed and placed on the aorta between the SMA and the CT, after which the cannula was advanced up to the SMA level, where its tip was fixed by ligation (Figure 1B). The next step included 2 manipulations: the occlusion of the distal part of the superior mesenteric vein (SMV) and the incision of the ileocecal SMV branch to secure the outflow of the perfusate from the intestinal circulation. The oxygenated HTS maintained at 37 ℃ was delivered through the SMA with an infusion pump (SPACE infusion system, B. Braun, Germany) at a constant rate of 15 mL/min. The flow rate was selected on the basis of our pilot experiments showing that the mean blood flow in the SMA of animals of comparable weight, having blood pressure (BP) in the range between 100 and 120 mmHg,

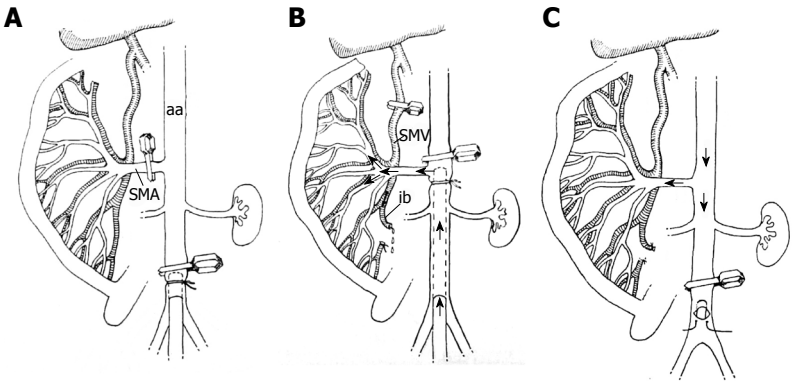

Figure 1 The sequential steps of the intestinal perfusion *in vivo*. A: The abdominal aorta (aa) is occluded by a clip, followed by cannulation of the vessel; the superior mesenteric artery (SMA) is clipped; B: The aortic clip is positioned above the SMA; the cannula is positioned at the level of SMA to allow its perfusion with hypertonic saline; the perfusate outflow is secured through the opened ileocecal branch (ib) of the superior mesenteric vein (SMV); the main trunk of SMV is clipped; C: The cannula is removed to allow blood flow through the SMA to resume simultaneously with the following procedures: the aortic clip is positioned as in Figure 1A; the clip occluding the SMV is removed, and bleeding from the ileocecal branch of the SMV is prevented by ligation.

was approximately 10% higher than 15 mL/min. Any appearance of blood in the perfusate was checked by visual inspection. At the end of the perfusion, the ileocecal SMV branch was ligated and the main trunk of the SMV was reopened. The aortic cannula was removed and the aortic incision closed with a suture (Figure 1C), so that blood flow was restored in both the aorta and the mesenteric vascular bed.

Experimental protocol: The animals were randomly allocated into 2 groups: (1) Controls (n = 6): intestinal ischemia by SMA occlusion for 30 min, followed by 30-min reperfusion with blood; and (2) HTS^{365mOsm} (n = 5): intestinal ischemia by SMA occlusion for 30 min, followed by 5-min reperfusion with HTS^{365mOsm}, followed by 25-min reperfusion with blood.

Blood pressure measurement: Arterial BP was measured in the common carotid artery using a miniature pressure transducer (Baxter, United States), to be monitored using PhysExp X4 software (Cardioprotect Ltd., St. Petersburg, Russian Federation). BP values were registered under the following conditions: at baseline, prior to SMA occlusion, 15 and 30 min after SMA occlusion, and at the 15th and 30th min of reperfusion.

Histological studies: At the end of reperfusion, intestinal samples were taken and routinely fixed in 10% buffered formaldehyde solution, embedded in paraffin, cut into 5 μm sections, and stained with hematoxylin and eosin. The slides were analyzed under a light microscope by the pathologist blinded to the treatment mode used in each group. The results were scored from 0 to 5 according to the scale of Chiu *et al*[17]. The method is based on the well established relationship between the grade of intestinal injury, if any, and its structural manifestation (grade 0: normal villi; grade 1: development of subepithelial space; grade 2: moderate lifting of subepithelial layer; grade 3: massive epithelial lifting with a few tips denuded; grade 4: denuded villi with lamina propria and dilated capillaries exposed; grade 5: digestion and disintegration of lamina propria with ulceration). In the present study, we rigorously followed this gradation as applied in similar trials by several groups[10,18,19].

Statistical analysis

Data were analyzed with SPSS 11.0 (SPSS Inc. Software, Chicago, IL, United States). All values are expressed as mean ± SD. Differences in the intestinal motility and IPF rate were tested by repeated-measures analysis of variance, followed by a Tukey post-hoc test. The non-parametric Mann-Whitney U test was used to determine the inter-group differences in AN value and histopathological score. $P \leqslant 0.05$ were considered significant.

RESULTS

Ex vivo experiments

Exclusions: Two animals were excluded from the final analysis (one in the control group and one in the HTS^{415mOsm} group) because the luminal flow exceeded 0.5 mL/min.

Intestinal perfusate flow rate: There were no inter-group differences in the IPF rate during the stabilization period (Figure 2). The IPF values remained unchanged over the entire 110-min perfusion period in the sham-treated group. In contrast, intestinal ischemia-reperfusion resulted in a reduction in the IPF rate during reperfusion (P < 0.05 *vs* sham). This reduction could have been due to IRI in the intestinal microcirculatory bed, resulting in no-reflow. The postischemic recovery of the IPF rate did not differ between the controls and the HTS^{365mOsm} group. In the HTS^{415mOsm} group, postischemic IPF rates were lower than in the controls and the HTS^{365mOsm} group (P < 0.05).

Area of necrosis: TTC-negative intestinal tissue was not present in the samples obtained from the sham-treated group. In the controls, AN averaged 14.8% ± 5.07% of the total tissue volume within the analyzed intestinal seg-

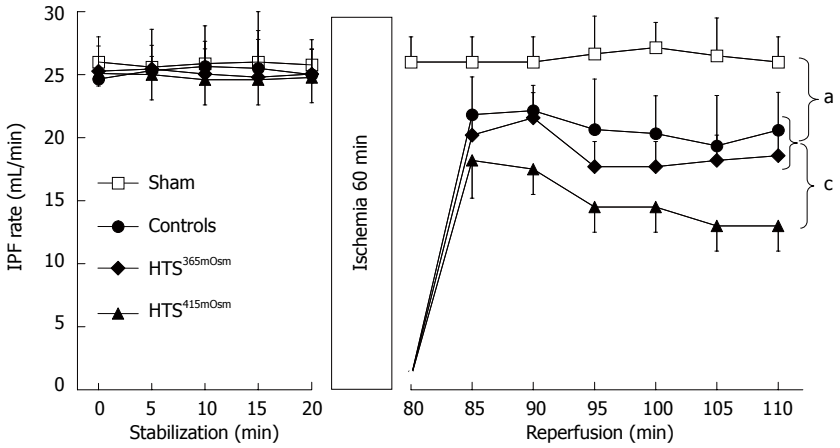

Figure 2 Intestinal perfusate flow rate in the *ex vivo* rat experiments with 60-min ischemia followed by varying conditions of reperfusion. The groups tested were: sham-operated (no ischemia at all, $n = 6$); controls (30 min reperfusion with normotonic buffer, $n = 5$); 2 groups with either HTS^{365mOsm} ($n = 5$) or HTS^{415mOsm} ($n = 5$), both subjected to 5-min reperfusion with the respective hypertonic solution followed by 25 min reperfusion with normotonic buffer. The pre-ischemic values of Intestinal perfusate flow (IPF) rate were similar in all groups. No changes were apparent in the shams over the whole period, whereas IPF values were lower in all the ischemia-reperfusion groups, although the groups showed different patterns. HTS^{415mOsm} resulted in the most prominent decrease in the IPF rate, with a further progressive fall to the end of the experiment. In contrast, IPF rate was less affected in the control and HTS^{365mOsm} groups, to a similar degree in both, while an appreciable degree of stabilization took place towards the end of the postischemic period. Postischemic IPF rate recovery did not differ between the controls and the HTS^{365mOsm} group, whereas postischemic IPF rate values were significantly lower in the HTS^{415mOsm} group, compared to both the controls and the HTS^{365mOsm} group. Data are mean ± SD. $^{a}P < 0.05$ *vs* sham; $^{c}P < 0.05$ *vs* controls.

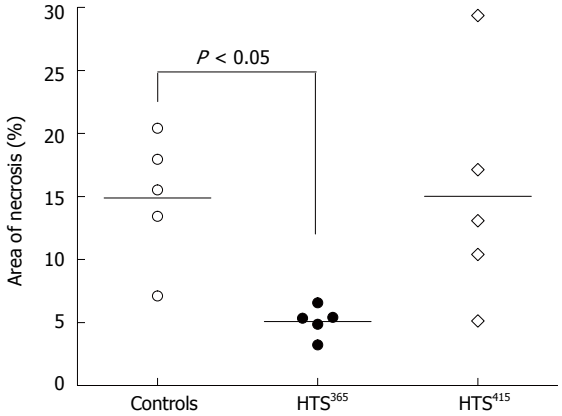

Figure 3 Area of necrosis within the isolated rat intestine after 60-min ischemia followed by the varying conditions of reperfusion. The area of necrosis (AN) is defined as the percentage of 2.3.5-triphenyltetrazolium chloride-negative in relation to the total intestinal tissue volume. Controls (reperfusion with normotonic saline) and the HTS^{415mOsm} group did not differ statistically regarding AN volume, whereas HTS^{365mOsm} administration resulted in a significantly lower value of AN. In both hypertonic saline (HTS) groups, the 5-min reperfusion with the appropriate hyperosmotic saline was followed by 25-min normotonic reperfusion. The data are presented as dot plots with median values.

ment (Figure 3). Administration of HTS^{365mOsm} for 5 min after 60-min ischemia resulted in decrease in AN (5.1% ± 1.20% *vs* controls, $P < 0.01$). However, perfusion of the intestine with the HTS of greater osmolarity (HTS^{415mOsm}) failed to protect the intestine from irreversible injury (AN = 15.0% ± 9.12% *vs* controls, $P > 0.05$).

Intestinal motility: The rate of intestinal contraction-relaxation pulses, which was used as a measure of intestinal motility in the present study, was similar at baseline in all groups (Figure 4). An increase in this parameter was observed during the first 10 min of reperfusion in the control group as compared to the sham-treated group, but no such increase was seen in the HTS^{365mOsm} group.

Histological data: Histological data from the intestinal samples obtained after 30-min reperfusion *ex vivo* are shown in Figure 5A. Sham-treated animals had normal or near-normal histology. The Chiu score was lower in the HTS^{365mOsm} group in comparison with controls (2.4 ± 0.54 *vs* 3.2 ± 0.44, $P = 0.042$, Figure 5A), while intestinal perfusion with HTS^{415mOsm} failed to improve the Chiu score (3.6 ± 0.54 *vs* controls, $P > 0.05$).

In vivo experiments

Exclusions: Although the experimental model suggested is highly invasive and complex, the extensive training prior to the beginning of the main experimental series has minimized the number of animals excluded due to technical reasons. Only one rat was excluded in the control group because of the severe bleeding and low BP.

Blood pressure values during intestinal perfusion *in vivo*: Baseline BP values did not differ between the controls and the HTS^{365mOsm} group (Table 1, Figure 6). SMA occlusion did not cause appreciable changes in BP level in either group. Aortic occlusion during the 5-min SMA perfusion period in the HTS^{365mOsm} group resulted in a trend to BP elevation. Reinstitution of blood flow in the aorta caused a temporary decrease in BP that lasted for only 2-3 min before it returned to the baseline values (Table 1). In contrast, SMA reperfusion in controls caused a decrease in BP level that persisted during the entire reperfusion period (Table 1, Figure 6A).

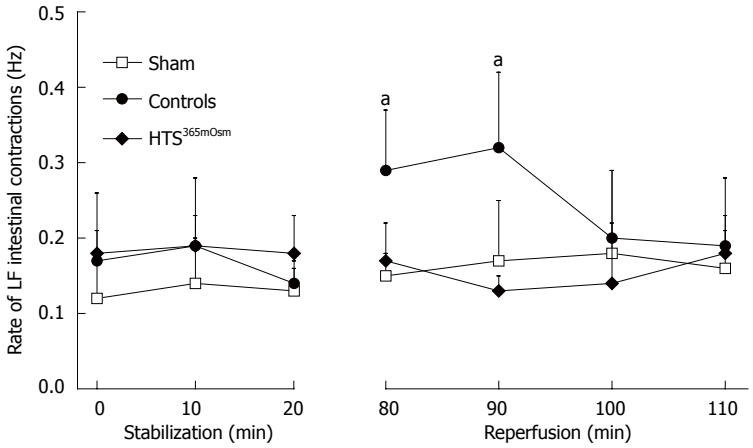

Figure 4 The rate of low frequency intestinal contractions in the *ex vivo* experiments. In shams, the intestine was continuously perfused for 110 min. In controls (60-min ischemia followed by 30-min reperfusion), intestinal contraction rate was significantly higher over the initial 10 min of reperfusion in comparison with the shams. Intestinal reperfusion with hyperosmotic saline in the HTS^{365mOsm} group prevented the increase in postischemic intestinal contraction rate. Data are mean ± SD. $^{a}P < 0.05$ *vs* sham. LF: Low frequency.

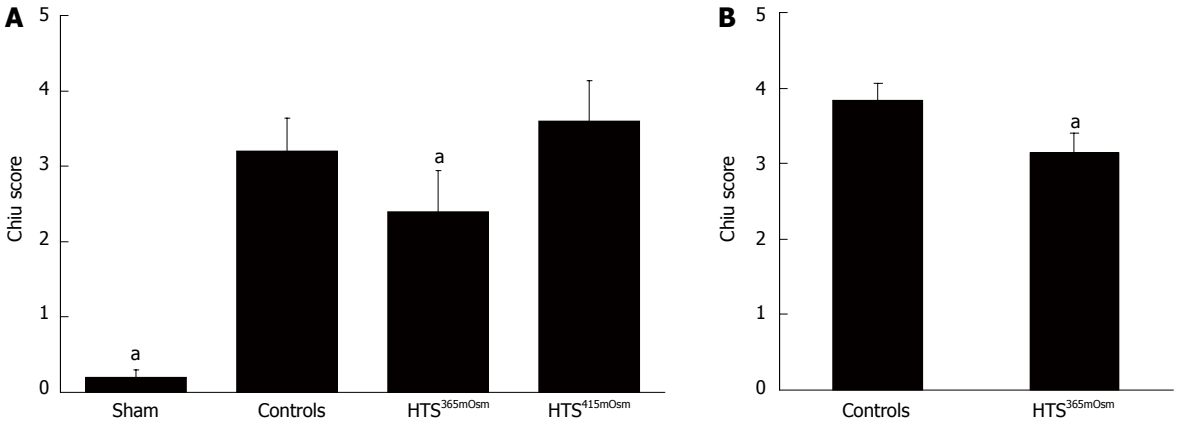

Figure 5 Histologically defined scores in the *ex vivo* (A) and *in vivo* (B) experiments. A, B: In the sham group (no ischemia at all, A) slight injury only is evident, probably due to the prolonged perfusion in the extracorporeal environment. Sixty-minute ischemia followed by 30-min normotonic reperfusion (controls) resulted in a significant injurious effect, which, however, was substantially less expressed in the HTS^{365mOsm} but not in the HTS^{415mOsm} group. The analogous difference between the controls and the HTS^{365mOsm} group is apparent in the *in vivo* experiments (B). The data are expressed as mean ± SD; $^{a}P < 0.05$ *vs* controls.

Table 1 Arterial blood pressure (mmHg) in the *in vivo* experiments (mean ± SD)

	Controls (n = 5)	HTS^{365mOsm} (n = 5)
Baseline	125 ± 8	132 ± 12
Before SMA occlusion	127 ± 10	128 ± 9
SMA occlusion		
15 min	118 ± 11	125 ± 7
30 min	121 ± 9	123 ± 6
SMA reperfusion		
15 min	68 ± 12	118 ± 13[a]
30 min	74 ± 8	126 ± 11[a]

SMA: Superior mesenteric artery. $^{a}P < 0.05$ *vs* controls.

Histology: Histological data from the intestinal samples obtained after 30-min reperfusion *in vivo* are shown in Figure 5B. The Chiu score was lower in the HTS^{365mOsm} group *vs* controls (3.1 ± 0.26 and 3.8 ± 0.22, $P = 0.0079$, respectively, Figure 5B), although the magnitude of the effect was lower than in the *ex vivo* series.

DISCUSSION

The results of this study indicate that the technique of isolated *in vivo* vascular perfusion of the intestine is reproducible and reliable. Using this approach, intestinal perfusion with HTS^{365mOsm} over the initial 5 min of reperfusion resulted in significant protection of the intestine from IRI, as demonstrated using morphological data (per both TTC and Chiu *et al*[17]). In order to evaluate both the experimental and also the possible clinical implications of this strategy, it seems appropriate to review in detail the relevant findings from systemic HTS administration. A salvation effect on the intestinal IRI, as well as limitation of remote organ injury, were observed in rats that were subjected to 60-min SMA occlusion followed by 6-h reperfusion, when intravenous administration of HTS was performed during the last 5 min of ischemia[10]. In parallel, an improvement in intestinal transit time, a reduction in mucosal injury, and limitation of intestinal myeloperoxidase activity were demonstrated using the same model; all effects were considered to be dependent on heme oxygenase-1 activity[18]. In addition, HTS infusion performed within the first 5 min of reperfusion after 120 min of SMA occlusion in pigs resulted in a better recovery of both mean arterial blood pressure and intestinal microvascular perfusion in comparison with conventional isotonic resuscitation[9]. In a somewhat different rat mod-

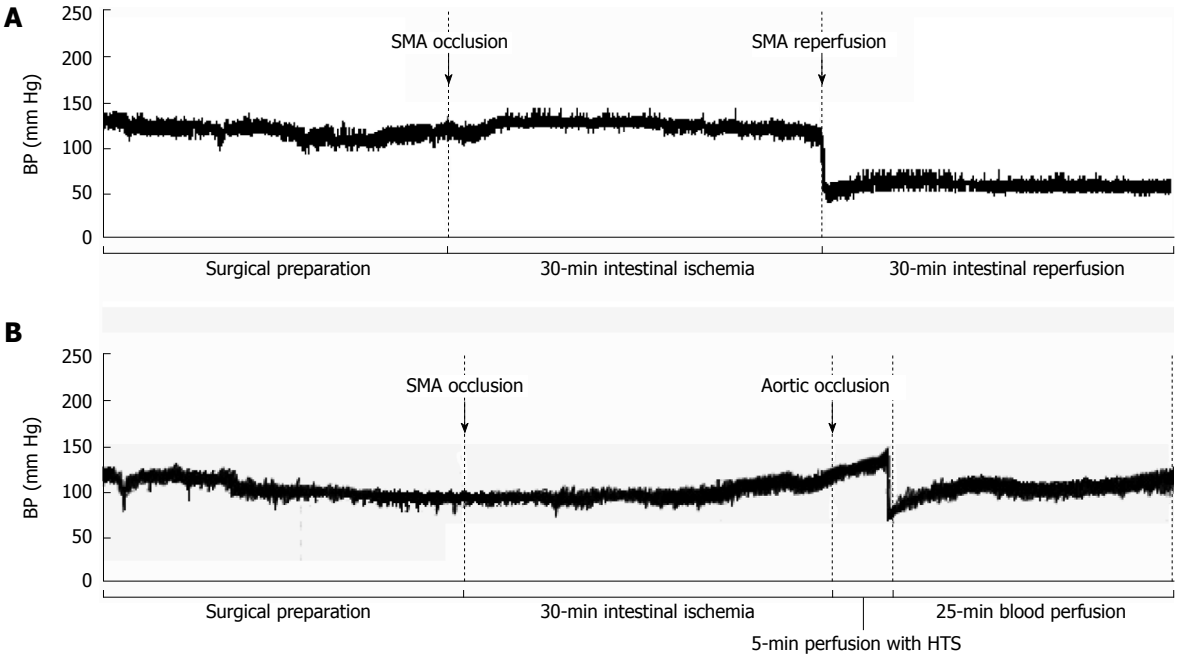

Figure 6 Representative samples of arterial blood pressure recordings in anesthetized rats subjected to intestinal ischemia-reperfusion *in vivo*. A: Thirty-minute superior mesenteric artery (SMA) occlusion followed by 30-min reperfusion (controls) resulted in a persistent blood pressure (BP) decrease over the entire reperfusion period; B: Thirty-minute SMA occlusion finalized by aortic clamping followed immediately by 5-min SMA perfusion with hypertonic saline (365 mOsm/L), with a subsequent 25-min reperfusion with blood, resulted in a transient drop with a rapid restoration of BP up to the initial values. HTS: Hypertonic saline.

el of intestinal IRI, this time induced by hemorrhagic shock followed by blood reinfusion, the administration of HTS for 25 min after the return of shed blood into circulation resulted in better post-resuscitation blood flow and improved endothelial function of the intestinal vasculature[12]. It also suppressed the apoptotic death of intestinal epithelial cells[20] and prevented intestinal mucosal injury[21]. As to the remote effects of systemic HTS treatment, a more complete and stable restoration of BP[12] as well as the prevention of systemic oxidative stress[21] have been observed. However, in general, systemic HTS administration has several intrinsic drawbacks, including an increased risk of hypernatremia, plasma hyperosmolarity, cardiac arrhythmias, and heart failure[12]. Apart from being free of the limitations inherent to systemic HTS administration, the technique of local HTS delivery seems to have 2 more additional advantages over a variety of protocols attempted previously[22,23]. First, the delivery of perfusate directly into the SMA, with outflow from the opened vein, completely prevents the otherwise inevitable generalized spread throughout the systemic circulation of the metabolites and bacterial toxins accumulated during ischemia. Second, the pharmacological agents recommended for strengthening the protective effect of HTS[24,25]-which could thus be potentially helpful to HTS administration *via* a local route-could be used at higher doses and therefore with higher efficiency, analogously with the strategy applied for regional perfusion in chemotherapy for certain neoplasms[26].

The question might arise as to whether the HTS osmolarity value used in the present study was optimal. While the osmolarity of HTS for hypertonic resuscitation averages approximately 7.5% NaCl, in our local delivery model we tested solutions with much lower osmolarities. Previous studies have tried values ranging from normal (300-310 mOsm/L) to nearly 400 mOsm/L for local organ anti-ischemic protection, particularly the heart; the optimal osmolarity was found to be 334 mOsm/L[27]. Interestingly, in *ex vivo* experiments, osmolarity values above 400 mOsm/L apparently produced a negative effect; that is, HTS delivery resulted in a significant deterioration in postischemic IPF rate recovery as compared to controls (Figure 2). Although different mechanisms might be involved in the observed effect, the most likely are endothelial injury and impaired microvascular function; however, this matter requires further investigation. Based on these findings, in this study a solution with a lower osmolarity ($HTS^{365mOsm}$) was preferred and was found to be effective for the preservation of intestinal viability, not only *ex vivo*, but also in the *in vivo* model. A higher HTS osmolarity value than that optimal for the heart could be justified by the higher hydrophilicity of the intestinal tissue, which would thus also require more potent anti-edema protection. It should be noted, however, that further experimentation will be needed in order to determine more precisely the optimal HTS parameter(s) for the anti-IRI protection of intestinal tissue.

Although not fully understood, the mechanism of the intestine protection with HTS may include such factors as dilatation of precapillary arterioles[12], inhibition of neutrophil-mediated reperfusion injury[11], or prevention of intestinal edema due to reperfusion-induced water influx into the cells[28]. At the molecular level, the major factor underlying the removal of excess water from the cells

has been shown to be the up-regulation of aquaporin 4 within the plasma membrane of the mucosal cells[29].

As mentioned before, protective effects of local HTS delivery on reperfusion injury have also been found in the heart, suggesting that common mechanisms may be involved. The concept of controlled cardiac reperfusion was originally proposed in the late 1980s to protect the myocardium from IRI by modifying the composition and/or delivery rate of the perfusion fluid[30]. One of the major modifications was the increased osmolarity of the perfusate, which was shown to decrease the incidence of reperfusion-induced arrhythmias, to attenuate tissue injury, and to reduce edema in the isolated hearts of pig and rabbit[27,31]. This promising approach is still awaiting implementation in clinical practice, initially in open heart surgery.

Local infusion of saline before reperfusion was also found to be protective in the brain. It was found that flushing the ischemic area of the brain with isotonic saline prior to reperfusion resulted in a reduced infarct volume and improved cerebral blood flow in the rat model[32]. Since normotonic saline was used in this study, the authors attributed its protective effect to the local clearance of proinflammatory cytokines and metabolic end-products.

In the present study, the spontaneous intestinal motility was assessed with a technique that has not yet been employed in the experiments simulating IRI. The observation of significant augmentation of the intestinal contraction rate over the first 10 min of intra-arterial perfusate reflow after 60-min *in vitro* ischemia (Figure 4) is in general agreement with data showing a transient increase in both the contraction rate and baseline tension observed upon reoxygenation of the jejunal rings after they were subjected to 60-min *in vitro* hypoxia[33]. In both situations, the increased motor activity of the intestine within the early postischemic period might be explained, in gross analogy with the postischemic heart response[34], by sudden oxygen-evoked re-energizing of the cells immediately after severe hypoxia. The subsequent excessive activation of contractile machinery might, in turn, contribute to the progression of mucosal injury, most likely due to the adverse effect of hypermotility. Its complete prevention with HTS perfusion (Figure 4) may point to the solution of the relevant clinical problem.

One obvious limitation of our *in vivo* model is the need to advance the perfusion cannula through the infrarenal aorta, rather than directly into the SMA. As a result, the kidneys and hindlimbs were exposed to an additional 5 min of ischemia. However, this limitation is imposed by the small size of the animal and the need to restore blood flow in the mesenteric vascular bed. In order to make the results comparable, control animals also received 5-min occlusion of the infrarenal aorta. Future studies are needed to verify the protective effect of local HTS infusion in large animal models. For instance, the study might be repeated in pigs using an endovascular approach with percutaneous puncture in the femoral artery and balloon occlusion of the SMA.

As to further extrapolations useful for clinical practice, the findings from the present study imply that intra-arterial HTS administration could contribute to the prevention, or at least amelioration, of 2 seemingly inevitable negative effects that would otherwise arise; that is, if "unprotected" reperfusion is applied. The first of these is reperfusion-induced intestinal damage, while the second includes whole-body disturbances, such as the systemic inflammatory response syndrome[4], hemodynamic impairments[35], and remote organ injuries[36], all of which are associated with the massive washout of metabolites and bacterial toxins from the ischemia-affected intestine into the systemic circulation. Our *in vivo* model could lead to the development of a novel therapy that combines local perfusion of the ischemic region with mechanical restoration of the blood flow and/or the administration of pharmacological agents. As to the clinical translation of the model, it might be speculated that perfusion of the SMA in patients with AMI could be performed after laparotomy, either *via* a catheter directly placed in the SMA or during percutaneous intervention. The perfusate outflow could be ensured through the punctured SMV. It should be noted that the suggested technique delayed restoration of blood flow to the intestine by 5 min; however, it is unlikely that perfusion with an oxygen- and glucose-enriched solution could cause any ischemic/hypoxic injury to the intestine during this period. It is also of importance to determine the optimal osmolarity value of the HTS, since the results of the present study have demonstrated the negative effect of HTS with excessive osmolarity, i.e., 415 mOsm/L.

In conclusion, the results of our experiments show that isolated intestinal perfusion with HTS at the beginning of the reperfusion period can appreciably protect the intestine from IRI. Despite the positive effect of intra-arterial HTS delivery, aimed in the present study at establishing the validity of this approach in principle, its further improvement by optimization of the particular parameters during all steps of the procedure will be required before clinical trials can be contemplated.

COMMENTS

Background

Acute mesenteric ischemia (AMI) is a life-threatening vascular emergency with high mortality rate. The major treatment approach for AMI is rapid intestinal revascularization. Unfortunately, even after timely revascularization, its positive effect can be compromised, at least in part, by the development of intestinal reperfusion injury.

Research frontiers

Systemic administration of hypertonic saline (HTS) was shown to be protective in the experimental models of AMI. In the present study, the authors investigated the effect of local intra-arterial HTS administration during the early reperfusion phase on the functional and structural manifestations of intestinal injury.

Innovations and breakthroughs

The authors showed for the first time that isolated intestinal perfusion with HTS at the beginning of the reperfusion period can appreciably protect the intestine from ischemia-reperfusion injury. In addition, the technique of isolated *in vivo* vascular perfusion of the intestine is suggested and experimentally validated.

Applications

This *in vivo* model could lead to the development of a novel therapy that combines local perfusion of the ischemic intestine with mechanical restoration of the blood flow and/or the administration of pharmacological agents.

Terminology

Intestinal ischemia-reperfusion injury (IRI) develops because of the temporal complete or partial cessation of blood supply to the intestine. HTS is crystalloid or colloid solution with osmolarity value exceeding physiological. Potential benefits of HTS in the settings of AMI include prevention of edema, improvement in the microcirculation, and washout of potentially toxic metabolites.

Peer review

This paper investigated the effect of local intestinal perfusion with HTS on intestinal IRI in both *ex vivo* and *in vivo* rat models, to find that brief intestinal postischemic perfusion with $HTS^{365mOsm}$ through the SMA followed by blood flow restoration was a protective procedure that could be used for the prevention of intestinal IRI. It is well written and organized.

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ORIGINAL ARTICLE

Characterization of focal liver masses using acoustic radiation force impulse elastography

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Abstract

AIM: To investigate the diagnostic performance of acoustic radiation force impulse (ARFI) elastography for characterizing focal liver mass by quantifying their stiffness.

METHODS: This prospective study included 62 patients with a focal liver mass that was well visualized on conventional ultrasonography performed in our institution from February 2011 to November 2011. Among them, 12 patients were excluded for ARFI measurement failure due to a lesion that was smaller than the region of the interest and at an inaccessible location (deeper than 8 cm) (n = 7) or poor compliance to hold their breath as required (n = 5). Finally, 50 patients with valid ARFI measurements were enrolled. If a patient had multiple liver masses, only one mass of interest was chosen. The masses were diagnosed by histological examination or clinical diagnostic criteria. During ultrasonographic evaluation, stiffness, expressed as velocity, was checked 10 times per focal liver mass and the surrounding liver parenchyma.

RESULTS: After further excluding three masses that were non-diagnostic on biopsy, a total of 47 focal mass lesions were tested, including 39 (83.0%) malignant masses [24 hepatocellular carcinomas (HCC), seven cholangiocellular carcinomas (CCC), and eight liver metastases] and eight (17.0%) benign masses (five hemangiomas and three focal nodular hyperplasias, FNH). Thirty-seven (74.0%) masses were confirmed by histological examination. The mean velocity was 2.48 m/s in HCCs, 1.65 m/s in CCCs, 2.35 m/s in metastases, 1.83 m/s in hemangiomas, and 0.97 m/s in FNHs. Although considerable overlap was still noted between malignant and benign masses, significant differences in ARFI values were observed between malignant and benign masses (mean 2.31 m/s *vs* 1.51 m/s, P = 0.047), as well as between HCCs and benign masses (mean 2.48 m/s *vs* 1.51 m/s, P = 0.006). The areas under the receiver operating characteristics curves (AUROC) for discriminating the malignant masses from benign masses was 0.724 (95%CI, 0.566-0.883, P = 0.048), and the AUROC for discriminating HCCs from benign masses was 0.813 (95%CI, 0.649-0.976, P = 0.008). To maximize the sum of sensitivity and specificity, an ARFI value of 1.82 m/s was selected as the cutoff value to differentiate malignant from benign liver masses. Furthermore, the cutoff value for distinguishing HCCs from benign masses was also determined to be 1.82 m/s. The diagnostic performance of the sum of the ARFI values for focal liver masses and the surrounding liver parenchyma to differentiate liver masses improved (AUROC = 0.853;

95%CI, 0.745-0.960; P = 0.002 in malignant liver masses *vs* benign ones and AUROC = 0.948; 95%CI, 0.896-0.992, P < 0.001 in HCCs *vs* benign masses).

CONCLUSION: ARFI elastography provides additional information for the differential diagnosis of liver masses. However, our results should be interpreted in clinical context, because considerable overlap in ARFI values existed among liver masses.

Key words: Acoustic radiation force impulse; Focal liver mass; Hepatocellular carcinoma; Hemangioma; Focal nodular hyperplasia; Cholangiocellular carcinoma; Liver metastasis

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INTRODUCTION

Focal liver masses are being discovered at increasing rates due to the wide accessibility of modern high resolution imaging procedures. Conventional ultrasonography (US) is typically used as a first imaging modality to evaluate a focal liver mass. The differential diagnosis of a focal liver mass using imaging studies is based on the characteristics of the surrounding liver parenchyma and underlying clinical conditions such as cirrhosis and the characteristics of the mass itself. Although contrast-enhanced US, computed tomography (CT), and magnetic resonance imaging (MRI) can assess the morphology of a focal liver mass and its vasculature with a high level of diagnostic accuracy[1-5], patients are exposed to potential risks, including contrast medium-induced side-effects and irradiation hazards[6], particularly when repeated examinations are required.

Acoustic radiation force impulse (ARFI) elastography has been introduced as a new ultrasound imaging modality to evaluate tissue stiffness using the radiation forced-based imaging method. The tissue response to the radiation force is observed using conventional B-mode imaging pulses, and it is possible to display the quantitative shear wave velocity (m/s) of the ARFI image[7,8]. Because velocity is directly related to tissue stiffness, ARFI imaging can be applied to evaluate tissue elasticity. Generally, the stiffer the tissue, the faster the shear wave propagates[6,9-13]. Although several reports have indicated a good correlation of ARFI elastography with the other elastography systems such as transient elastography[14,15] and with histological fibrosis grade[14,16,17], ARFI elastography differs from other elastography systems which apply pressure manually to the surface of the organs or a mechanical vibration to induce an elastic shear wave with only M-mode US imaging. Instead, ARFI uses short-duration acoustic pulses generated from a probe under real-time B-mode imaging to produce localized displacements in tissue[18-20]. Furthermore, because ARFI elastography uses elastography with a flexible metering box of the region of the interest (ROI), it is the only elastography method suitable for quantifying focal liver mass stiffness.

Few reports have investigated the applicability of ARFI elastography to evaluate focal liver masses[6,20-25]. Thus, we prospectively recruited patients with a focal liver mass and investigated the diagnostic performance of ARFI elastography to discriminate malignant liver masses and hepatocellular carcinoma (HCC) from benign masses by quantifying their stiffness.

MATERIALS AND METHODS

Subjects

From February 2011 to November 2011, a total of 62 patients with a focal liver mass that was well visualized on conventional US were prospectively recruited for this study. The subjects were referred to our institute for further evaluation of a focal liver mass from primary or secondary clinics, or had been diagnosed with a focal liver mass during a surveillance examination at our institute. Of these, 12 patients were excluded for the following reasons: (1) ARFI measurement failure due to a lesion that was smaller than the ROI and at an inaccessible location (deeper than 8 cm) (n = 7) or (2) poor compliance or inability to hold their breath as required (n = 5). Finally, 50 patients with valid ARFI measurements were enrolled. If a patient had multiple liver masses, only one mass of interest was chosen.

This study was approved by the institutional review board at Severance Hospital in Seoul, South Korea, and conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Written informed consent was obtained from all participants.

Histological confirmation of the liver masses

We performed a targeted biopsy on masses when a confirmatory histological diagnosis was needed or the radiological diagnosis was not confirmative. Among the 50 masses in our study, 37 (74.0%) received targeted biopsy for histological confirmation. Masses that had any component of cholangiocellular carcinomas (CCC) or combined HCC (CHCC) were classified as CCC and the others as HCC.

Clinical diagnosis of the liver masses

A clinical diagnosis of HCC was made according to the American Association for the Study of Liver Disease recommendation[26]. Briefly, patients were diagnosed with HCC if they had a tumor with a maximum diameter > 2 cm and exhibited typical features of HCC on dynamic CT, defined as enhancement in the arterial phase, early washout on the portal phase, and an α-fetoprotein level

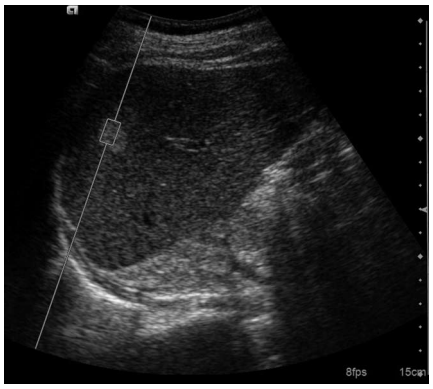

Figure 1 Measurement of acoustic radiation force impulse value within the region of interest of a focal liver mass. The shear wave velocity measured when the region of interest box was placed within the mass.

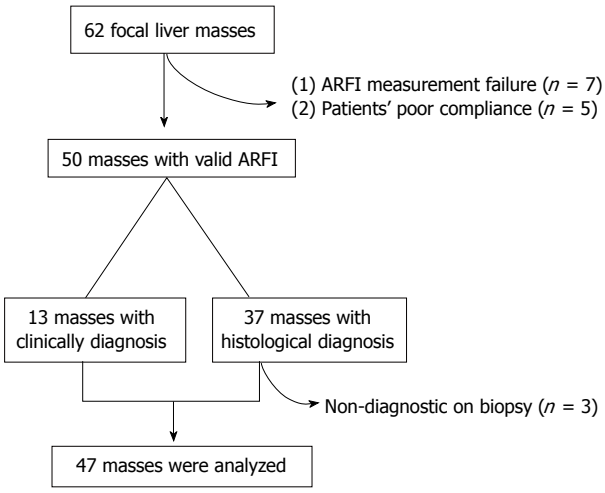

Figure 2 Recruitment algorithm.

> 200 ng/mL. Overall, nine patients satisfied these criteria sufficiently to be diagnosed with HCC without a histological examination.

The presence of a hemangioma was diagnosed clinically in four patients, based on a combination of typical findings, determined using CT or MRI, and a lack of growth for at least 12 mo[22]. A hemangioma appears as a mass with hypoattenuation on unenhanced CT, very high signal intensity on T2-weighted images, peripheral nodular enhancement in the arterial phase, and progressive filling-in of enhancement with no washout in later phases[20].

ARFI elastography

ARFI elastography was performed with an Acuson S2000 ultrasound system (Siemens, Erlanger, Germany), using a 4-1 MHz curved array probe. ARFI elastography was performed by a single physician in all patients (2 years of experience with US and more than 100 examinations with ARFI elastography) who was blinded with regard to the clinical and biochemical data. For patients who underwent liver biopsy, ARFI elastography was performed just before the biopsy, on the same day. For patients with a solitary liver mass that was clinically diagnosed, ARFI elastography was performed at the time of enrollment in this study.

Details of the technical background and examination procedure have been described previously[27]. Briefly, a B-mode US image of the lesion was identified utilizing a ROI, characterized by a box with a fixed dimension of 1 cm × 0.5 cm and a maximum depth of 8 cm. The ROI was entirely located in the lesion, and the ROI location was changed to cover large masses as much as possible without including any vascular or biliary structures (Figure 1). The potential presence of degeneration, such as necrotic, cystic, or calcified portions, was not included in the ROI. To evaluate background liver status of the focal mass, measurements were also taken in the surrounding liver parenchyma with the ROI within 2-3 cm from the target mass, taking care not to comprise any vascular or biliary structure. To ensure quality of the ARFI measurement, 10 measurements were performed for each mass and surrounding liver parenchyma.

Statistical analysis

Continuous variables are expressed as medians and ranges. The χ^2 test or Fisher's exact test and the Mann-Whitney test were used to compare categorical and continuous variables, respectively. Receiver operating characteristics (ROC) curves and areas under the ROC curves (AUROC) were used to estimate diagnostic performance. The cutoff ARFI value for maximal diagnostic accuracy was selected by considering the highest sum of sensitivity and specificity. A P-value < 0.05 was considered statistically significant. The statistical analysis was performed using SPSS software ver. 18.0.0 (Chicago, IL, United States).

RESULTS

Final diagnosis of focal liver masses

We further excluded three masses that were non-diagnostic on biopsy due to a lack of sufficient tissue or ineffective targeting of the mass. Thus, 47 masses (34 histologically confirmed and 13 clinically diagnosed) were evaluated (Figure 2).

Overall, 15 HCCs, eight metastases (three from colorectal cancers, two from gallbladder cancer, one from pancreatic cancer, one from a gastrointestinal stromal tumor, and one from cervical cancer), seven CCCs, three focal nodular hyperplasias (FNH), and one hemangioma were diagnosed. Figure 3 describes pre-biopsy and post-biopsy diagnoses: 21 (61.8%) masses were consistent with the pre-biopsy diagnosis, and 10 masses diagnosed as HCC or CHCC at the time of pre-biopsy were finally confirmed as six HCCs and four CCCs through the biopsy. The histological diagnosis of the other three masses changed (two FNHs to one hemangioma and one HCC; one poorly-differentiated malignancy or metastatic mass to CCC).

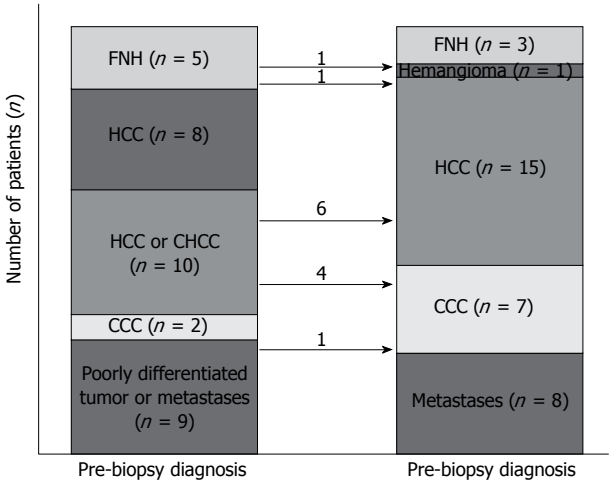

Figure 3 The flow of histological diagnosis. The values on arrows indicate the number of patients who obtained diagnoses differing from those pre-biopsy diagnoses after liver biopsy.

Characteristics and ARFI values of focal liver masses

The depth, location (right *vs* left lobe), and size of each focal liver mass are described in Table 1. ARFI values of HCCs and metastases were higher (2.48 and 2.35 m/s) than those of hemangiomas (1.83 m/s), CCCs (1.65 m/s), and FNH (0.97 m/s). ARFI values for CCCs, hemangiomas, and metastases were significantly higher than those for their surrounding liver parenchyma (mean 1.65 *vs* 1.07 m/s in CCCs; 1.83 m/s *vs* 1.10 m/s in hemangiomas; 2.35 m/s *vs* 1.45 m/s in metastases; all $P < 0.05$), whereas ARFI values for HCCs were similar to those of surrounding liver parenchyma (2.48 m/s *vs* 2.14 m/s, $P = 0.134$).

When HCCs, CCCs, and metastases were categorized into a malignant liver mass group ($n = 39$) and the others were stratified into a benign mass group ($n = 8$), a significant difference appeared in the between the malignant and benign liver masses (2.31 ± 1.05 m/s *vs* 1.51 ± 0.69 m/s, $P = 0.047$) (Figure 4A). However, considerable overlap in ARFI values was noted between malignant and benign masses. Additionally, a significant difference in the ARFI values was observed between the HCCs and the benign masses (2.48 ± 0.84 m/s *vs* 1.51 ± 0.69 m/s, $P = 0.006$) (Figure 4A). To address this overlap, we attempted further analysis taking into consideration the ARFI values of surrounding liver parenchyma as well as those of focal liver masses. When ARFI values of focal liver masses and their surrounding liver parenchyma were combined, the differences in ARFI values for each tumor type became more prominent (4.29 ± 1.22 m/s *vs* 2.92 ± 0.35 m/s for malignant masses *vs* benign masses, $P < 0.001$; 4.62 ± 0.96 m/s *vs* 2.92 ± 0.35 m/s for HCCs *vs* benign masses, $P < 0.001$) (Figure 4B).

Discrimination of malignant liver masses and HCCs from benign masses

The AUROC for discriminating the malignant masses from benign masses was 0.724 (95%CI, 0.566-0.883, $P = 0.048$), and the AUROC for discriminating HCCs from benign masses was 0.813 (95%CI, 0.649-0.976, $P = 0.008$) (Table 2). To maximize the sum of sensitivity and specificity, an ARFI value of 1.82 m/s was selected as the cutoff value to differentiate malignant from benign liver masses. Furthermore, the cutoff value for distinguishing HCCs from benign masses was also determined to be 1.82 m/s (Table 2). Additionally, the diagnostic performance of the sum of the ARFI values for focal liver masses and the surrounding liver parenchyma to differentiate liver masses improved (AUROC = 0.853; 95%CI, 0.745-0.960; $P = 0.002$ in malignant liver masses *vs* benign ones and AUROC = 0.948; 95%CI, 0.896-0.992, $P < 0.001$ in HCCs *vs* benign masses) (Table 2).

DISCUSSION

ARFI elastography has been proposed as a new method for assessing liver stiffness[7-9]. Although ARFI elastography uses shear wave velocity (m/s) to assess liver stiffness, which is similar to FibroScan®, it exhibits several unique properties. First, ARFI elastography allows for the evaluation of deep tissue by generating a shear wave without the need for exertional compression[7,28]. Second, ARFI elastography has the distinct advantage of being integrated into a conventional US system and can provide additional real-time information during a conventional US study[14,15]. Third, ARFI elastography can be performed regardless of the presence of impediments such as ascites, although the reproducibility in this setting should be further investigated[19,29]. Finally, ARFI elastography offers a flexible metering box at variable depths, allowing the examination of specific liver areas[21,30]. Due to these characteristics, the clinical applicability of ARFI elastography has expanded to characterize and distinguish focal liver masses beyond a simple assessment of liver fibrosis[6,20-25].

According to previous studies[22,24,25], malignant liver masses are stiffer than benign masses, as reflected by higher ARFI values. Consistent with this finding, the ARFI values of malignant liver masses (2.31 m/s) or HCCs (2.48 m/s) in our cohort were significantly higher than those of benign masses (1.51 m/s) (all $P < 0.05$). However, when we consider that two types of malignant liver masses (HCCs and CCCs) with different ARFI values were stratified into one malignant mass group in our study, the accuracy of ARFI elastography for identifying malignant liver masses would change according to the proportion of HCCs and CCCs. Thus, an exact comparison between the ARFI values of HCCs and CCCs as well as those of benign masses should be performed in future, larger-scale studies. Additionally, the respective ARFI values of HCCs and metastatic masses in our study were 2.48 m/s and 2.35 m/s, which were similar to data from previous studies (2.45 to 2.63 m/s in HCC and 2.18 to 2.88 m/s in metastasis)[22,23].

ARFI values for hemangiomas vary among reports[6,20,22,23]. The ARFI values of hemangiomas reported

Table 1 Characteristics and acoustic radiation force impulse values of focal liver masses

Variables	Total masses (n = 47)	HCC (n = 24)	CCC (n = 7)	Metastases (n = 8)	Hemangioma (n = 5)	FNH (n = 3)
Histologic confirmation	34 (72.3)	15 (62.5)	7 (100)	8 (100)	1 (20.0)	3 (100)
Size, cm	5.0 (1.4-20.5)	4.9 (1.4-18.3)	9.4 (7.4-18.8)	8.0 (1.6-20.5)	1.9 (1.5-4.7)	2.7 (2.0-3.0)
Depth, cm	5.6 (2.7-7.8)	5.7 (2.7-7.8)	6.4 (4.8-7.2)	4.9 (3.5-7.6)	5.3 (4.5-6.5)	5.6 (2.8-7.8)
Right/left lobe	34 (72.3)/13 (27.7)	19 (79.2)/5 (20.8)	5 (71.4)/2 (28.6)	5 (62.5)/3 (37.5)	4 (80.0)/1 (20.0)	1 (33.3)/2 (66.7)
ARFI value, m/s						
Masses	2.23 ± 0.98	2.48 ± 0.84	1.65 ± 1.43	2.35 ± 1.18	1.83 ± 0.62	0.97 ± 0.48
Surrounding parenchyma	1.83 ± 0.73	2.14 ± 0.59	1.07 ± 0.49	1.45 ± 0.51	1.10 ± 0.14	1.63 ± 0.40
P value	0.029	0.134	0.015	0.043	0.013	0.581

Data was expressed as median (range) or n (%). HCC: Hepatocellular carcinoma; CCC: Cholangiocellular carcinoma; FNH: Focal nodular hyperplasia; ARFI: Acoustic radiation force impulse.

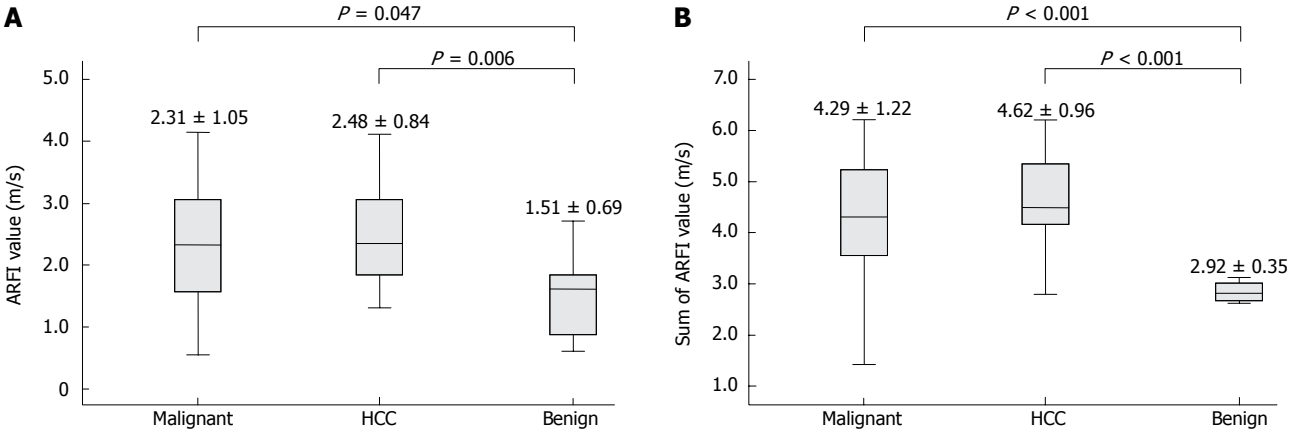

Figure 4 Acoustic radiation force impulse values of malignant masses, hepatocellular carcinoma, and benign masses. A: Acoustic radiation force impulse (AFRI) value of a focal liver mass; B: Sum of the ARFI values of liver mass and the surrounding liver parenchyma. HCC: Hepatocellular carcinoma.

Table 2 Optimal cutoff acoustic radiation force impulse values and corresponding diagnostic indices for discriminating malignant masses and hepatocellular carcinoma from benign liver masses

	Malignant *vs* benign		HCC *vs* benign	
	Masses	Masses with surrounding liver parenchyma	Masses	Masses with surrounding liver parenchyma
Cutoff ARFI value, m/s	1.82[1]	3.72[2]	1.82[1]	3.79[2]
AUROC (95%CI)	0.724 (0.566-0.883)	0.853 (0.745-0.960)	0.813 (0.649-0.976)	0.948 (0.896-0.992)
Sensitivity, %	71.8	71.8	79.2	87.5
Specificity, %	75.0	100	75.0	100
Positive predictive value, %	93.3	100	90.5	100
Negative predictive value, %	35.3	42.1	54.5	72.9

[1]ARFI value of a focal liver mass; [2]Sum of ARFI values of focal liver mass and surrounding liver parenchyma. ARFI: Acoustic radiation force impulse; HCC: Hepatocellular carcinoma; AUROC: Area under the receiver operating characteristics; CI: Confidence intervals.

in two previous studies (1.51 and 1.75 m/s, respectively) were comparable to our data (1.83 m/s)[20,22,23], whereas the ARFI value for a hemangioma in another study was rather higher (2.36 m/s)[22,23]. The variability in hemangioma ARFI values has been explained based on the amount of fibrotic septa that divide the dilated vascular spaces[22]. That is, hemangiomas are composed of multiple vascular channels filled with blood so they would not be expected to be stiff, and therefore would have low ARFI values; in contrast, those including pathological patterns such as sclerosis, thrombosis of the vessels, or calcification, would be stiff and have high ARFI values[20]. Because relatively few hemangioma cases were analyzed in these studies, this discrepancy will need to be investigated *via* further study including a larger number of cases.

Some researchers have attempted to suggest a cutoff ARFI value for distinguishing malignant liver masses from benign masses. We obtained a high PPV (malignant masses *vs* benign masses, PPV 93.3%; HCC *vs* benign masses, 90.5%) using the cutoff ARFI value of 1.82 m/s, which maximized the sum of the sensitivity and specificity for distinguishing masses. Our cutoff value of 1.82 m/s for identifying malignant liver masses was slightly lower than those in previous studies (2.0 to 2.5 m/s)[22,24,25],

which can be partially explained by the different methods used to categorize the masses. In one study, which reported a rather high cutoff value of 2.5 m/s, only metastases that had a high ARFI value of 4.18 m/s were categorized into a malignant group and compared with benign masses[24], whereas another study that reported 2.22 m/s as a cutoff value for discriminating malignant masses from benign masses included FNHs, adenomas, and focal fatty change lesions in the benign mass group as well as hemangiomas[25]. Thus, in this regard, further study is needed to prevent potential bias due to heterogeneity of the masses and to confirm the clinically applicable cutoff values for ARFI elastography. Although our cutoff value may be useful to physicians who encounter focal liver masses during routine US evaluation, it should be interpreted cautiously in the clinical context for several reasons. First, although our cutoff value would correctly characterize HCC and metastasis, it would mischaracterize CCC with a relatively low ARFI value and hemangioma with a relatively high ARFI value. Second, there was considerable overlap of ARFI values between malignant and benign masses.

Underlying fibrosis of the liver is a consideration for the differential diagnosis of a focal liver mass. Several previous studies have proposed that HCCs generally appear softer (lower ARFI values) than the surrounding liver[21], whereas metastases and hemangiomas generally appear harder than that of the surrounding liver despite some controversies among studies[21]. Similarly, we found that CCCs, hemangiomas, and metastases had higher ARFI values than those of surrounding liver parenchyma, whereas ARFI values of HCCs were statistically equivalent to those of the surrounding liver parenchyma. Because hemangiomas and metastases were evaluated in patients without chronic liver disease in most studies[6,25], ARFI values seemed consistently higher than those of a background liver. In contrast, because HCCs were evaluated in patients with chronic liver disease and diverse degrees of background liver fibrosis, the comparative results between ARFI values of HCCs and those of background liver differed among studies based on the characteristics of each study cohort[6,21,25]. That is, simultaneously measuring ARFI values of focal liver mass and the surrounding liver should focus on assessing the respective characteristics of the hepatic mass and surrounding fibrosis to prevent a misdiagnosis of the hepatic mass using the correlation of ARFI values between the liver mass and background liver parenchyma. However, when we used the sum of ARFI values of focal liver mass and the surrounding liver parenchyma, the diagnostic performance in terms of distinguishing liver mass improved. Thus, these controversial findings concerning the simultaneous measurement of liver masses and their surrounding liver parenchyma should be further investigated in future larger-scale studies.

Although most cases (72.3%) were histologically confirmed in our study, the relative small sample size of our cohort and inclusion of patients with high ALT, which has the potential of overestimating influences on ARFI values[30,31], are potential limitations. Although ARFI elastography can freely locate the ROI box in a specific area within a mass and measure its stiffness, morphological characteristics of liver masses including heterogeneous components such as HCC with hemorrhage and fatty metamorphosis[6,20,32] and lesion shapes were not considered in our study, which is a limitation of this study. Further investigation of how to evaluate such heterogeneous or morphologically varying liver masses using ARFI elastography and their influences on ARFI values of liver masses should be conducted.

In our study, we demonstrated the potential clinical utility of ARFI elastography for characterization of focal liver masses. Although this study had limitations and should be interpreted cautiously, our findings provide a useful reference for the differential diagnosis of a focal liver mass and will provide additional information to clinicians who are confronted with a need for an immediate diagnosis of a focal liver mass during a routine US examination before a further diagnostic imaging study such as contrast-enhanced US, CT or MRI. However, further studies with larger numbers of cases are warranted to assess the utility of ARFI elastography in the clinic.

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COMMENTS

Background

Acoustic radiation force impulse (ARFI) elastography can quantify tissue elasticity. Compared to previous elastography techniques, one of the distinguishing advantages of ARFI elastography is that it offers a flexible metering box at variable depths, which allows an examination of specific liver areas. With using this characteristic of ARFI elastography, authors investigated its applicability to evaluate focal liver masses.

Research frontiers

According to previous studies, malignant liver masses are stiffer than benign masses, as reflected by higher ARFI values. However, previous studies have showed somewhat controversial results with various median ARFI values for each focal liver mass and they have had limitations, such as most subjects not being supported by adequate histological confirmation.

Innovations and breakthroughs

Authors prospectively investigated 50 focal liver masses found during routine ultrasonography (US) study, and measured the stiffness of each focal liver mass using ARFI elastography. Focal liver masses included hepatocellular carcinomas (HCCs), cholangiocellular carcinomas, metastases, hemangiomas, and focal nodular hyperplasia. The advantage of our study over other previous studies is that majority of our subjects (72.3%) are confirmed with histological examinations.

Applications

Using ARFI elastography, physicians who are confronted with a need for an immediate diagnosis of a focal liver mass during a routine US examination can get additional information for the differential diagnosis of liver masses in clinical practice. Further future studies with larger numbers of cases are warranted to assess the utility of ARFI elastography.

Terminology

ARFI elastography is an emerging examination which can quantify tissue elasticity. ARFI elastography has the distinct advantage of being integrated into a conventional US system and can be checked simultaneously during a conventional US study. Furthermore, because ARFI elastography uses elastography with a flexible metering box of the region of the interest, it is suitable for quantifying focal liver mass stiffness.

Peer review

Although this study had limitations and should be interpreted cautiously, their results show the clinical applicability of ARFI elastography as a complementary diagnostic tool for the differential diagnosis of liver masses.

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ORIGINAL ARTICLE

Inositol-requiring enzyme 1α is required for gut development in *Xenopus lavies* embryos

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Abstract

AIM: To investigate the role of inositol-requiring enzyme 1α (IRE1α) in gut development of *Xenopus lavies* embryos.

METHODS: *Xenopus* embryos were obtained with *in vitro* fertilization and cultured in 0.1 × MBSH. One and half nanogram of IRE1α, 1 ng of IRE1α-GR mRNA, 1 ng of IRE1αΔC-GR mRNA, and 50 ng of IRE1α morpholino oligonucleotide (MO) or XBP1(C)MO were injected into four blastomeres at 4-cell stage for scoring the phenotype and marker gene analysis. To rescue the effect of IRE1α MO, 1 ng of IRE1α-GR mRNA was co-injected with 50 ng of MO. For the activation of the GR-fusion proteins, dexamethasone was prepared as 5 mmol/L stock solutions in 100% ethanol and applied to the mRNA injected embryos at desired stages in a concentration of 10 μmol/L in 0.1 × MBSH. Embryos were kept in dexamethasone up to stage 41. Whole-mount *in situ* hybridization was used to determine specific gene expression, such as IRE1α, IRE1β, Xbra and Xsox17α. IRE1α protein expression during *Xenopus* embryogenesis was detected by Western blotting.

RESULTS: In the whole-mount *in situ* hybridization analysis, xenopus IRE1α and IRE1β showed quite different expression pattern during tadpole stage. The relatively higher expression of IRE1α was observed in the pancreas, and significant transcription of IRE1β was found in the liver. IRE1α protein could be detected at all developmental stages analyzed, from stage 1 to stage 42. Gain-of-function assay showed that IRE1α mRNA injected embryos at tailbud stage were nearly normal and the expression of the pan-mesodermal marker gene Xbra and the endodermal gene Xsox17α at stage 10.5 was not significantly changed in embryos injected with IRE1α mRNA as compared to uninjected control embryos. And at tadpole stage, the embryos injected with IRE1α-GR mRNA did not display overt phenotype, such as gut-coiling defect. Loss-of-function assay demonstrated that the IRE1α MO injected embryos were morphologically normal before the tailbud stages. We did not observe a significant change of mesodermal and endodermal marker gene expression, while after stage 40, about 80% of the MO injected embryos exhibited dramatic gut defects in which the guts did not coil, but other structures outside the gastrointestinal tract were relatively normal. To test if the phenotypes were specifically caused by the knockdown of IRE1α, a rescue experiment was performed by co-injection of IRE1α-GR mRMA with IRE1α MO. The data obtained demonstrated that the gut coiling defect was rescued. The deletion mutant of IRE1α was constructed, consisting of the N-terminal part without the C-terminal kinase and RNase domains named IRE1αΔC, to investigate the functional domain of IRE1α. Injection of IRE1αΔC-GR mRNA caused similar morphological alterations with gut malformation by interfering with the function of endogenous xIRE1α. In order to investigate if IRE1α/

XBP1 pathway was involved in gut development, 50 ng of XBP1 MO was injected and the results showed that knockdown of XBP1 resulted in similar morphological alterations with gut-coiling defect at tadpole stage.

CONCLUSION: IRE1α is not required for germ layer formation but for gut development in *Xenopus lavies* and it may function *via* XBP1-dependent pathway.

Key words: Inositol-requiring enzyme 1α; XBP1; *Xenopus lavies*; Gut; Development

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INTRODUCTION

The endoplasmic reticulum (ER) plays a central role in the synthesis and modification of secretary and membrane proteins in all eukaryotic cells[1-3]. Under normal conditions, these proteins are correctly folded and assembled in the ER. However, when cells are exposed to disturbed environment, such as overproduction of ER proteins, viral infection, and glucose deprivation, these proteins accumulate as unfolded or misfolded forms in the ER lumen and, consequently, cause ER stress. To maintain cellular homeostasis, cells induce some adaptive responses to ER stress. One of them is the unfolded protein response (UPR), which up-regulates the transcription of various genes to increase the protein-folding and protein-degradation activity in the ER[4-7]. Inositol requiring enzyme-1 (IRE1) is an ER-located type I transmembrane protein with a kinase domain and RNase domain in the cytosolic region and has a unique function of relieving ER stress in cells. When the amino-terminal luminal region senses perturbations in the ER environment, *via* trans-autophosphorylation and activation of its RNase domain, IRE1 induces unconventional splicing of mRNA coding, a specific transcription factor for activating the UPR[8-10]. IRE1 is highly conserved from yeast to humans, and two IRE1 paralogues have been reported in mammals: IRE1α and IRE1β[11-13]. Upon activation by ER stress, IRE1 performs an unconventional cytoplasmic splicing of XBP1 pre-mRNA, thus allowing the synthesis of active XBP1, which activates UPR target genes to restore the homeostasis of the ER[10,14,15]. The spliced XBP1 mRNA is translated into a functional transcription factor to up-regulate gene expression for ER quality control. IRE1 is also reported to activate proapoptotic JNK signaling under ER stress conditions[16].

IRE1α is also known to be expressed ubiquitously in fetal and adult mice and to be essential for mammalian developmental processes[17]. Therefore, IRE1α inactivation results in widespread developmental defects, leading to embryonic death after 12.5 d of gestation in mice[18]. However, the cause of this embryonic lethality is not fully understood. These lines of evidence suggest that IRE1α has a unique function in mammalian developmental processes, but it has been hitherto unclear in which tissues and how IRE1α functions during embryogenesis.

Xenopus lavies is an excellent model system for studying organ development[19]. However, little information is available about the function of the IRE1/XBP1 pathway during embryogenesis, although loss of function studies revealed that IRE1 or XBP1 is absolutely required for embryonic development of C. elegans[20], Drosophila[21] or mouse[22]. In *Xenopus*, although the transcripts of two isoforms of IRE1, IRE1α and IRE1β show similar spatial expression in pre-neurula embryos, they are also differentially expressed following the onset of neurulation[23]. IRE1α is localized to the nervous system and mesoderm or endoderm-derived organs, such as pronephros and pancreas. However, the role of IRE1α in organogenesis is still unclear. In the present study, both gain and loss of function analyses revealed that IRE1α is required for gut development in *Xenopus*.

MATERIALS AND METHODS

Embryos

Wild type *Xenopus lavies* eggs were obtained by injecting 1000 IU of human chorionic gonadotrophin into the dorsal lymph sacs of adult females 6-8 h before egg collection. Eggs were fertilized *in vitro* with minced testes, dejellied with 2% cysteine hydrochloride (pH 7.8-8.0) 30 min after fertilization, and cultured in 0.1 × MBSH (8.8 mmol/L NaCl, 0.24 mmol/L $NaHCO_3$, 0.1 mmol/LKCl, 0.082 mmol/L $MgSO_4$, 0.041 mmol/L $CaCl_2$, 0.033 mmol/L $Ca(NO_3)_2$, 1 mmol/L HEPES, pH 7.4). Staging of *Xenopus lavies* embryos was according to Nieuwkoop and Faber (1967).

Plasmids and constructs

IRE1α ORF was amplified from a cDNA pool consisting of st.1, st.8, st.10, st.15, st.20 and st.28 cDNAs and subcloned to $pCS2^+$ vector. The construct was named $pCS2^+$-IRE1α. To make expression constructs, complete coding region and the N-terminal region (aa 1-479) were amplified from the $pCS2^+$-IRE1α using PCR and subcloned to the BamHI-EcoRV sites on $pCS2^+$-GR. The resulting constructs were designated as $pCS2^+$IRE1α-GR and $pCS2^+$IRE1αΔC-GR.

In vitro RNA synthesis, antisense morpholino oligonucleotides and microinjection

Plasmids $pCS2^+$IRE1α, $pCS2^+$IRE1α-GR and $pCS2^+$IRE1αΔC-GR were linearized with *Not*I. Capped mRNA for microinjection was synthesized with SP6 mMessage mMachine™ kit (Ambion) and cleaned up with RNeasy kit (Qiagen). The antisense morpholino

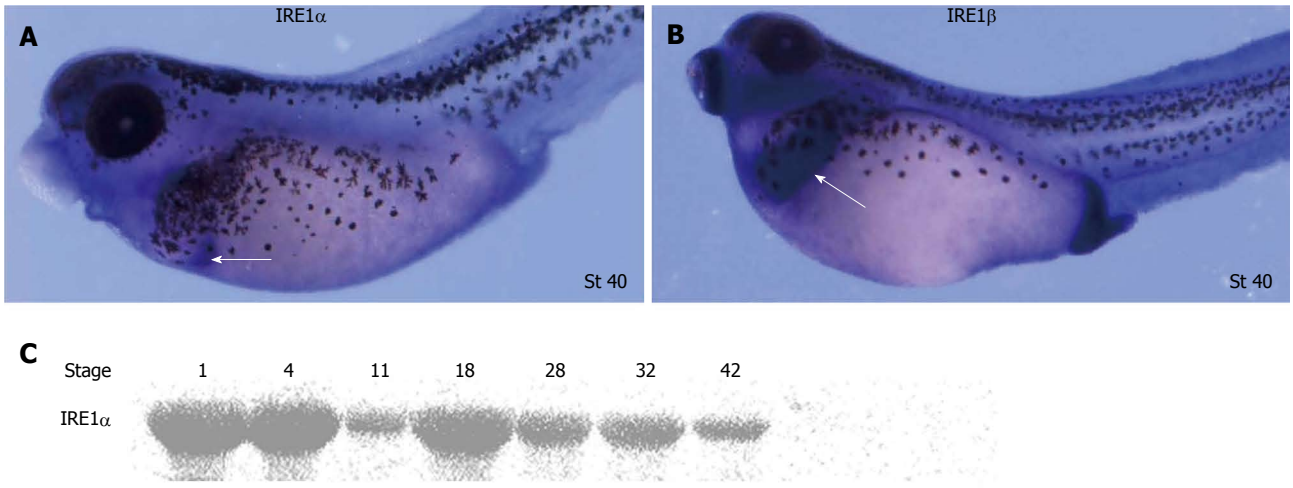

Figure 1 Expression pattern of xIRE1 in *Xenopus lavies* during development. A, B: Whole-mount *in situ* hybridizations revealed relatively high expression of inositol-requiring enzyme (IRE) 1α in pancreas (white arrow in A) and relatively high expression of IRE1β in liver (white arrow in B); C: Western blotting revealed temporal expression of IRE1α during *Xenopus* embryogenesis.

oligonucleotide (Gene Tools) used for IRE1α functional knockdown (IRE1α MO) was: 5'-AAGAGAACCGC-CAGAGGC GCCATG T-3'; and an antisense morpholino oligonucleotide XBP1(C) MO designed to inhibit the cytoplasmic splicing of xXBP1 was: 5'-GACATCT-GGGCCTGCTC CTGC TGCA-3'. One and half nanogram of IRE1α, 1 ng of IRE1α-GR mRNA, 1 ng of IRE1αΔC-GR mRNA, and 50 ng of IRE1α MO or XBP1(C)MO were injected into four blastomeres at 4-cell stage for scoring the phenotype, whole-mount *in situ* hybridization and marker gene analysis. For the activation of the GR-fusion proteins, dexamethasone (Sigma) was prepared as 5 mmol/L stock solutions in 100% ethanol and applied to the control and mRNA injected embryos at desired stages in a concentration of 10 μmol/L in 0.1 × MBS. Embryos were kept in dexamethasone up to stage 41. To rescue the effect of blocking xXBP1 splicing by IRE1α MO, 1 ng of IRE1α-GR mRNA was co-injected with 50 ng of MO.

In vitro translation

In vitro protein translation was performed with TNT coupled Reticulocyte Lysate Systems (Promega) to test the efficiency of IRE1α MO for blocking protein translation. One μg of pCS2$^+$IRE1α plasmid was used either alone or together with 20 μg IRE1α MO for *in vitro* translation. Translation products were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and chemiluminescent detection.

Whole-mount in situ hybridization

Whole-mount *in situ* hybridization was performed according to the protocol described elsewhere[24]. The digoxigenin-labeled antisense probes were prepared as follows: pDrive-IRE1α cut with *Hin*dⅢ, transcribed with T7 RNA polymerase; pCS2$^+$XIRE1β cut with *Xba*I, transcribed with T3 RNA polymerase; pBS-Xbra cut with *Sal*I, transcribed with T7 RNA polymerase; and pCS2$^+$XSox17α cut with *Cla*I, transcribed with T7 RNA polymerase.

Protein extraction and Western blotting

Embryos were homogenized in RIPA lysis buffer (20 mmol/L Tris, pH 8; 2 mmol/L EDTA, pH 8; 0.5% NP-40; 25 mmol/L β glycerophosphate; 100 mmol/L NaF; 100 mmol/L PMSF and phosphatase inhibitor cocktail). Lysates were centrifuged at 4 ℃, 15 000 × *g*, for 20 min, and the supernatants were added to 5 × SDS loading buffer. Proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane (Millipore, Billerica, MA). Immunoblotting membranes were blocked with 5% milk in TBST. After several washes in TBST, membranes were incubated overnight with the first antibody [anti-IRE1α antibody (Santa Cruz Biotechnology, Santa Cruz, CA), 1:500]. Loading controls of presumably constantly expressed proteins such as β-actin were used; however, their variability and increase in development precluded their use[25]. Detection was then done with HRP-labeled secondary antibodies and enhanced chemiluminescence (ECL).

RESULTS

Expression of xIRE1α in Xenopus lavies during development

Two forms of IRE1 genes, IRE1α and IRE1β, exist in *Xenopus lavies*. Whole-mount *in situ* hybridization revealed that IRE1α and IRE1β were expressed in a similar pattern from egg to gastrulation. In tailbud embryos, IRE1α was detected in a domain that probably represented the dorsal pancreas anlage. IRE1β was only observed in the hatching gland and cement gland until the hatched tadpole stage[23]. To further explore the spatial expression patterns of IRE1α and IRE1β in *Xenopus* embryos at later stages, whole-mount *in situ* hybridizations were carried out. During tadpole stages, relatively high expression of IRE1α was observed in the pancreas (Figure 1A), and significant transcription of IRE1β was observed in the

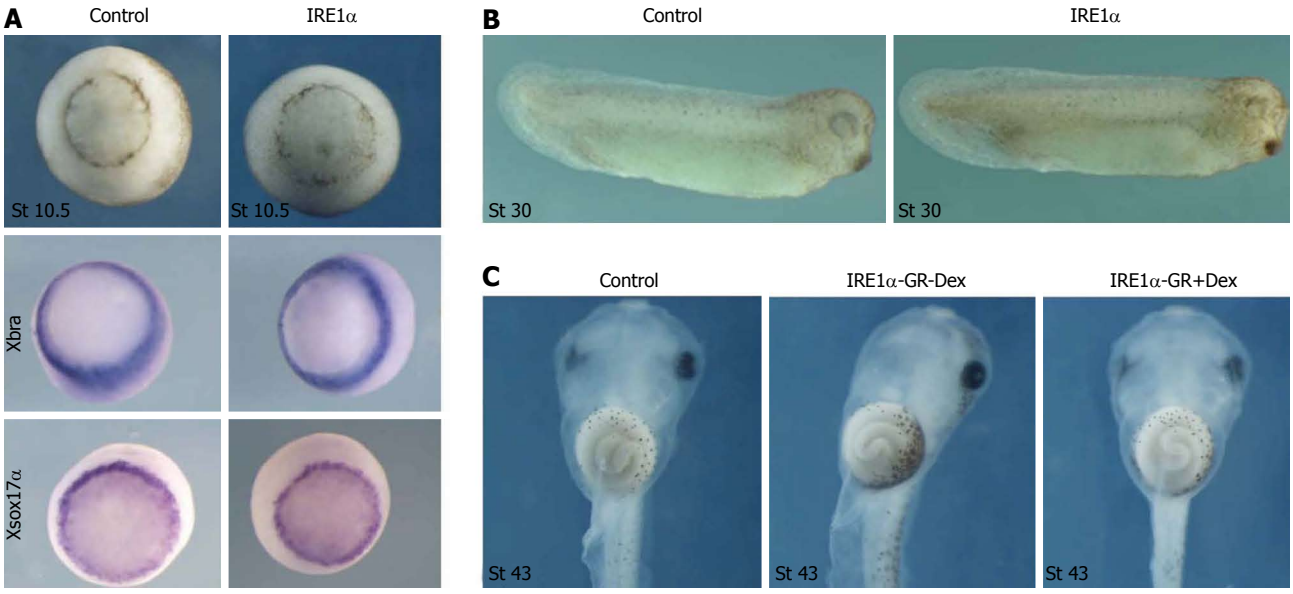

Figure 2 Overexpression of inositol-requiring enzyme 1α affected neither mesoderm and endoderm formation nor gut development. A: Overexpression of inositol-requiring enzyme (IRE) 1α did not change the phenotype and the expression of Xbra and Xsox17α at stage 10.5; B: IRE1α mRNA injected embryos at tailbud stage were nearly normal; C: Embryos injected with IRE1α-GR mRNA did not display any defect until tadpole stage.

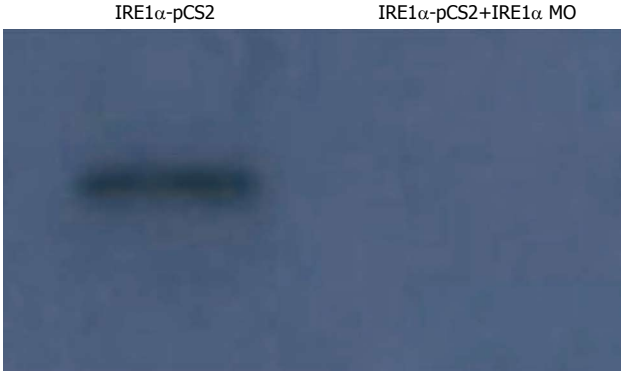

Figure 3 Inositol-requiring enzyme 1α morpholino oligonucleotide blocks inositol-requiring enzyme 1α translation in an *in vitro* transcription/translation assay. In this assay, xIRE1α was effectively transcribed/translated from a pCS2+xIRE1α construct. However, translation was dramatically decreased by addition of IRE1α morpholino oligonucleotide (MO). IRE: Inositol-requiring enzyme.

liver (Figure 1B). IRE1α protein could be detected at all developmental stages analyzed (Figure 1C).

IRE1α gain of function

Different doses of IRE1α mRNA were injected into all blastomeres at 4-cell stage. Even at a dose of 1.5 ng, injected embryos at tailbud stage were nearly normal (Figure 2B). Whole-mount *in situ* hybridization of embryos injected with IRE1α mRNA revealed that the expression of the pan-mesodermal marker gene Xbra and the endodermal gene Xsox17α at stage 10.5 was not significantly changed as compared to uninjected control embryos (Figure 2A). To test the function of IRE1α in the later stage of development, the mRNA encoding a dexamethasone inducible variant of IRE1α, referred to as IRE1α-GR, was injected into all four blastomeres at 4-cell stage *Xenopus* embryos. For the activation of the GR-fusion proteins, dexamethasone treatment was administered at embryonic stage 27. No apparent change of phenotype was observed even at tadpole stage (Figure 2C). Therefore, gain of function of IRE1α did not lead to an apparent change of phenotype and we did not observe a significant change of mesodermal and endodermal marker gene expression.

IRE1α MO blocking IRE1α translation

To further explore the function of xIRE1α during embryonic development, we performed a loss of function (LOF) analysis using an antisense morpholino oligonucleotide (IRE1α MO) directed against xIRE1α. *In vitro* protein translation was performed with TNT coupled Reticulocyte Lysate Systems to test the efficiency of IRE1α MO for blocking protein translation. As shown in Figure 3, IRE1α MO totally blocked IRE1α translation.

IRE1α loss of function

The IRE1α MO-injected embryos were morphologically normal before the tailbud stages (Figure 4B) and we did not observe a significant change of mesodermal and endodermal marker gene expression (Figure 4A), but after stage 40, about 80% of the MO (50 ng)-injected embryos exhibited dramatic gut defects in which the guts did not coil, while other structures outside the gastrointestinal tract developed normally (Figure 4). To test if the phenotypes were specifically caused by the knockdown of IRE1α, a rescue experiment was performed by co-injection of IRE1α-GR mRMA with MO. The results indicated that the phenotype could be rescued (Figure 5). Taken together, these data indicate that IRE1α is re-

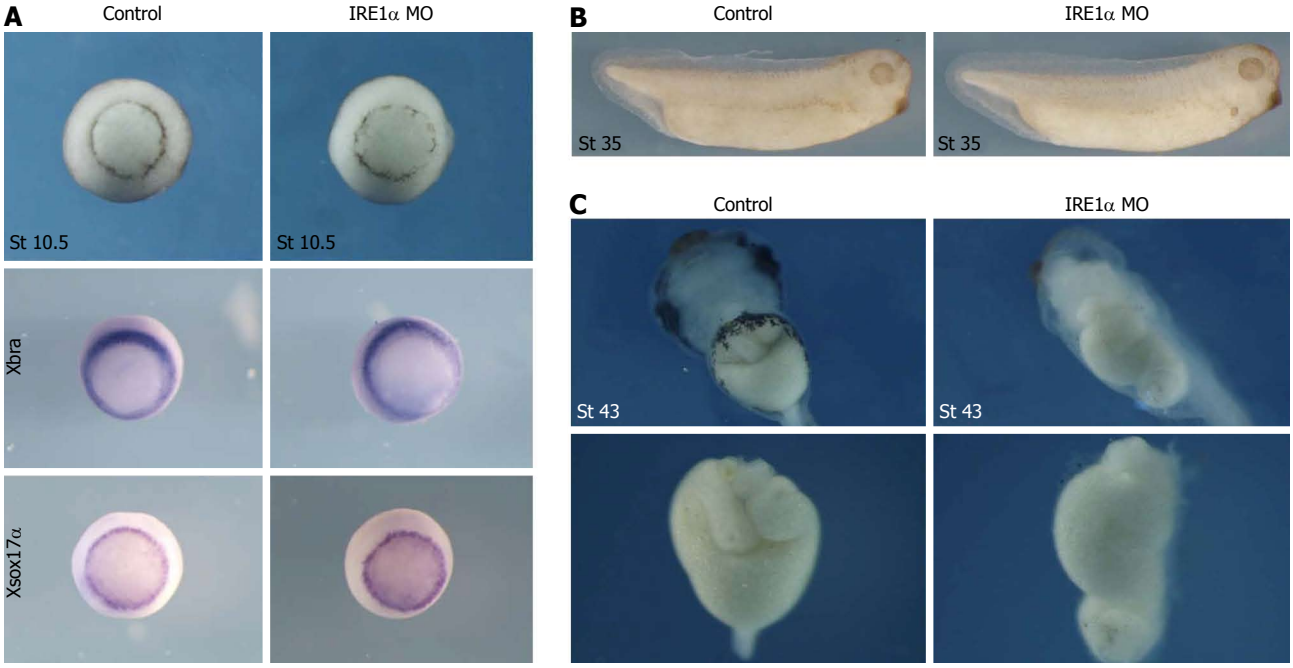

Figure 4 *Xenopus* inositol-requiring enzyme 1α is required for gut development. A: Knockdown of x IRE1α did not change the phenotype and the expression of Xbra and Xsox17α at stage 10.5; B: The inositol-requiring enzyme (IRE) 1α morpholino oligonucleotide (MO)-injected embryos were morphologically normal before the tailbud stages; C: IRE1α knockdown upon injection of 50 ng of MO resulted in a gut defective phenotype. Surgically-resected guts from embryos were shown in C under panel. Coiled structure of gut was not detected in IRE1α MO-injected embryos at stage 43. MO injection was repeated 5 times in a total of 278 embryos.

Figure 5 Rescue of xIRE1α knockdown with IRE1α-GR mRNA. Gut defective phenotype caused by 50 ng of morpholino oligonucleotide (MO) could be rescued by co-injection of 1 ng of inositol-requiring enzyme (IRE) 1α-GR mRNA. Co-injection was done 3 times in a total of 258 embryos.

quired for proper gut development in *Xenopus*.

Deletion mutant of IRE1α

IRE1α is a Ser/Thr protein kinase and endoribonuclease that, upon activation, initiates the unconventional splicing of the mRNA of XBP1[12]. The deletion mutant was constructed consisting of the N-terminal part without the C-terminal kinase and RNase domains (Figure 6A). Injection of IRE1αΔC-GR mRNA caused similar morphological alterations with gut malformation by interfering with the function of endogenous xIRE1α (Figure 6B-D). These data further confirmed the notion that IRE1αΔC represents a dominant-negative form of IRE1α.

XBP1 loss of function

IRE1α is the most evolutionarily conserved branch of the UPR, and upon activation, initiates the unconventional splicing of the mRNA encoding the transcriptional factor XBP1 to attenuate ER stress by mediating UPR. In other words, IRE1α mediates XBP1 splicing. IRE1α MO could knockdown IRE1α expression, and then XBP1 splicing would be repressed. To test if knockdown of XBP1 might cause similar phenotype with IRE1α knockdown, 50 ng of XBP1(C)MO was injected into four blastomeres at 4-cell stage for scoring the phenotype. As shown in Figure 7, injection of XBP1(C)MO caused similar phenotype with gut-coiling defect.

DISCUSSION

In eukaryotic cells, ER is responsible for the early steps in the maturation of most proteins in the secretory pathway, such as folding of the newly synthesized polypeptide chains and post-translational modifications that are essential for protein function[26,27]. Nascent polypeptides are translocated to the ER lumen in an unfolded state, where

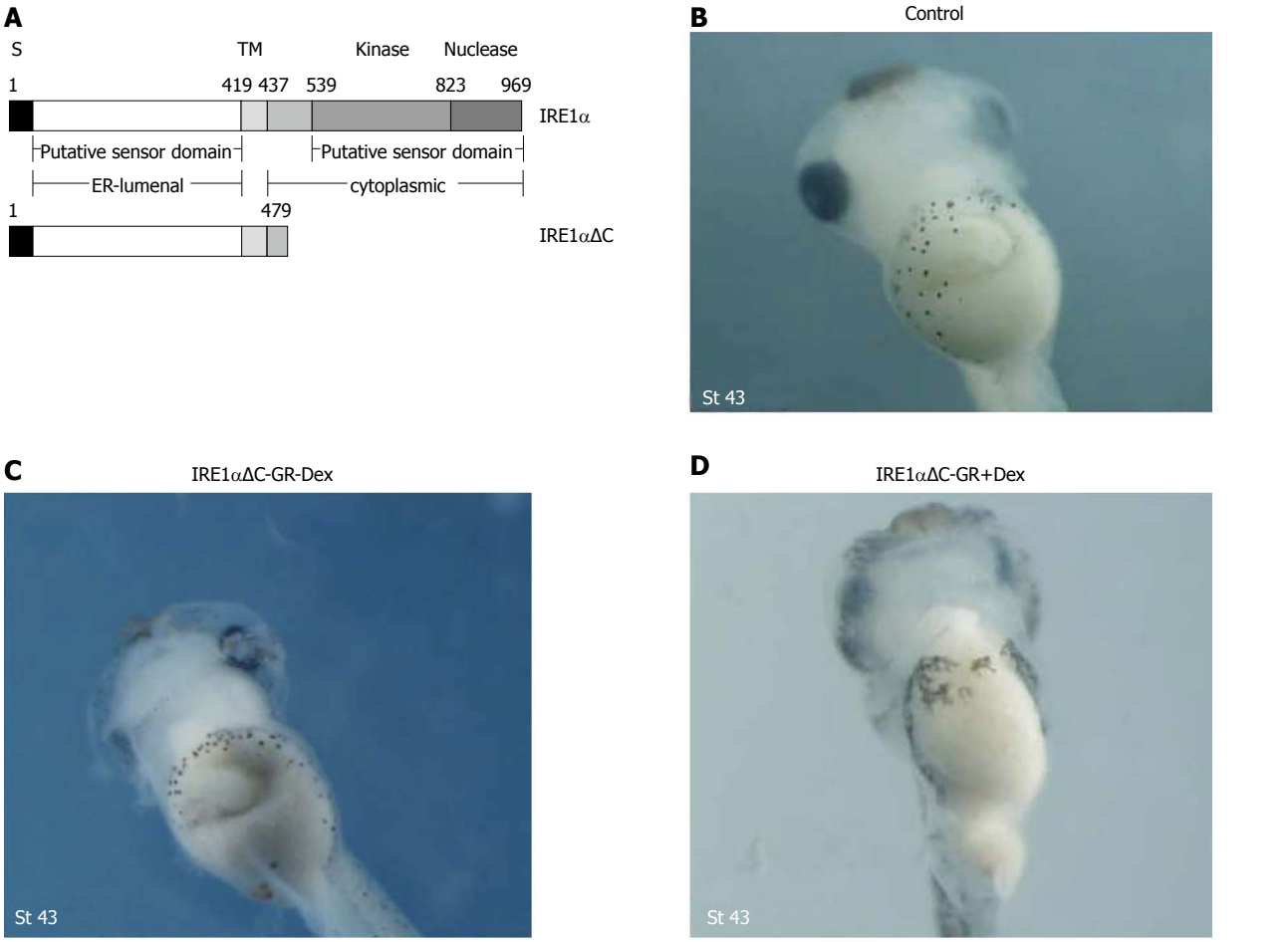

Figure 6 Injection of IRE1αΔC-GR mRNA caused morphological alterations with gut malformation. A: Diagram depicting the construction of deletion mutants. S and TM denote the signal peptide and the transmembrane domain; B-D: Gut defects were also observed with a dominant-negative mutant, IRE1αΔC, lacking the cytoplasmic kinase and RNase domains.

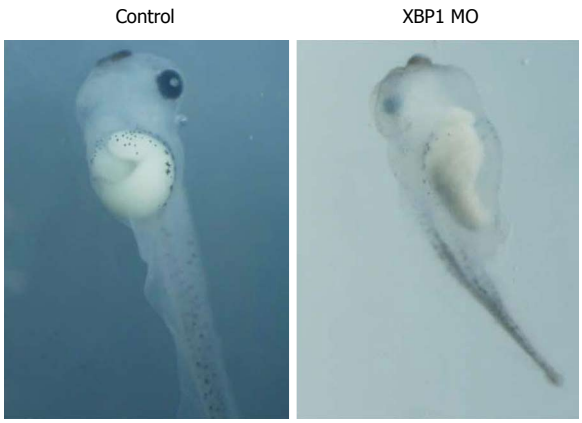

Figure 7 XBP1 knockdown affected gut development. Injection of XBP1(C) morpholino oligonucleotide (MO) caused similar phenotype with gut-coiling defect at stage 43.

they are processed for folding. However, the function of ER will be disrupted when the inflow of unfolded polypeptide chains exceeds the folding or processing capacity of the ER[28,29]. This ER stress in turn leads to the activation of a series of adaptive pathways known as UPR to maintain ER homeostasis[30]. The adaptive process, UPR, has at least two distinct components[4]. The first consists of the rapid and transient attenuation of new protein synthesis and can be considered to be an attempt on the part of the cell to limit the load on the folding apparatus in the ER. The second component consists of the upregulation of expression of genes whose products promote protein folding in the ER and degradation of malfolded proteins. The latter UPR signaling pathways are transduced by three ER resident transmembrane proteins IRE1, PERK and ATF6 upon activation[14], among which IRE1 functions as an endoribonuclease (RNase) to process XBP1 pre-mRNA to a mature form. Spliced XBP1 is a transcriptional activator that plays a fundamental role in the activation of a wide variety of UPR target genes[31,32]. Two mammalian homologues of yeast IRE1 have been identified: IRE1α and IRE1β. IRE1α is expressed in most cells and tissues, with highest levels of expression in the pancreas and placenta[17]. IRE1β expression is prominent only in intestinal epithelial cells[13].

Accumulation of malfolded proteins in the ER occurs under many pathophysiological conditions, in addition, it also takes place during embryonic development. During embryogenesis of vertebrates, such as *Xenopus*, germ layer induction, pattern formation, and morphoge-

netic movement are known to be mediated by secreted proteins, including fibroblast growth factors (FGFs)[33], TGF-β/nodal/BMPs, and Wnts[34,35]. Therefore, dysfunction of the ER should also interfere with the secretion of these proteins and consequently disrupt early embryonic development[36].

Although the effect of ER stress on cellular physiology has been extensively investigated, little is known so far about how it affects early embryonic development. The previous study has verified the conservation of IRE1/XBP1 pathway in *Xenopus* embryos and the importance of IRE1β for mesoderm formation in *Xenopus* embryos[23]. In this study, we demonstrated that (1) IRE1α mainly expressed in pancreas at tadpole stages and IRE1α protein could be detected at all developmental stages analyzed (Figure 1); (2) although it did not cause overt phenotypes upon overexpression (Figure 2), specific knockdown of IRE1α (Figure 4) or XBP1 (Figure 7) led to a gut-coiling defect, and injection of xIRE1αΔC-GR mRNA caused similar morphological alterations with gut malformation (Figure 6); and (3) IRE1α-GR mRNA can rescue IRE1 knockdown phenotypes (Figure 5).

In conclusion, our loss and gain of function data support the notion that IRE1α is required for gut development in *Xenopus* embryos. Our results demonstrate that homeostasis of ER and xIRE1α functions are required for gut development in *Xenopus* embryos. We infer from our results that IRE1 regulates gut development through IRE1α-XBP1 pathway.

COMMENTS

Background

Inositol-requiring enzyme 1α (IRE1α) is an endoplasmic reticulum (ER)-located type I transmembrane protein with a kinase domain and RNase domain in the cytosolic region. IRE1α induces the unconventional splicing of XBP1 mRNA under ER stress condition. However, a XBP1-independent IRE1α function also exists. IRE1α is expressed ubiquitously in fetal and adult mice and is essential for mammalian developmental processes. However, the function of IRE1α in specific organs and tissues remains incompletely understood.

Research frontiers

IRE1α is confirmed to be essential during mammalian development. However, the IRE1α conventional knockout mice showed embryonic lethality, and it has been reported that during development IRE1α is required for B-cell differentiation, placental development and embryonic viability. And the function of IRE1α in specific organs and tissues deserves to be illustrated.

Innovations and breakthroughs

Compared with previous studies, this study used the *Xenopus lavies* as animal model to study the function of IRE1α during their development. After overexpressing and knockdown of the IRE1α, the phenotype could be analyzed. And the results showed that at early stage, IRE1α did not play a significant role in germ layer formation, however, at the stage of organogenesis, knockdown of IRE1α or XBP1 caused gut-coiling defect. The results suggested that IRE1α is not required for germ layer formation, but for gut development in *Xenopus lavies* and it may function *via* XBP1-dependent pathway.

Applications

The results in this study showed that IRE1α does play a role in organogenesis, however, two questions remain to be answered: which organs or tissues in gut are affected and what is the underlying mechanism.

Peer review

This is an interesting article. The authors show in a systematic manner that the role of IRE1a in the gut development of *Xenopus*. They also show that it acts through the XBP1 processing.

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BRIEF ARTICLE

Partially hydrolyzed guar gum in pediatric functional abdominal pain

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Abstract

AIM: To assess the effects of partially hydrolyzed guar gum (PHGG) diet supplement in pediatric chronic abdominal pain (CAP) and irritable bowel syndrome (IBS).

METHODS: A randomized, double-blind pilot study was performed in sixty children (8-16 years) with functional bowel disorders, such as CAP or IBS, diagnosed according to Rome Ⅲ criteria. All patients underwent ultrasound, blood and stool examinations to rule out any organic disease. Patients were allocated to receive PHGG at dosage of 5 g/d (n = 30) or placebo (fruit-juice n = 30) for 4 wk. The evaluation of the efficacy of fiber supplement included IBS symptom severity score (Birmingham IBS Questionnaire), severity of abdominal pain (Wong-Baker Face Pain Rating Score) and bowel habit (Bristol Stool Scale). Symptom scores were completed at 2, 4, and 8 wk. The change from baseline in the symptom severity scale at the end of treatment and at 4 wk follow-up after treatment was the primary endpoint. The secondary endpoint was to evaluate compliance to supplementation with the PHGG in the pediatric population. Differences within groups during the treatment period and follow-up were evaluated by the Wilcoxon signed-rank test.

RESULTS: The results of the study were assessed considering some variables, such as frequency and intensity of symptoms with modifications of the bowel habit**.** Both groups were balanced for baseline characteristics and all patients completed the study. Group A (PHGG group) presented a higher level of efficacy compared to group B (control group), (43% *vs* 5%, P = 0.025) in reducing clinical symptoms with modification of Birmingham IBS score (median 0 ± 1 *vs* 4 ± 1, P = 0.025), in intensity of CAP assessed with the Wong-Baker Face Pain Rating Score and in normalization of bowel habit evaluated with the Bristol Stool Scale (40% *vs* 13.3%, P = 0.025). In IBS subgroups, statistical analysis shown a tendency toward normalization of bowel movements, but there was no difference in the prevalence of improvement in two bowel habit subsets. PHGG was therefore better tolerated without any adverse effects.

CONCLUSION: Although the cause of pediatric functional gastrointestinal disorders is not known, the results show that complementary therapy with PHGG may have beneficial effects on symptom control.

Key words: Functional bowel disorders; Partially hydrolyzed guar gum; Pediatric chronic abdominal pain; Fiber diet

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INTRODUCTION

Functional gastrointestinal disorders (FGIDs) are defined as a variable combination of chronic or recurrent gastrointestinal (GI) symptoms; they are age dependent and not explained by structural or biochemical abnormalities[1]. Chronic abdominal pain (CAP) is the most common condition in FGIDs. It is usually functional without objective evidence of an underlying organic disorder. Apley *et al*[2] introduced the term recurrent abdominal pain (RAP) for the first time in pediatric literature, using it to describe a condition whereby children have experienced at least 3 bouts of pain, severe enough to affect activities, over a period of at least 3 mo. In 1999, the Pediatric Rome Working Group introduced standardized symptom-based criteria for pediatric FGIDs with the publication of the Rome Ⅱ criteria[3]. In 2006, the "Rome Committee"[4] defined new diagnostic criteria (Roma Ⅲ) for pediatric FGIDs, differentiating functional abdominal pain (FAP) from dyspepsia and irritable bowel syndrome (IBS) in that the pain is at a different site with normal bowel habits. The exact prevalence of CAP in children is not known. It seems to account for 2%-4% of all pediatric office visits[5]. Several studies suggested that 13% of middle-school students and 17% of high school students have weekly experience of abdominal pain[6,7]. According to these criteria, IBS is an FGID characterized by abdominal pain or discomfort, accompanied by altered bowel habits (constipation, IBS-c or diarrhea, IBS-d or alternating)[8]. It has been shown that some factors, especially psychological factors, dietary habits, and frequency of exercise, are associated with onset and course of IBS[9-11]. The term "functional" is not real but conceptual and the pathogenesis can be correlated with alterations of visceral sensitivity, increased intestinal permeability, chronic inflammation and presence of a genetic predisposition[12]. Pharmacotherapy generally cannot be recommended for children with FAP, except in the context of clinical trials. Drugs should only be given in exceptional cases[13,14]. Up to 40% of children undergo alternative or complementary therapy such as reassurance, phytotherapy, dietary restrictions or homeopathy[15]. The putative benefit of such methods has not been documented by controlled clinical trials.

The beneficial effects of water-soluble dietary fibers have received attention as complementary therapy in FGIDs, especially in FAP and IBS, for their ability to modify bowel pattern, accelerate oral-to-anal transit and decrease intracolonic pressure and alleviate pain[16,17]. Partially hydrolyzed guar gum (PHGG) is a vegetal, water-soluble, non-viscous, non-gelling dietary fiber that is derived from guar gum, a water-soluble, viscous, gelling polysaccharide found in the seeds of the guar plant. The saccharide component of guar gum is galactomannan[18]. Parisi *et al*[19] showed, in an adult open trial, that PHGG supplementation is followed by a decrease of IBS symptoms, such as abdominal pain and bowel habit. Feldman *et al*[20], in a small, prospective, randomized, double-blind, controlled trial, have revealed that fiber supplementation can improve symptoms in children with FAP. Despite this, there have been no recently available published randomized controlled trials (RCTs) to support the use of fiber in the treatment of CAP in a pediatric population.

The aim of this study was to assess the effect of PHGG diet supplement on CAP and IBS symptoms in paediatric population.

MATERIALS AND METHODS

Sixty patients were prospectively enrolled in the study and randomly assigned to one of 2 study arms (PHGG group or group A: 30 patients; placebo group or group B: 30 patients). Median age was 12.8 years (range 8-16 years) with a greater predominance of females (62% girls and 38% boys). CAP and IBS patients were defined according to the Rome Ⅲ criteria.

All patients were identified into two subgroups: 21/30 (70%) and 19/30 (63%) with constipation-predominant IBS in group A and B respectively; 9/30 (30%) and 11/30 (37%) with diarrhoea-predominant IBS in group A and B respectively. At baseline, the two groups were not statistically different, with respect to age, sex, alterations in bowel movements, incidence and intensity of self-reported symptoms. Subjects' overall baseline demographic and clinical characteristics are summarized in Table 1. All patients underwent ultrasound, blood and stool examinations to exclude organic disease. Seven days before joining the study, patients were asked to not use any medication. The study was performed in accordance with the Declaration of Helsinki and was approved by the local ethics committee. Informed consent was obtained for each patient. All patients completed the trial without any dropouts. Figure 1 is a flow diagram showing the subjects' progression through the study. Patients were consecutively recruited from November 2010 to May 2011, in the Pediatric Gastroenterology Unit of the University of Messina, and randomly assigned to two groups (1:1, PHGG group or group A: 30 patients; placebo group or group B: 30 patients) to receive either a beverage of PHGG (Benefibra, Novartis Consumer Health) at a dosage of 5 g/d in 50 mL of fruit-juice (n = 30) or matching placebo (fruit-juice, n = 30) for 4 wk. For technical reasons of non-laboratory reproducibility of an inert and odorless powder, the placebo consisted of a fruit juice. As in other studies, PHGG was mixed with fruit juice during meals or between meals.

The manufacturer had no role in the conception, design or conduct of the study or in the analysis or interpretation of the data. Randomization was based on a computer-generated list. Supplementation was stopped after 4 wk and patients were followed up for a further 4 wk. GI symptoms were assessed with the "Birmingham IBS Symptom Questionnaire", "Wong Baker Faces Pain Rating Score" and "Bristol Stool Scale". The Birmingham IBS symptoms score consists of 11 questions based

Table 1 Baseline demographic and clinical data

	PHGG group A	Placebo group B	*P* value
n	30	30	
Age (yr)	12.3 ± 2.0	13.1 ± 1.5	0.16
Sex (male/female)	12/18	11/19	0.88
Self-reported pain	3.7 ± 1.2	3.5 ± 1.5	0.15
c-IBS	21/30 (70%)	19/30 (63%)	0.75
d-IBS	9/30 (30%)	11/30 (37%)	0.64

Values are mean ± SD or *n* (%). PHGG: Partially hydrolyzed guar gum; IBS: Irritable bowel syndrome.

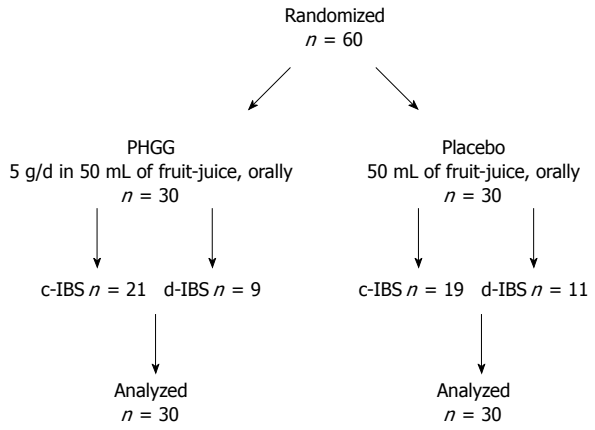

Figure 1 Flow diagram of the progress through the study. PHGG: Partially hydrolyzed guar gum; IBS: Irritable bowel syndrome.

on the frequency of IBS related symptoms. Each question had a standard response scale with symptoms all being measured on a 6-point Likert scale ranging from 0 = none of the time, to 5 = all of the time (Table 2). Wong-Baker Faces Pain Rating Score was used to evaluate CAP severity with a variable score from 0 = no hurt, to 5 = hurts worst. The Bristol Stool Scale classifies the form and consistency of stools into 7 categories (from separate hard lump to entirely liquid stools).

All patients were assessed clinically at 2, 4, and 8 wk (T1, T2, T3) by means of a physical examination and the scoring systems from baseline (Table 3). At T2, compliance with treatment (worse, unchanged, better) was also assessed. Adverse events or any use of other drugs were recorded. Primary outcome was reduction in the frequency and intensity of clinical symptoms and correlation with the improvement of character of stool. Secondary outcome was the evaluation of compliance and safety of PHGG in children.

Statistical analysis

Data are given as mean ± SD. Differences between groups were evaluated by the Kruskall-Wallis one-way analysis of variance (ANOVA). Nominal variables were analyzed with Pearson's chi-square test and Fisher's exact test when, in a 2 × 2 table, one cell had an expected frequency ⩽ 5. Differences within groups during the treatment period and follow-up were evaluated by the Wilcoxon signed-rank test. The statistical level of significance was set at the 5% level ($P < 0.05$). The system utilized was IBM SPSS Statistics Processor.

Table 2 Modified by Birmingham Score Questionnaire[36]

Constipation	Diarrhoea	Pain
Hard bowel motions	Loose, mushy or watery bowel motions	Discomfort or pain
Straining	Diarrhoea	Discomfort or pain after eating
Constipation	Leaked or soiled	Sleep problem
	Urgency	
	Mucus or slime	

Each question had a standard response scale with symptoms all being measured on a 6-point Likert Scale ranging from 0 = none of the time to 5 = all of the time.

RESULTS

The results of the study were assessed considering some main variables, such as improving frequency and intensity of the symptoms and modifications of the bowel habit.

Overall rating of frequency symptoms

At the enrolment visit (T0), mean score evaluation of principal IBS related symptoms (Birmingham Score) confirmed that symptoms were present almost every day, together with a strong functional disability in both groups. Overall, 15 of the 60 (24%) participants with IBS reported treatment success. Those in the PHGG group were more likely to have treatment success than those in the control group (43% *vs* 5%, $P = 0.025$). Responders with significant reduction of Birmingham IBS score (median 0 ± 1 *vs* 4 ± 1, $P = 0.025$) was shown in the PHGG group *vs* placebo at both 4 wk and 8 wk (Table 3). The total score and the three subscale scores for constipation, diarrhoea and pain symptoms of the Birmingham score were significantly improved at the 4 and 8 wk evaluations compared to the baseline in the PHGG group. The supplementation response was comparable both in IBS-d and IBS-c subgroups. In group B, no significant difference was found in comparisons at any evaluation time point for any subscale score.

Bowel habits

At baseline all patients were shown a wide range of alterations in bowel movements, evaluated with Bristol Stool Scale, without any difference in the two treatment groups. Effects of PHGG supplementation (5 g/d) for 4 wk on fecal output in IBS-d and IBS-c subsets *vs* placebo were also evaluated. In group A, there was a tendency toward normalization of bowel movements, which is highlighted by the progressive normalization of Bristol Stool Scale at type 3 or 4 (Table 3). In particular, 16 (26.6%) of 60 patients had normalized bowel habits: in the PHGG group the prevalence of improvement was 40% (12 patients), while it was 13.3% (4 patients) in the placebo group ($P = 0.025$). This result remained constant during the follow-up 4 wk. There was no difference in the prevalence of

Table 3 Outcome measures at baseline (T0), at 4 wk of supplementation (T2) and after 8 wk (mean ± SD)

	Group A PHGG 5g/d (n = 30)			Group B Placebo (n = 30)		
	Baseline	4 wk (T2)	8 wk (T3)	Baseline	4 wk (T2)	8 wk (T3)
Birmingham Score[a]	28.5 ± 7.16	24.3 ± 6.02	23.0 ± 6.15	29.5 ± 6.94	28.4 ± 8.39	28.7 ± 7.54
Bristol Stool Score[a]						
IBS-c	1.00 ± 1.02	2.02 ± 1.50	2.32 ± 1.50	1.16 ± 0.89	1.76 ± 1.04	1.65 ± 1.08
IBS-d	5.02 ± 0.63	4.01 ± 0.16	4.07 ± 0.12	5.54 ± 0.32	4.86 ± 0.96	4.89 ± 0.73
Wong-Baker Score[a]	2.15 ± 0.14	1.86 ± 0.14	1.63 ± 0.16	2.16 ± 0.17	2.04 ± 0.17	2.05 ± 0.19

[a]$P < 0.05$ *vs* placebo (Wilcoxon 2-sample test). PHGG: Partially hydrolyzed guar gum; IBS: Irritable bowel syndrome.

improvement in two bowel habit subsets ($P > 0.05$).

Intensity of the abdominal pain

There was no difference in pain intensity reported at baseline between the groups as Wong-Baker Face Pain Rating Score. During the course of study, there was a decrease in the intensity of pain in the group of children given PHGG, which was not seen in the placebo-supplemented group (Table 3). However, this result was not statistically significant ($P > 0.05$), compared with baseline at wk 4 and 8. Improvement of clinical symptoms in group A was correlated with a change of bowel habit and persisted 4 wk (T8) after cessation of PHGG supplementation. The clinical response was comparable both in IBS-d and IBS-c subgroups (Table 3). Analysis of the data confirmed optimal compliance and safety of PHGG dietary supplementation.

DISCUSSION

CAP is common in children and adolescents. In most children, CAP is functional without objective evidence of an underlying organic disorder. Children with CAP are more likely than children without CAP to have headache, joint pain, anorexia, vomiting, nausea and altered bowel habit assignable to IBS[21]. Physicians must decide whether to order diagnostic tests or use conservative management. The presence of alarm symptoms or signs suggests higher pretest probability and prevalence of organic disease and may justify the performance of diagnostic tests. CAP can cause long absences from school and markedly worsens quality of life of the children and parents[22,23].

In a recent American study, the diagnostic evaluation of CAP in a tertiary center in United States was found to cost approximately $6000 per patient[24]. The first treatment step is an age-appropriate assessment through the reassurance of the child and family on the absence of organic causes, but this does not mean that abdominal pain is not a real problem. Cognitive behavioral therapy, however, is an effective form of alternative treatment[25,26].

A thorough review of literature, with a focus on RCTs, revealed a paucity of studies examining effectiveness of pharmacologic and dietary interventions. Definitive statements concerning therapeutic efficacy are quite limited. Huertas-Ceballos *et al*[15], in a meta-analysis, failed to reveal any therapeutic benefit from a low-lactose or high-fiber diet for children with CAP. Therapeutic trials in adults with CAP associated with IBS symptoms have revealed a high rate of the placebo-response, confirming that non-pharmacological therapies alone are often adequate for many patients. PHGG is a soluble fiber with important properties, such as non-viscous texture, normal fermentation, non gelling, high hydrophilic potential and no interference with micronutrient absorption[27]. There is clear evidence that fiber decreases whole gut transit time, accelerates oral-to-anal transit, and decreases intracolonic pressure reducing abdominal pain. Fiber may represent a mainstay in the FAP and IBS therapeutic algorithm. Results of fiber supplementation in the adult population in patients with FAP and IBS has produced contrasting results, and the main reason for the variation is correlated with different types of fiber used[28,29]. The main distinction between soluble and insoluble fiber is essential as only soluble fiber such as PHGG dissolves in water and is widely metabolized in the large bowel, thus producing short-chain fatty acids, leading to selective stimulation of microbial growth[30,31]. PHGG may also act as prebiotic, thus modulating intestinal microbiota. Weaver *et al*[32], in experimental studies in rats, demonstrated that PHGG administration was accompanied by a rise in butyrate concentrations of colonocytes. Tuohy *et al*[33] showed that PHGG supplementation in healthy volunteers caused selective increase in the percentage of *Bifidobacteria* and *Lactobacilli* with beneficial modulation of microbiota that has been reported to ameliorate IBS symptoms, with a decrease in pain and flatulence[34].

Bijkerk *et al*[35] observed in an adult population that, although general fiber supplementation globally alleviates IBS symptoms, the beneficial effect is mainly associated with the use of soluble fiber rather than insoluble fiber. This study demonstrated that soluble fiber is effective in decreasing global IBS symptoms[36] but was no better than a placebo. Some of these above mentioned studies on the use of fiber in adult populations were biased as they confirmed that the placebo response in IBS patients ranged from 20%-50%. In our study, the placebo response was much lower than expected.

In 2012, a systematic review identified 3 RCTs evalu-

ating fiber supplementation in children with FGIDs. Patients were supplemented with different dietary fiber types for 4-6 wk[57]. Among these, the Feldman[20] study, a randomized, double-blind, placebo-controlled trial, is the only study in children with CAP (26 for group) recruited from primary care practices and supplemented with soluble fibers. Improvement of symptoms in treated patients with fiber not was significant *vs* the placebo-group. In patients with IBS symptoms with modification of the bowel habit, water-soluble fibers, such as PHGG, decrease symptoms also with a prebiotic effect, beneficial modification of the intestinal microflora and selective increase of *Lactobacilli* and *Bifidobacteria*[38]. PHGG was therefore tolerated and preferred by patients, indicating higher success of soluble fiber than bran or insoluble fiber. The present findings confirm the beneficial effects of PHGG at 5 g/d and in the short term (4 wk). Our study can be considered the first prospective, randomized, controlled, single-blind, clinical trial conducted with this particular fiber supplementation (PHGG) in pediatric CAP and IBS. Some limitations should caution against generalizing from the results of this study, such as the classification at baseline of CAP according to severity of symptoms (mild, moderate and severe) and lack of knowledge of dietary habits in patients enrolled. Given the good results obtained for the first time, it is important to confirm these preliminary data on a greater number of patients and also to consider the active role of liquid fiber in improvement of symptoms. The efficacy of this approach has proven how dietary management is more effective than pharmacological therapy in children with CAP and IBS.

In summary, fiber supplementation can be considered an important option in pediatric CAP and IBS. Water-soluble fiber, such as PHGG, is preferable to insoluble fiber. Moreover, initial studies have shown that fiber may act as a prebiotic, thus increasing the therapeutic benefits. Further placebo-controlled studies are needed to evaluate whether PHGG can also be seen as a maintenance therapy of CAP.

COMMENTS

Background

Functional bowel disorders, such as chronic abdominal pain (CAP), are frequent in children and similar to adult irritable bowel syndrome (IBS). Some children with CAP develop substantial disability and limitations in physical and psychosocial functions. There is little evidence of the efficacy of conventional medical treatment while there is a moderate evidence for the efficacy of complementary therapy (diet, fibers, low-lactose intake) in the adult population. Water-soluble fibers, such as oats, barley and gums in psyllium, can be safe in IBS symptoms.

Research frontiers

Water-soluble fibers are known for their ability to modify bowel patterns, accelerate oral-to-anal transit, decrease intracolonic pressure and alleviate pain. Functional abdominal disorders, such as abdominal pain and IBS, are frequent also in pediatric populations and should stimulate the trend to conservative therapy.

Innovations and breakthroughs

To date, there has been a limited number of studies regarding specific optional treatment in CAP and IBS. This is the first study in a pediatric population that showed a clinically significant improvement of the symptoms in pediatric functional gastrointestinal disease with dietary manipulation. The small sample size and a low placebo effect may indicate a requirement for request powered and well designed randomized controlled trials on the clinical effectiveness and safety of dietary treatment.

Applications

The findings in this study indicate that fiber supplementation can be considered an important therapeutic option in pediatric IBS.

Peer review

It is very well written and the topic is very interesting for the readers. This is an important topic for gastroenterologists, clinicians, surgeons, Critical Care doctors and nutritionists.

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BRIEF ARTICLE

Atorvastatin and rosuvastatin do not prevent thioacetamide induced liver cirrhosis in rats

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Abstract

AIM: To examine whether the administration of atorvastatin and rosuvastatin would prevent experimentally-induced hepatic cirrhosis in rats.

METHODS: Liver cirrhosis was induced by injections of thioacetamide (TAA). Rats were treated concurrently with TAA alone or TAA and either atorvastatin (1,10 and 20 mg/kg) or rosuvastatin (1, 2.5, 5, 10 and 20 mg/kg) given daily by nasogastric gavage.

RESULTS: Liver fibrosis and hepatic hydroxyproline content, in the TAA-treated group was significantly higher than those of the controls [11.5 ± 3.2 *vs* 2.6 ± 0.6 mg/g protein (P = 0.02)]. There were no differences in serum aminotransferase levels in the TAA controls compared to all the groups treated concomitantly by statins. Both statins used in our study did not prevent liver fibrosis or reduce portal hypertension, and had no effect on hepatic oxidative stress. Accordingly, the hepatic level of malondialdehyde was not lower in those groups treated by TAA + statins compared to TAA only. *In vitro* studies, using the BrdU method have shown that atorvastatin had no effect of hepatic stellate cells proliferation. Nevertheless, statin treatment was not associated with worsening of liver damage, portal hypertension or survival rate.

CONCLUSION: Atorvastatin or rosuvastatin did not inhibit TAA-induced liver cirrhosis or oxidative stress in rats. Whether statins may have therapeutic applications in hepatic fibrosis due to other etiologies deserve further investigation.

Key words: Liver cirrhosis; Statins; Thioacetamide

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INTRODUCTION

Liver cirrhosis is one of the leading causes of morbidity and mortality worldwide. Hepatic stellate cells (HSC) play a major role in the pathogenesis of hepatic fibrosis[1]. Injury to hepatocytes results in generation of lipid peroxides, which may have a direct stimulatory effect on matrix production by activated HSC[2]. It has been suggested that aldehyde-protein adducts, including products of lipid peroxidation, modulate collagen gene expression in human fibroblasts[3,4] and may be a link between tissue injury and hepatic fibrosis[2,5]. Several

studies demonstrated that activation of HSC in culture is provoked by generation of free radicals and is blocked by anti-oxidants. This activation may involve the transcription factor c-myb and nuclear factor kappa B (NF-κB)[6,7]. Accordingly, antioxidants have been suggested as therapeutic modalities in experimental models[8-10], and in patients with chronic liver injury[11].

3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) are used extensively to reduce serum cholesterol in an effort to reduce atherosclerotic cardiovascular morbidity and mortality. In addition to their cholesterol-lowering effect, statins demonstrate other biological effects (pleiotropic effects) that some of them may lead to clinical benefits. Those include anti-inflammatory[12] and antioxidant effects[13], inhibition of PDGF-stimulated proliferation and upregulation of tumor growth factor (TGF)-β signaling in murine mesangial cells and cultured heart cells[14-16].

Over the past decade the potential effect of statins as anti-fibrotic agents has received increasing attention. The rationale for the anti fibrotic efficacy is based on the ability of statin compounds to: (1) decrease the growth of human Ito cells in vitro, independently of their effect on cholesterol synthesis[17]; (2) inhibit the proliferation rate of HSC and reduction of the collagen protein steady state levels by both simvastatin and lovastatin[18]; (3) inhibit steatosis, hepatic fibrosis and carcinogenesis in a rat model of non-alcoholic steatohepatitis (NASH)[19]; and (4) enhance hepatic nitric oxide production and decrease hepatic vascular resistance in patients with cirrhosis and portal hypertension[20]. In addition to inhibiting stellate cell activation, the anti-oxidative activity and the attenuation of inflammation by specific statin derivatives may also contribute to the inhibition of fibrosis. Despite the existing data suggesting that statin derivatives can inhibit fibrosis by various mechanisms, treatment with simvastatin or pravastatin did not decrease fibrosis-induced by bile duct ligation (BDL) or carbon tetrachloride (CCl_4) in rats[21,22]. However, one recent study showed that very early atorvastatin treatment inhibits HSC activation and fibrosis in the BDL model *in vivo*[23].

To further elucidate the anti-fibrotic activity of statins in the liver, we examined the effects of both atorvastatin and rosuvastatin in a well characterized model of chronic thioacetamide (TAA)-induced administration in which cirrhosis is mainly produced *via* the formation of reactive oxygen species. TAA undergoes an extensive metabolism to acetamide shortly after administration, and to the hepatotoxic reactive metabolite thioacetamide-S-oxide by the mixed function oxidase system[24-26]. We hypothesized that inhibition of HSC activity in addition to the anti inflammatory and anti oxidative effects induced by statins may prevent the hepatic damage induced by TAA in rats.

Our results indicate that both atorvastatin and rosuvastatin did not diminish neither oxidative stress nor the development of TAA-induced cirrhosis in rats, and also had no effect on the proliferation of cultured HSC.

MATERIALS AND METHODS

Materials and animals

TAA, atorvastatin and rosuvastatin was purchased from Sigma (Sigma Chemical Co., St. Louis, MO). Male Wistar rats (250-300 g), obtained from Tel-Aviv University Animal Breeding Center, were kept in the animal breeding house of the E. Wolfson Medical Center and fed a Purina chow *ad libitum*. Animals were kept with a 12-h light-dark cycle at constant temperature and humidity and the rats had free access to tap water during the study period. Use of animals was in accordance with the National Institutes of Health Policy on the care and use of laboratory animals and was approved by the Animal Use and Care Committee of the E. Wolfson Medical Center.

Induction of liver cirrhosis

For induction of liver cirrhosis, rats were given intraperitoneal injections of thioacetamide, 200 mg/kg, twice a week for 12 wk, as previously described[27]. Control rats were treated with intraperitoneal injections of NaCl 0.9%.

Analysis of liver histopathology

The rats were sacrificed at the completion of the treatment protocols, their livers were removed, and midsections of the left lobes of the livers were processed for light microscopy. This processing consisted of fixing the specimens in a 5% neutral formol solution, embedding the specimens in paraffin, making sections of 5 μm thickness, and staining the sections with hematoxylin and eosin and Masson Trichrome. The tissue slices were scanned and scored blindly by two expert pathologists. The degree of fibrosis was expressed as the mean of 10 different fields in each slide, which had been classified on a scale of 0-4 according to Batts and Ludwig[28].

Measurement of hepatic hydroxyproline levels

Quantitative determination of hepatic hydroxyproline content was performed as previously described[29].

Measurement of hepatic malondialdehyde

For the determination of the hepatic content of malondialdehyde, liver tissue (5 g) was cut into small pieces using a razor blade, and homogenized after dilution in H_2O 1:10 w/v. Liver homogenate was centrifuged in 900 g for 5 min, and then the supernatant was collected and centrifuged in 20 000 rpm in Sorvall for 30 min using plastic tubes. The clear supernatant was obtained and malondialdehyde was measured and expressed as nmole/g wet tissue using the thiobarbituric acid method[30]. Briefly, to 1 mL of 10% liver homogenate with 1.15% KCl were added 2 mL of fresh solution 15% w/v TCA, 0.375% w/v thiobarbituric acid, 0.25 mL/L HCl. The mixture was heated at 95 ℃ for 15 min. The solution was cooled to room temperature using tap water and centrifuged at 300 rpm for 10 min. Absorption of the pink supernatant was determined spectrophoto-

metrically at 532 nm.

Effect of atorvastatin on proliferation of primary hepatic stellate cells

Hepatic stellate cells were isolated from male rat using sequential pronase-collagenase digestion followed by Nycodenz (Sigma-Aldrich, Inc., St. Louis, MO, United States) density gradient centrifugation essentially as described previously with minor modifications[1]. Briefly, the liver of male rat Wistar (300-400 g) was minced with scalpels and incubated with 100 mL of freshly prepared and filtered-GBSS with 65 mg pronase (Roche Molecular Biochemicals, Indianapolis, IN, United States), 50 mg collagenase (Worthington Biochemical Corporationv, Lakewood, NJ, United States), and 0.5mL 2.7%$CaCl_2$ for 15 min at 37 ℃ with 200 rpm shaking. Then 50 mL of freshly prepared and filtered-GBSS was added containing 12.5 mg pronase, 12.5 mg collagenase, 20 μg/mL DNAse I (Sigma-Aldrich, Inc., St. Louis, MO, United States) and 0.25 mL 2.7% $CaCl_2$ for 30 min at 37 ℃ with 200 rpm shaking. The digested tissue was filtered through a sterile 150-μm metal mesh, and the cells were centrifuged at for 2000 rpm for 7 min. The digested liver hepatic stellate cells were isolated on a 17.5% Nycodenz gradient centrifuged at 2700 rpm for 20 min. A stellate cell-enriched fraction was present in the upper layer. Cells were washed twice by centrifugation (1200 rpm, 4 ℃, 5 min) in DMEM with 10% fetal calf serum (FCS), 100 μg/mL penicillin and 100 μg/mL of streptomycin.

Reagents

Atorvastatin (Sigma-Aldrich, Inc., St. Louis, MO, United States) stock solution was dissolved in DDW to a concentration of 5×10^{-4} mol/L. PDGF-BB (Peprotech Inc. NJ, United States) was dissolved in DDW to a concentration of 1μg/mL. All the reagents were aliquot and stored in -20 ℃ until use.

Proliferation assays

The proliferation of HSC was examined by BrdU method (Exalpha biological, Inc. Watertown, MA, United States). Primary HSC were cultured for 14 d, after which they were trypsinized and plated at a density of 20 000 cell/well in 96 well plates in DMEM containing 10% FCS. The cells were incubated for 24 h, and then they were serum starved in DMEM + 0.5% FCS overnight. At the following day, the various stimuli were added in medium containing 0.5% FCS. HSC were exposed to either 30 ng/mL of PDGF (Peprotech, Inc. NJ, United States), and different concentration of Atorvastatin (5×10^{-8} mol/L, 10^{-7} mol/L, 5×10^{-7} mol/L) alone, or combination of the two. After 24 h the cells were tested for proliferation according to the manufacture instruction.

Western blotting

HSCs were plated on 100 mm plates at a density of 2×10^6 cells/plate. After 24 h, the medium was changed to starvation medium (DMEM + 0.5% FCS) overnight. The following day, cells were incubated for 24 h with the different treatments according to which experiments were performed. Total proteins were extracted by incubating the cells for 30 min on ice in RIPA buffer containing a 1:100 dilution of a protease inhibitor cocktail (Sigma-Aldrich, Inc., St. Louis, MO, United States). After 20 min centrifugation at 14 000 rpm at 4 ℃, extracts were normalized to total protein content, determined using the BCA Reagent (Sigma-Aldrich, Inc., St. Louis, MO, United States). Equal amounts of total protein-were separated in 4%-12% bis-tris (BT) gels (NuPAGE, Gibco-BRL Life Technologies, Grand Island, NY), blotted onto Hybond C extra membranes, blocked overnight in 5% milk, and incubated with antibodies against α smooth muscle actin (αSMA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, Santa Cruz, CA, United States) and then incubated with horseradish peroxidase-conjugated secondary antibody. Afterwards, signals were detected by chemiluminescent. Expression of proteins was normalized to the expression of GAPDH.

Experimental design (in vivo experiments)

Control groups: (1) normal controls: injections of NaCl 0.9%; and (2) cirrhotic controls: TAA (200 mg/kg *ip*, twice weekly) injections for 12 wk.

Atorvastatin groups: (1) TAA + Atorvastatin 1 mg/kg daily; (2) TAA + Atorvastatin 10 mg/kg daily; and (3) TAA+ Atorvastatin 20 mg/kg daily.

Rosuvastatin groups: (1) TAA + Rosuvastatin 2.5 mg/kg daily; (2) TAA + Rosuvastatin 5 mg/kg daily; (3) TAA + Rosuvastatin 10 mg/kg daily; and (4) TAA + Rosuvastatin 20 mg/kg daily.

Each subgroup included 6-7 rats. The statins were given *po*, started concurrently with TAA and continued during the study. When the treatments were completed after 12 wk the rats were sacrificed, their livers were removed and the weights of their spleens measured.

Statistical analysis

The data are presented as median (range). The significance of differences among different groups was determined by a student's *t*-test.

RESULTS

Induction of liver cirrhosis by thioacetamide

Intraperitoneal administration of TAA for 12 wk, resulted in a uniform coarse granulation of the surface of the rats' liver. Microscopic analysis revealed liver cirrhosis, characterized by mixed-sized fibrotic nodules in these TAA-treated rats. Neither atorvastatin nor rosuvastatin had any effect on liver enzymes when given alone or in addition to TAA.

Table 1 Effects of rosuvastatin and atorvastatin treatment on oxidative stress and liver fibrosis in thioacethamide-treated rats

	ALT	MDA nmole/g liver	Hydroxyproline (mg/g protein)	Fibrosis score (0-4)	Spleen weight (mg)
Rosuvastatin dose (mg/kg)					
Control	59 (44, 69)	30.6 (20.5, 31.1)	2.67 (1.9, 3.3)	0	1200 (1100, 1400)
Ros 2.5	52 (47, 58)	24.1 (19.8, 33.9)	2.7 (2.2, 3.5)	0	1020 (950, 1150)
Ros 5	50 (41, 57)	31.3 (26.9, 36.7)	2.8 (2.3, 3.1)	0	1100 (950, 1300)
R 10	56 (46, 64)	33.7 (27.7, 37.8)	2.3 (1.8, 3.7)	0	1000 (800, 1200)
R 20	44 (41, 47)	29.4 (23.9, 39.6)	3.9 (2.4, 4.2)	0	1200 (900, 1200)
TAA	63 (37, 79)	48.7 (38.4, 50.2)	11.2 (4.5, 15.4)	4 (1.5, 4)	1900 (1600, 2200)
TAA + R 2.5	53 (47, 63)	22 (19, 31.2)	7.5 (3.7, 13.1)	2.5 (1, 4)	1450 (1050, 1950)
TAA + R 5	54 (48, 67)	36.5 (24.8, 52.1)	11.0 (4.7, 16.2)	3.25 (1, 4)	1230 (1000, 1350)
TAA + R 10	51 (43, 60)	37.8 (27.5, 44.9)	11.8 (8.3, 16.2)	3 (2, 4)	1200 (1100, 1500)
TAA + R 20	51.5 (48, 55)	39.2 (29.6, 46.8)	12.3 (3.1, 14.3)	3.5 (1, 4)	1050 (900, 1300)
Atorvastatin dose (mg/kg)					
Control	59 (44, 69)	30.6 (20.5, 31.1)	2.67 (1.9, 3.3)	0	1200 (1100, 1400)
Ato 1	61 (55, 81)	32.8 (27.4, 33.6)	2.5 (2.1, 3.1)	0	1300 (1100, 1400)
Ato 10	56 (53, 59)	32.9 (29.1, 35.2)	3.4 (1.6, 3.7)	0	1200 (1100, 1300)
Ato 20	58 (48, 73)	30.1 (29.8, 35.3)	2.3 (1.95, 3)	0	1100 (1100, 1200)
TAA	63 (37, 79)	48.7 (38.4, 50.2)	11.2 (4.5, 15.4)	4 (1.5, 4)	1900 (1600, 2200)
TAA + Ato 1	49 (22, 70)	46.6 (35.8, 63.1)	9.1 (6.7, 16.1)	3 (2, 4)	1600 (900, 3000)
TAA + Ato 10	53 (47, 62)	43.4 (36.2, 53.1)	12.5 (6.9, 15.4)	4 (2, 4)	1500 (1400, 1700)
TAA + Ato 20	57 (43, 67)	48.2 (34.2, 58.5)	10.9 (5.3, 16.8)	4 (3, 4)	1500 (1300, 1700)

n = 7, in the groups that received thioacethamide (TAA), n = 4 in the control, rosuvastatin only treated groups and atorvastatin only treated groups. TAA, 200 mg/kg *ip* twice weekly for 12 wk. Rosuvastatin and atorvastatin had given daily by nasogastric gavage. Ros: Rosuvastatin; ALT:Alanine transaminase; MDA: Malondialdehyde.

Inhibition of thioacetamide-induced liver cirrhosis by statins

Compared to the rats which received TAA only, neither atorvastatin nor rosuvastatin had protective effect on the histopathologic score after 12 wk and TAA-induced liver cirrhosis was not inhibited by all the doses that were examined. Hepatic fibrosis was also quantitated by the measurement of hepatic hydroxyproline levels. The mean hydroxyproline levels of the TAA-treated group were similar to those of the TAA plus atorvastatin or rosuvastatin at all doses used.

Spleen weights

An indirect measure of portal hypertension was obtained by measuring the weights of the rats' spleens at the end of the experiment. Characteristic hemodynamic changes have previously been shown after 3 mo of TAA administration, i.e., upon TAA-induced liver cirrhosis[27]. These changes include portal hypertension and hyperdynamic circulation which are accompanied with a significant increase in spleen weight. After 12 wk, the mean spleen weight of rats receiving TAA was about 30% higher than those receiving injections of 0.9% NaCl or statins only. The mean spleen weight of rats that received statins in addition to TAA was not lower than that of TAA alone (Table 1).

Hepatic levels of malondialdehyde and lipid peroxides

The hepatic levels of malondialdehyde measured after 12 wk, were not significantly different in the rats treated with TAA and statins compared to TAA only. Table 1 summarize the rosuvastatin and atorvastatin treatment effects on oxidative stress and liver fibrosis in TAA-treated rats [median (range)], n = 6-7.

Effect of Atorvastatin on hepatic stellate cells proliferation and smooth muscle actin expression

Atorvastatin in different concentrations had no effect on HSC proliferation as examined by the BrdU method (Figure 1) and no effect on the expression of smooth muscle actin determined by western blot analysis (Figure 2).

DISCUSSION

Our major finding in the present study is that atorvastatin and rosuvastatin do not have a therapeutic value as a potential anti-oxidant or anti-fibrotic agents targeting increased oxidative stress or liver fibrosis induced by TAA in rats.

There are various experimental observations regarding the direct anti-fibrotic activity of the statins by the inhibition of stellate cell proliferation; lovastatin inhibits pancreatic stellate cell activation and alpha-smooth muscle actin expression[31] and both simvastatin and lovastatin interferes with HSC activation in vitro[20,21]. Fluvastatin reduces renal fibroblast proliferation and collagen type Ⅲ production[32] and suppresses oxidative stress and kidney fibrosis after ureteral obstruction[33]. It is also possible that the anti-fibrotic effects of statins are mediated through mechanisms that stimulate fibroblast apoptosis *in vitro* and *in vivo* as it was shown in lung and renal fibroblasts[32,34]. Alternatively, the anti-fibrotic effects of statins may be mediated through their newly recognized anti inflammatory[12] and antioxidant mechanisms[35-37]. These include the inhibition of myeloperoxidase derived and nitric oxide derived oxidants[36],

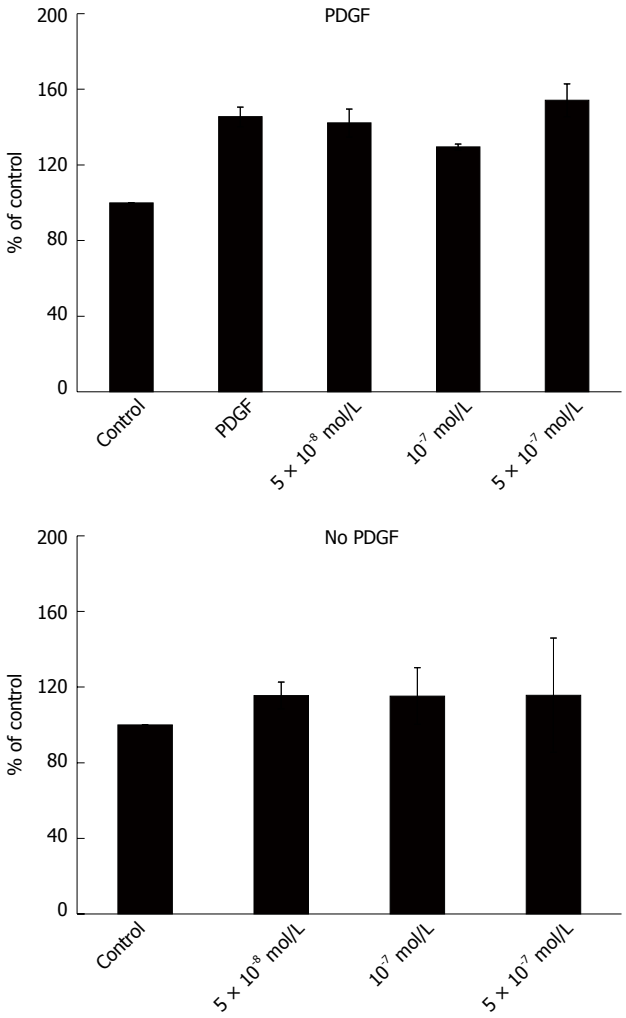

Figure 1 Effect of atorvastatin on hepatic stellate cells proliferation. PDGF: Platelet derived growth factor.

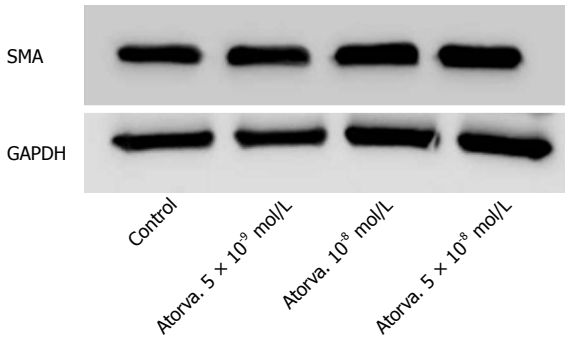

Figure 2 Atorvastatin effect on the expression of smooth muscle actin. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; SMA: Smooth muscle actin.

S-nitrosylation and activation of thioredoxin in endothelial cells[37], decreased expression of essential NAD(P)H oxidase subunits and upregulation of catalase expression in vascular smooth muscle cells[35]. Statins also induce the expression of a protein with antioxidant and anti-inflammatory functions, heme oxygenase-1 (HO-1), *in vitro* and *in vivo*[33,38]. However, this effect is tissue specific demonstrating significant increase in liver HO-1 with simvastatin and lovastatin but not atorvastatin and rosuvastatin. The involvement of hydroxyl radicals and oxidative stress in TAA induced cirrhosis[39], the anti-oxidative effects of different statins, including atorvastatin, and the antifibrotic effect of atorvastatin in a rat model if BDL as was reported recently by Trebicka *et al*[23], provided a good rationale for the assumption that statins may reduce or prevent fibrogenesis in this specific animal model.

The explanation for the unexpected failure of atorvastatin and rosuvastatin to inhibit fibrosis in this model of cirrhosis are not clear. Two studies demonstrated anti fibrogenic effects of atorvastatin in a CCl_4 induced fibrosis[40,41]. Gardner *et al*[41] have shown that atorvastatin exhibited statistically significant, although modest, suppression of CCl_4-induced fibrogenesis after 3 wk of treatment. This was shown only using a novel technique for measuring hepatic collagen synthesis *in vivo* through metabolic labeling with heavy water (^2H_2O). Histopathology of the same tissues revealed no significant differences in fibrosis scores among groups that received co-treatment with atorvastatin, emphasizing the importance of the fibrosis measuring method.

Nevertheless the evidence that fibrosis was not inhibited by atorvastatin and with a more potent cholesterol metabolism pathway inhibitor, rosuvastatin, suggests that this effect is not selective and occurs independently of their HMG-CoA inhibition. Since we used much higher doses than those used clinically (up to 20 mg/kg) it does not seem that we are dealing with under dose treatment. Appropriate timing for the administration of the statins may also be critical. Later therapy in the BDL model, for example, lacked significant effects on fibrosis with no change in hepatic inflammation[23]. The statin treatment in our study began in parallel to the administration of TAA. Although, it is possible that pretreatment by statins would be more effective, the fact that there was no improvement, neither in MDA levels nor in fibrosis after three months, do not support this hypothesis. Another possibility to explain these negative results might be the selection of the wrong statins. Indeed, the two statins that inhibited HSC proliferation in vitro were lovastatin and simvastatin[18] and atorvastatin *in vivo*. In addition pitavastatin was the one that inhibited hepatic fibrosis in a choline-deficient L-amino acid defined diet liver fibrosis[19]. However, despite these effects, even the latter mechanisms were not efficient to prevent or inhibit liver fibrosis, as it was demonstrated with simvastatin or pravastatin in two different animal models of cirrhosis (bile duct ligation and CCl_4), in rats[21,22]. Moreover, antioxidant therapy in human clinical trials also lack or have minimal effect in chronic liver disease[42,43]. Finally, statin-induced protein kinase C (PKC) activation in activated HSCs may interrupt statin-induced HSC apoptosis, thereby reducing its antifibrotic efficacy. Indeed Yang *et al*[44] recently demonstrated that simultaneous treatment with pravastatin and PKC inhibitor may

synergistically enhance antifibrotic efficacy in hepatic fibrosis induced by intraperitoneal injection of carbon tetrachloride or thioacetamide in mice. Finally, we also have to consider the possibility that the negative results may be due to the small numbers (type 2 error).

To further explore the effect of statins on liver fibrosis we examined the effect of several atorvastain concentrations on the proliferation of primary HSC. We observed that atorvastatin had no inhibitory effect on HSC proliferation either in the presence or in the absence of PDGF (Figure 1). This finding is in discordance with several previous studies that demonstrated decreased HSC activation by statins[17,18]. Moreover, using western blot analysis we found that atorvastatin (5×10^{-8}-10^{-9} mol/L) had no effect on the expression of α smooth muscle actin further supporting the lack of effect shown in the proliferation assay.

It is of interest that treatment with atorvastatin or rosuvastatin in all doses did not reduce spleen weights, a parameter of portal hypertension. This is in contrast to the recently described therapeutic effects of simvastatin in patients with cirrhosis and portal hypertension. In patients with cirrhosis and portal hypertension simvastatin enhanced hepatic nitric oxide production and decreased hepatic resistance[20]. Similarly, in cirrhotic rats, induced by BDL, atorvastatin has been shown to lower intrahepatic resistance and to decrease portal hypertension[45].

Finally, oral atorvastatin and rosuvastatin were both well tolerated with no side effects, toxicity or mortality. Furthermore, transaminase levels, hepatic MDA and hydroxyproline and liver histopathology score did not aggravate. These data suggest that the use of atorvastatin and rosuvastatin is not associated with an increased risk of hepatoxicity in damaged liver.

In summary, our results show that atorvastatin and rosuvastatin have no effect on TAA-induced liver cirrhosis. Obviously further studies are required to evaluate whether statins may have therapeutic applications, in the development of hepatic fibrosis induced by other etiologies.

COMMENTS

Background

Liver cirrhosis is one of the leading causes of morbidity and mortality worldwide. Hepatic stellate cells (HSC) play a major role in the pathogenesis of hepatic fibrosis. Several studies demonstrated that activation of HSC in culture is provoked by generation of free radicals. Accordingly, antioxidants have been suggested as therapeutic modalities in experimental models, and in patients with chronic liver injury.

Research frontiers

Previous studies mainly focused on the potential effect of statins as anti-fibrotic agents. However, whether the anti-oxidative activity and the attenuation of inflammation by specific statin derivatives may also contribute to the inhibition of stellate cell activation and fibrosis remain unclear. The authors hypothesized that inhibition of HSC activity in addition to the anti inflammatory and anti oxidative effects induced by statins may prevent the hepatic damage induced by TAA in rats.

Innovations and breakthroughs

To further elucidate the anti-fibrotic activity of statins in the liver, the authors examined the effects of both atorvastatin and rosuvastatin in thioacetamide (TAA)-induced liver fibrosis in which cirrhosis is mainly produced *via* the formation of reactive oxygen species. The major finding was that both statins do not have a therapeutic value as a potential anti-oxidant or anti-fibrotic agents targeting increased oxidative stress or liver fibrosis induced by TAA.

Applications

The results show that atorvastatin and rosuvastatin have no effect on cirrhosis induced mainly by oxidative stress. Further studies are required to evaluate clarify the mechanism by which statins may have therapeutic applications, in hepatic fibrosis induced by other etiologies.

Terminology

3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) are used extensively to reduce serum cholesterol in an effort to reduce atherosclerotic cardiovascular morbidity and mortality. In addition to their cholesterol-lowering effect, statins demonstrate also anti-inflammatory and antioxidant effects. TAA-induced cirrhosis is mainly produced *via* the formation of reactive oxygen species. TAA undergoes an extensive metabolism to acetamide shortly after administration, and to the hepatotoxic reactive metabolite thioacetamide-S-oxide by the mixed function oxidase system.

Peer review

Well written manuscript which presented that atorvastatin or rosuvastatin did not inhibit the formation of TAA-induced liver cirrhosis in rats. Further, no induction of oxidative stress or effect on HSC proliferation have been noted. The model is well presented, the experiments seems to be correct. Even if results are negative, and in partial or total contrast with previous studies, the study appears well conducted and the results discussed with honesty, and caution.

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BRIEF ARTICLE

Hyperglycemia is a significant prognostic factor of hepatocellular carcinoma after curative therapy

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Abstract

AIM: To evaluate whether metabolic factors are related to distant recurrence of hepatocellular carcinoma (HCC) and survival after curative treatment.

METHODS: This retrospective study included 344 patients whose HCC was treated curatively by radiofrequency ablation (RFA) therapy. The mean age was 67.6 years and the mean observation period was 4.04 years. The etiological background of liver disease was hepatitis B virus infection in 30, hepatitis C virus infection in 278, excessive alcohol drinking in 9, and other in 27 patients. The Child-Pugh classification grade was A (n = 307) or B (n = 37). The number of HCC nodules was one in 260, two in 61, and three in 23 patients. For surveillance of HCC recurrence after curative therapy with RFA, patients were radiologically evaluated every 3 mo. Factors associated with distant recurrence of HCC or survival were studied.

RESULTS: Inadequate maintenance of blood glucose in diabetic patients was associated with higher incidence of distant recurrence. The 1-, 2-, and 3-year recurrence rates were significantly higher in diabetic patients with inadequate maintenance of blood glucose compared with the others: 50.6% *vs* 26.8%, 83.5% *vs* 54.4%, and 93.8% *vs* 73.0%, respectively (P = 0.0001). Inadequate maintenance of blood glucose was an independent predictor of distant recurrence [adjusted relative risk 1.97 (95%CI, 1.33-2.91), (P = 0.0007)] after adjustment for other risk factors, such as number of HCC nodules [2.03 (95%CI, 1.51-2.73), P < 0.0001] and initial level of serum alpha fetoprotein (AFP) [1.43 (95%CI, 1.04-1.97), P = 0.028]. Obesity was not an independent predictor of recurrence. The incidence of distant recurrence did not differ between diabetic patients with adequate maintenance of blood glucose and non-diabetic patients. Among 232 patients who had HCC recurrence, 138 had a second recurrence. The 1-, 2-, and 3-year rates of second recurrence were significantly higher in diabetic patients with inadequate maintenance of blood glucose than in the others: 9.0% *vs* 5.9%, 53.1% *vs* 24.3%, and 69.6% *vs* 42.3%, respectively (P = 0.0021). Inadequate maintenance of blood glucose in diabetic patients [1.99 (95%CI, 1.23-3.22), P = 0.0049] and presence of multiple HCC nodules [1.53 (95%CI, 1.06-2.22), P = 0.024] were again significantly associated with second HCC recurrence. Inadequate maintenance of blood glucose in diabetic

patients was also a significant predictor of poor survival [2.77 (95%CI, 1.38-5.57), P = 0.0046] independent of excessive alcohol drinking [6.34 (95%CI, 1.35-29.7), P = 0.019], initial level of serum AFP [3.40 (95%CI, 1.88-6.18), P < 0.0001] and Child-Pugh classification grade B [2.24 (95%CI, 1.12-4.46), P = 0.022]. Comparing diabetic patients with inadequate maintenance of blood glucose *vs* the others, the 1-, 2-, and 3-year survival rates were significantly lower in diabetic patients with inadequate maintenance of blood glucose: 92% *vs* 99%, 85% *vs* 96%, and 70% *vs* 92%, respectively (P = 0.0003).

CONCLUSION: Inadequate maintenance of blood glucose in diabetic patients is a significant risk factor for recurrence of HCC and for poor survival after curative RFA therapy.

Key words: Hyperglycemia; Hepatocellular carcinoma; Recurrence; Radio frequency ablation; Survival

Hosokawa T, Kurosaki M, Tsuchiya K, Matsuda S, Muraoka M, Suzuki Y, Tamaki N, Yasui Y, Nakata T, Nishimura T, Suzuki S, Ueda K, Nakanishi H, Itakura J, Takahashi Y, Izumi N. Hyperglycemia is a significant prognostic factor of hepatocellular carcinoma after curative therapy. *World J Gastroenterol* 2013; 19(2): 249-257 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i2/249.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i2.249

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide[1] and its incidence has been increasing in many countries[2]. Surgical resection, liver transplantation, and local ablation therapy, such as radiofrequency ablation (RFA) therapy, have been considered as efficient curative therapies for HCC. RFA therapy is now widely performed in patients with small HCC[3] and a randomized controlled study demonstrated that the survival rates were similar in patients with small HCC receiving RFA or surgical resection[4]. A characteristic of HCC is its high rate of recurrence after curative resection or local ablation therapy, reaching approximately 80% within 5 years[5-7]. Identification of factors related to recurrence of HCC and therapeutic intervention targeting these factors may lead to prevention of frequent recurrence of HCC and improved survival.

Tumor factors, such as the number of HCC nodules and their size, are associated with the recurrence of HCC and survival prognosis[8-10]. Another factor that is associated with the recurrence of HCC and survival is the hepatic reserve function at the time of HCC therapy[8,10,11]. Hepatitis C virus (HCV) and hepatitis B virus (HBV) infection are the major causes responsible for 80% of HCC cases[2] and antiviral therapy targeting HCV[12,13] or HBV[14] has been shown to decrease HCC recurrence, and improve hepatic reserve function and survival. Non-alcoholic steatohepatitis (NASH) has also received attention as a cause of HCC[15]. Metabolic factors, such as obesity and diabetes, are closely linked to the etiology of NASH. These metabolic factors have also been identified as risk factors for several other types of cancer. Obesity is associated with increased mortality rates of several cancers[16,17] and diabetes is also reported as a risk factor for liver, pancreatic, renal, and colon cancers[18,19]. If these metabolic factors are related to the recurrence of HCC, therapeutic intervention targeting these factors may lead to prevention of frequent recurrence of HCC and improved survival. The impact of diabetes on the recurrence of HCC after treatment has been discussed, but with conflicting results[20-23].

In this study, factors contributing to the recurrence and prognosis of HCC after curative treatment were analyzed. We found that inadequate maintenance of blood glucose was related to the high rate of HCC recurrence and poor survival.

MATERIALS AND METHODS

Patients whose HCC was treated by RFA at the Musashino Red Cross Hospital were studied retrospectively for factors associated with recurrence of HCC and survival. The inclusion criteria were as follows: (1) HCC treated curatively with RFA at the Musashino Red Cross Hospital between 1999 and 2007; (2) maximum diameter of HCC nodule $\leqslant$ 3 cm; (3) number of HCC nodules $\leqslant$ 3; (4) no previous history of treatment for HCC; and (5) follow-up observation for at least 6 mo after RFA therapy. 344 patients met these criteria, including 140 women and 204 men, with a mean age of 67.6 years and mean observation time of 4.04 years. The clinical characteristics of the patients are summarized in Table 1. The etiological background of liver disease was HBV infection in 30, HCV infection in 278, excessive alcohol drinking (intake of ethanol $\geqslant$ 60 g/d for $\geqslant$ 5 years continuously) in 9, and non-B non-C non-alcoholic etiology in 27 patients. The Child-Pugh classification grade was either A (n = 307) or B (n = 37). The number of HCC nodules was one in 260, two in 61, and three in 23 patients. Thus, 260 patients had a single lesion, and 84 had multiple lesions. The maximum diameter of HCC nodules was 19.9 ± 0.3 mm.

Obesity was defined as a body mass index > 25 kg/m^2 according to the definition of the Japan Society for the Study of Obesity[24]. Blood glucose was measured monthly for 6 mo after HCC treatment and the average value was determined. Inadequate maintenance of blood glucose was defined as an average value of blood glucose $\geqslant$ 200 mg/dL. The level of hemoglobin A1c (HbA1c) was not used in the present study because the lifespan of erythrocytes is shortened due to hypersplenism in patients with chronic hepatitis or cirrhosis, leading to lower HbA1c levels relative to the blood glucose level[25]. Diagnosis of type 2 diabetes was made according to the

Table 1 Characteristics of patients undergoing curative radiofrequency ablation for hepatocellular carcinoma *n* (%)

Variable	Value
Sex (male/female)	204/140
Age(yr)	67.6 ± 8.4
Etiology of liver disease: HBV/HCV/NBNC	30/278/36
AST (IU/L)	84.0 ± 34.5
ALT (IU/L)	73.2 ± 36.5
GGT (IU/L)	82.9 ± 96.8
T-Chol (mg/dL)	157.8 ± 32.0
TG (mg/dL)	112.3 ± 55.7
Mean blood sugar (mg/dL)	139.3 ± 44.0
Diabetes mellitus	159 (48)
BMI > 25 kg/m^2	86 (25)
Maximum diameter of HCC nodule (mm)	19.9 ± 0.3
Number of HCC nodules: single/2 or 3	260/84
AFP (ng/mL)	214 ± 1025
Alcohol drinking > 60 g/d	9 (2.6)
Child-Pugh grade: A/B	307/37

HBV: Hepatitis B virus; HCV: Hepatitis C virus; NBNV: Neither HBV nor HCV; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: γ-glutamyltransferase; T-Chol: Total cholesterol; TG: Triglyceride; BMI: Body mass index; AFP: α-fetoprotein; HCC: Hepatocellular carcinoma.

American Diabetes Association criteria of a fasting blood glucose level ≥ 126 mg/dL (≥ 7.0 mmol/L) and/or HbA1c level ≥ 6.5[26]. After initial treatment of HCC by RFA, the ablated area was confirmed by contrast-enhanced computed tomography (CT) within one week. If the ablated area was not sufficient, then RFA therapy was repeated until the HCC nodule was completely ablated.

HCC surveillance and diagnosis of recurrence

Diagnosis of HCC was based on abdominal ultrasonography, contrast-enhanced CT, magnetic resonance imaging (MRI), or angiography. Classical HCC was diagnosed for tumors showing vascular enhancement with washout on at least two types of diagnostic imaging. Tumor biopsy was used to diagnose tumors with non-classical imaging findings.

For surveillance of HCC recurrence after curative therapy with RFA, patients were evaluated by abdominal ultrasonography, contrast-enhanced CT, or contrast-enhanced MRI every three months. Recurrence of HCC was diagnosed based on a new lesion detected by ultrasonography showing vascular enhancement with washout on CT or MRI. If the tumor was not hypervascular, a tumor biopsy was performed to confirm the diagnosis.

Statistical analysis

For analysis of survival and recurrence, the time of initial RFA treatment was defined as day zero. Survival rate and recurrence rate were analyzed by the Kaplan-Meier method and log rank test. Multivariate analysis was performed using a Cox proportional hazard model. Data were analyzed using StatView Version 5.0 (SAS Institute Inc, Cary, North Carolina, United States) and IBM-SPSS statistics version 18 (IBM SPSS Inc, Chicago, IL, United States). Statistical significance was set at $P < 0.05$.

RESULTS

Factors associated with HCC recurrence

Of the 344 patients whose HCC was curatively treated by RFA, 232 had HCC recurrence. The 1-, 2-, and 3-year recurrence rates were 29.3%, 57.5%, and 75.2%, respectively. On univariate analysis, inadequate maintenance of blood glucose, higher initial level of serum AFP and multiple HCC nodules were significantly associated with HCC recurrence. Obesity ($P = 0.06$) and diabetes ($P = 0.65$) were not significantly associated with HCC recurrence.

Thirty-seven patients had diabetes with inadequate maintenance of blood glucose, 122 patients had diabetes with adequate maintenance of blood glucose, and 185 patients did not have diabetes. The HCC recurrence rate was significantly higher in diabetic patients with inadequate maintenance of blood glucose than in the others ($P = 0.0001$) (Figure 1A).

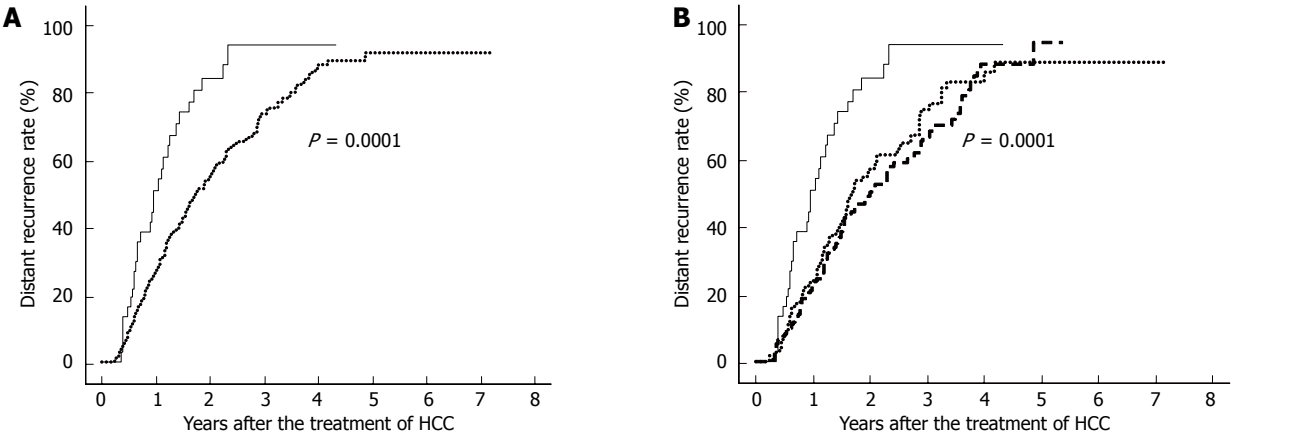
Comparing patients with diabetes ($n = 159$) and patients who did not have diabetes ($n = 185$), there was no significant difference in the recurrence rate ($P = 0.65$). Upon comparison of the three groups, i.e., the diabetes with inadequate maintenance of blood glucose group, the diabetes with adequate maintenance of blood glucose group, and the non-diabetes group, the recurrence rate was significantly higher in the diabetes with inadequate maintenance of blood glucose group than in the other two groups ($P = 0.0001$) (Figure 1B). On the other hand, there was no significant difference in the HCC recurrence rate between the diabetes patients with adequate maintenance of blood glucose group and the non-diabetes group.

In terms of the number of HCC nodules, namely, single ($n = 260$) *vs* multiple ($n = 84$), the recurrence rate was significantly higher in patients with multiple HCC nodules ($P = 0.0001$). Within each subgroup of patients with single and multiple HCC nodules, diabetes with inadequate maintenance of blood glucose was significantly associated with recurrence of HCC (single, $P = 0.006$; multiple, $P = 0.025$) (Figure 2A, B). In terms of the initial level of serum AFP ≥100 ng/mL ($n = 70$) *vs* < 100 ng/mL ($n = 274$), the recurrence rate was significantly higher in patients with AFP ≥ 100 g/mL ($P = 0.018$). Within each subgroup of patients with AFP ≥ 100 ng/mL and < 100 ng/mL, diabetes with inadequate maintenance of blood glucose was associated with a higher rate of recurrence (AFP ≥ 100 ng/mL, $P = 0.005$; AFP < 100 ng/mL, $P = 0.017$) (Figure 2C, D).

Independent risk factors for distant recurrence of HCC on multivariate analysis were inadequate maintenance of blood glucose in diabetic patients [adjusted relative risk, 1.97 (95%CI, 1.33-2.91), $P = 0.0007$], multiple HCC nodules [2.03 (1.51-2.73), $P < 0.0001$], and AFP ≥ 100 ng/mL [1.43 (1.04-1.97), $P = 0.028$] (Table 2).

Factors associated with second recurrence

Among the 232 patients who had HCC recurrence, 138 had a second recurrence. Regarding second recurrence, inadequate maintenance of blood glucose in diabetic pa-



Recurrence rate at	1-year	2-year	3-year
Inadequate maintenance of blood glucose	50.6%	83.5%	93.8%
Others	26.8%	54.4%	73.0%

Recurrence rate at	1-year	2-year	3-year
Diabetes with inadequate maintenance of blood glucose	50.6%	83.5%	93.8%
Diabetes with adequate maintenance of blood glucose	21.9%	48.7%	66.5%
Non-diabetes	23.6%	55.5%	74.4%

Figure 1 Kaplan-Meier curves showing a higher rate of hepatocellular carcinoma recurrence in diabetic patients with hyperglycemia. A: The cumulative incidence of the recurrence of hepatocellular carcinoma (HCC) was significantly higher in diabetic patients with inadequate maintenance of blood glucose (blood glucose ⩾ 200 mg/dL solid line) than in the others (dotted line) (P = 0.0001); B: The HCC recurrence rate was significantly higher in diabetic patients with inadequate maintenance of blood glucose (solid line) than in diabetic patients with adequate maintenance of blood glucose (blood glucose < 200 mg/dL, broken line) or non-diabetic patients (dotted line) (P = 0.0001). There was no significant difference in HCC recurrence rate between diabetic patients with adequate maintenance of blood glucose and non-diabetic patients.

Table 2 Multivariate analysis of factors associated with recurrence of hepatocellular carcinoma

Factors	Odds ratio (95%CI)	*P*-value
First recurrence		
Inadequate maintenance of blood glucose	1.97 (1.33-2.91)	0.0007
Multiple HCC nodules	2.03 (1.51-2.73)	< 0.0001
AFP ⩾ 100 ng/mL	1.43 (1.04-1.97)	0.028
Second recurrence		
Inadequate maintenance of blood glucose (mg/dL)	1.99 (1.23-3.22)	0.0049
Multiple HCC nodules	1.53 (1.06-2.22)	0.024

Inadequate maintenance of blood glucose was defined as an average of casual blood glucose of ⩾ 200 mg/dL. HCC: Hepatocellular carcinoma; AFP: α-fetoprotein.

tients and multiple HCC nodules were again significantly associated with HCC recurrence. Obesity (P = 0.18), diabetes (P = 0.31) and initial level of serum AFP (P = 0.08) were not associated with second recurrence. In terms of the number of HCC nodules, namely, single *vs* multiple, the 1-, 2-, and 3-year recurrence rates were significantly higher in patients with multiple lesions (6.4% *vs* 6.1%, 39.3% *vs* 23.1%, and 52.5% *vs* 42.3%, respectively, P = 0.013). Upon comparing diabetic patients with inadequate maintenance of blood glucose *vs* the others, the rate of second recurrence was significantly higher in diabetic patients with inadequate maintenance of blood glucose (P = 0.0021) (Figure 3A). Upon comparing patients with diabetes *vs* patients who did not have diabetes, the rates of second recurrence were not significantly different (P = 0.31). Upon comparison of the three groups, i.e., the diabetes with inadequate maintenance of blood glucose group, the diabetes with adequate maintenance of blood glucose group, and the non-diabetes group, the second recurrence rate was again significantly higher in the diabetes with inadequate maintenance of blood glucose group than in the other two groups (P = 0.0035) (Figure 3B). On the other hand, there was no significant difference in the second recurrence rate between the diabetes with adequate maintenance of blood glucose group and the non-diabetes group.

Independent risk factors for second recurrence of HCC on multivariate analysis were inadequate maintenance of blood glucose [1.99 (95%CI, 1.23-3.22), P = 0.0049] and multiple HCC nodules [1.53 (95%CI, 1.06-2.22), P = 0.024] (Table 2).

Factors associated with survival

There were 52 HCC-related or hepatic failure deaths. On univariate analysis, inadequate maintenance of blood glucose, excessive alcohol drinking, higher initial level of serum AFP and Child-Pugh classification grade B were significantly associated with survival. Obesity (P = 0.81) and diabetes (P = 0.11) were not significantly associated with survival.

Upon comparing diabetic patients with inadequate maintenance of blood glucose *vs* the others, the survival rate was significantly lower in patients with inadequate maintenance of blood glucose (P = 0.0003) (Figure 4A). Upon comparing diabetic patients *vs* non-diabetic patients, the survival rates were not significantly different

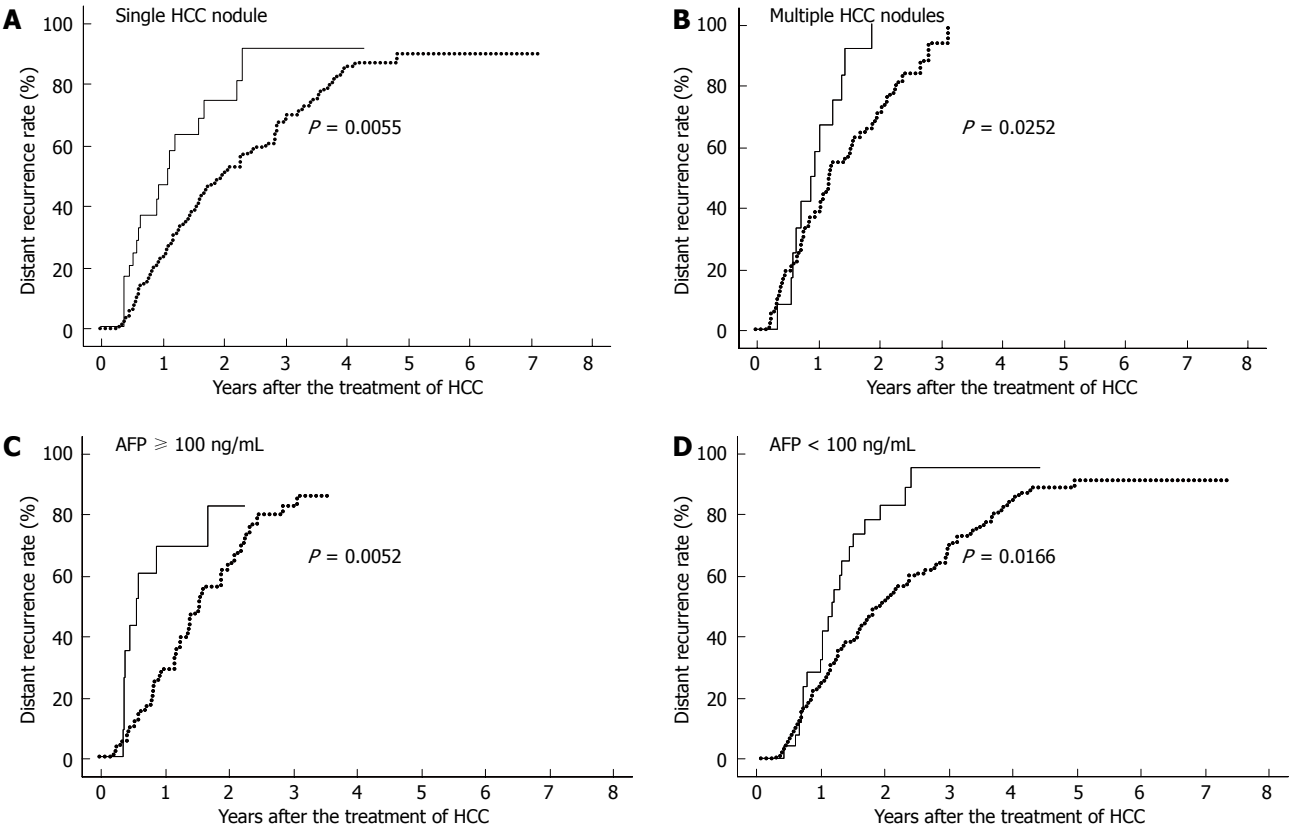


Figure 2 Diabetic patients with inadequate maintenance of blood glucose have higher rate of hepatocellular carcinoma recurrence after stratification by other risk factors. A: $P = 0.006$ for single hepatocellular carcinoma (HCC) nodule; B: $P = 0.025$ for multiple HCC nodules; C: $P = 0.005$ for AFP ⩾ 100 ng/mL; D: $P = 0.017$ for α-fetoprotein (AFP) < 100 ng/mL. The cumulative incidence of the recurrence of HCC was significantly higher in diabetic patients with inadequate maintenance of blood glucose (solid line) than in the others (dotted line). after stratification by number of HCC nodules and by initial level of AFP.

Table 3 Multivariable analysis of factors associated with survival

Factors	Odds ratio (95%CI)	*P*-value
Inadequate maintenance of blood glucose	2.77 (1.38-5.57)	0.0046
Alcohol drinking ⩾ 60 g/d	6.34 (1.35-29.7)	0.019
Child Pugh grade B	2.24 (1.12-4.46)	0.022
AFP ⩾ 100 ng/mL	3.40 (1.88-6.18)	< 0.0001

Inadequate maintenance of blood glucose was defined as an average of casual blood glucose of ⩾ 200 mg/dL. AFP: α-fetoprotein.

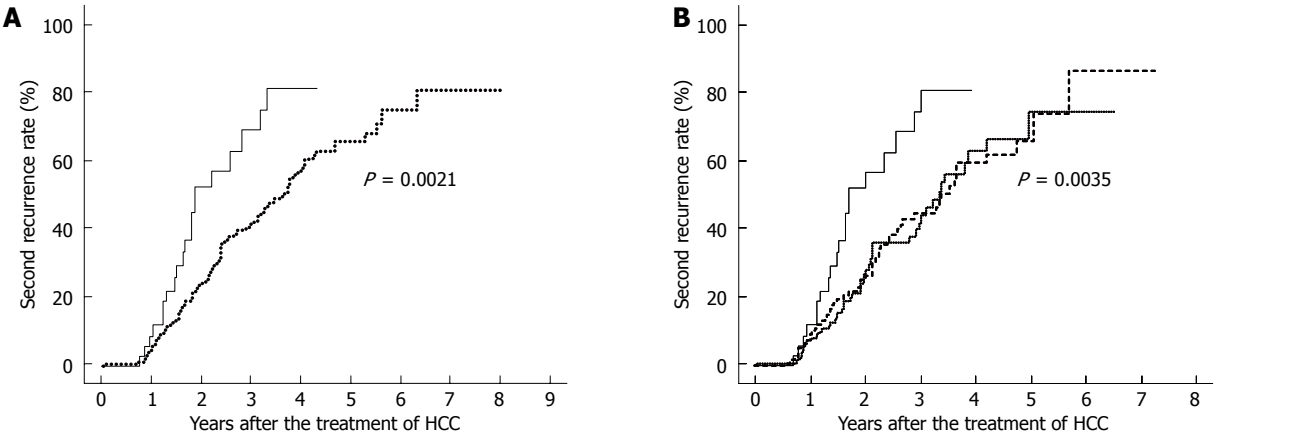
($P = 0.11$). of the survival rate was compared among the three groups, i.e., the diabetes with inadequate maintenance of blood glucose group, the diabetes with adequate maintenance of blood glucose group, and the non-diabetes group. The survival rate was significantly poorer in the diabetes with inadequate maintenance of blood glucose group than in the other two groups ($P = 0.0003$) (Figure 4B), while it did not differ between the diabetes with adequate maintenance of blood glucose group and the non-diabetes group.

The number of HCC nodules, which was a significant factor for HCC recurrence, was not related to survival ($P = 0.34$). Patients with excessive alcohol drinking had poor survival prognosis compared to those with non-excessive or no alcohol drinking ($P = 0.046$). Survival was better in patients in Child-Pugh A class than in patients in Child-Pugh B class ($P = 0.0082$). AFP ⩾ 100 ng/mL was associated with poor survival compared with AFP < 100 ng/mL ($P < 0.0001$).

On multivariate analysis, inadequate maintenance of blood glucose was a significant predictor of poor survival [2.77 (95%CI, 1.38-5.57), $P = 0.0046$] independent of excessive alcohol drinking [6.34 (95%CI, 1.35-29.7), $P = 0.019$], initial level of serum AFP ⩾ 100 ng/mL [3.40 (95%CI, 1.88-6.18), $P < 0.0001$] and Child-Pugh classification grade B [2.24 (95%CI, 1.12-4.46), $P = 0.022$] (Table 3).

DISCUSSION

The impact of metabolic factors, such as hyperglycemia, diabetes and obesity, on distant recurrence and survival after curative RFA therapy for HCC was analyzed retrospectively. We identified that inadequate maintenance of blood glucose in diabetic patients was a significant and independent risk factor for early recurrence of HCC and a risk factor for poor survival, whereas obesity and diabetes were not. Diabetic patients with inadequate maintenance of blood glucose had a higher rate of HCC recurrence and poorer survival compared with diabetic patients with adequate maintenance of blood glucose and non-diabetic patients. In other words, even in patients with diabetes, if



Second recurrence rate at	1-year	2-year	3-year
Inadequate maintenance of blood glucose	9.0%	53.1%	69.6%
Others	5.9%	24.3%	42.3%

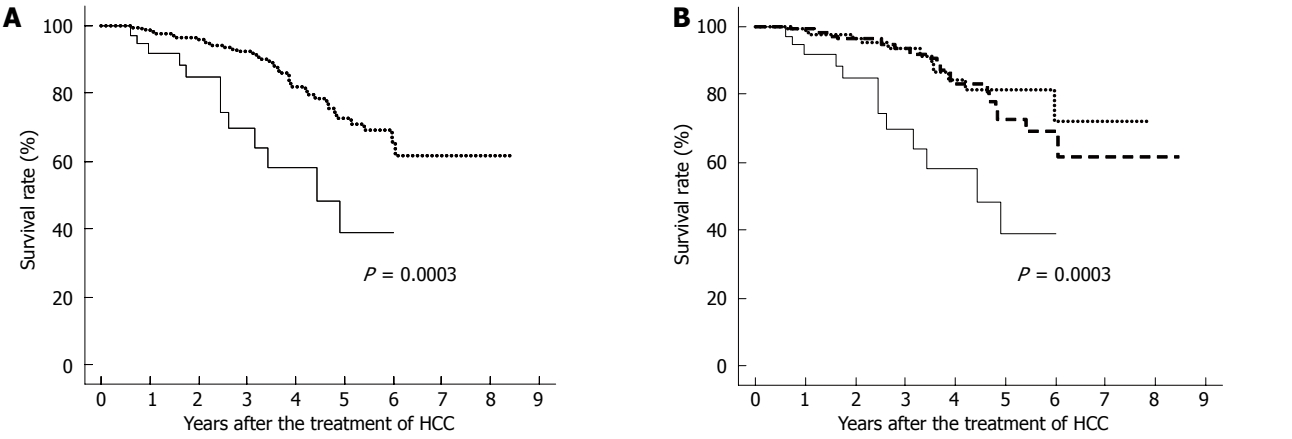
Second recurrence rate at	1-year	2-year	3-year
Diabetes with inadequate maintenance of blood glucose	9.0%	53.1%	69.6%
Diabetes with adequate maintenance of blood glucose	5.9%	22.1%	43.7%
Non-diabetes	5.6%	21.5%	36.5%

Figure 3 Kaplan-Meier curves showing a higher rate of second recurrence of hepatocellular carcinoma in diabetic patients with inadequate maintenance of blood glucose. A: The cumulative incidence of the second recurrence of hepatocellular carcinoma (HCC) was significantly higher in diabetic patients with inadequate maintenance of blood glucose (blood glucose ⩾ 200 mg/dL solid line) than in the others (dotted line) (P = 0.002); B: The rate of second recurrence of HCC was significantly higher in diabetic patients with inadequate maintenance of blood glucose (solid line) than in diabetic patients with adequate maintenance of blood glucose (blood glucose < 200 mg/dL, broken line) or non-diabetic patients (dotted line) (P = 0.004). There was no significant difference in the rate of second recurrence of HCC between diabetic patients with adequate maintenance of blood glucose and non-diabetic patients.

the blood glucose was adequately maintained, the HCC recurrence rate and survival did not differ significantly compared with those in non-diabetic patients. These results indicate the possibility that adequate management of hyperglycemia may lead to reduction in the risk of HCC recurrence and improvement of overall survival.

The contribution of diabetes to the development of HCC has been confirmed in several reports[27-30]. The impact of diabetes on the recurrence of HCC after treatment has also been discussed, but with conflicting results[20-22]. A recent study from Taiwan demonstrated that diabetes may not affect the intra-hepatic HCC recurrence and survival after RFA[23]. The results of the present study also indicated that diabetes itself is not a significant risk factor if the level of blood glucose is adequately managed. Rather, hyperglycemia was a significant risk factor for the recurrence of HCC. There may be several mechanisms involved in the relationship between hyperglycemia and HCC recurrence. Hyperglycemia promotes cancer cell proliferation in pancreatic cancer cells and breast cancer cells[31-33] through accelerated cell cycle progression or through the production of reactive oxygen species, leading to activation of protein kinase C and increased DNA synthesis in cancer cells[34]. A previous study in hepatitis C patients indicated that hyperglycemia after challenge with 75-g oral glucose tolerance test was associated with the risk for HCC while hyperglycemia at fasting was not[35]. A possible reason for this result may be that patients with post-challenge hyperglycemia may have higher fluctuations in daily glucose levels that lead to oxidative stress[35], because it was reported that acute fluctuations in blood glucose levels cause greater oxidative stress than sustained chronic hyperglycemia[36]. Taken together, a possible mechanism for the relationship between higher level of casual blood glucose and development of HCC in the present study may be that daily fluctuations in serum glucose levels caused greater oxidative stress. Alternatively, hyper-insulinemia or increased level of insulin-like growth factor, which are caused by hyperglycemia, may be related to carcinogenesis[37-39]. Insulin levels were not measured in our study; therefore, the effects of insulin could not be identified.

Discussions are now taking place on methods of treating diabetes from the standpoint of cancer prevention. Control of hyperglycemia could reduce cancer incidence, which means that hyperglycemia could directly contribute to the development of cancer[39]. The results of our study also showed that adequate management of hyperglycemia may lead to reduction in the risk of HCC recurrence and improvement of overall survival. Improvement in insulin resistance is undoubtedly the most important factor for the treatment of diabetes, but glycemic control is often difficult to achieve with dietary therapy, exercise, or insulin resistance-improving drugs alone. It was reported that metformin may be associated with a lower risk of cancer[38] and there is a theoretical concern that exogenous insulin may be associated with an increased risk of cancer[40]. In fact, a recent study reported that insulin therapy in patients with HCV infection is linked with the development of HCC[41]. On the other hand, with insulin treatment, concomitant use of metformin has been reported to offset the carcinogenic risk of insulin[42]. Whether glycemic control should be a priority, or whether avoiding hyper-insulinemia because



Second recurrence rate at	1-year	2-year	3-year
Inadequate maintenance of blood glucose	91.7%	84.6%	69.7%
Others	98.7%	95.9%	92.4%

Second recurrence rate at	1-year	2-year	3-year
Diabetes with inadequate maintenance of blood glucose	91.7%	84.6%	69.7%
Diabetes with adequate maintenance of blood glucose	99.2%	96.3%	93.7%
Non-diabetes	99.2%	96.4%	93.3%

Figure 4 Patients with inadequate maintenance of blood glucose have a lower survival rate. A: The survival rate after curative local ablation therapy for hepatocellular carcinoma (HCC) was significantly lower in diabetic patients with inadequate maintenance of blood glucose (blood glucose ≥ 200 mg/dL solid line) than in the others (dotted line) (P = 0.0003); B: The survival rate was significantly lower in diabetic patients with inadequate maintenance of blood glucose (solid line) than in diabetic patients with adequate maintenance of blood glucose (blood glucose < 200 mg/dL, broken line) or non-diabetic patients (dotted line) (P = 0.0003). There was no significant difference in survival rate between diabetic patients with adequate maintenance of blood glucose and non-diabetic patients.

of therapy should be a priority, is an issue for future investigation.

In terms of survival of HCC patients, associations with liver function and tumor factors have been reported[10], but conflicting results have been reported for the relationship with diabetes[20,21]. These two studies involved heterogeneous groups of HCC patients treated with various therapies, including surgery, local ablation therapy and transcatheter arterial embolization. This heterogeneity may have led to the conflicting results, because the survival of HCC patients may be strongly affected by the initial treatment. Our study involved a homogeneous patient population, i.e., all patients were initially treated curatively by RFA. The results of our study suggest that glycemic control in diabetic patients, more so than diabetes itself, plays a role in survival. The mechanism by which glycemic control and survival are related is unknown, but frequent recurrence of HCC in hyperglycemic patients and the accumulation of damage in liver function because of repeated treatment intervention for HCC may lead to worsening survival.

In conclusion, inadequate maintenance of blood glucose in diabetic patients was a significant and independent risk factor for early recurrence of HCC and for poor survival. Adequate management of hyperglycemia in diabetic patients may lead to reduction in the risk of HCC recurrence and improvement in overall survival.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide. Radiofrequency ablation (RFA) therapy is an efficient curative therapy for HCC, but long-term survival is limited because of the high rate of distant recurrence of approximately 80% within 5 years. Identification of factors related to recurrence of HCC and therapeutic intervention targeting these factors may lead to prevention of frequent recurrence of HCC and improved survival.

Research frontiers

Metabolic factors, such as obesity and diabetes, have been identified as risk factors for several types of cancer, such as cancer of the liver, pancreas, kidney, and colon. These metabolic factors may be related to recurrence of HCC. The impact of diabetes on the recurrence of HCC after treatment has been discussed, but with conflicting results.

Innovations and breakthroughs

The authors identified that inadequate maintenance of blood glucose in diabetic patients was a significant and independent risk factor for early recurrence of HCC and a risk factor for poor survival, whereas diabetes was not. In other words, even in patients with diabetes, if the blood glucose was adequately maintained, then the HCC recurrence rate and survival did not differ significantly from those in non-diabetic patients.

Applications

The results of the present study indicate the possibility that adequate management of hyperglycemia in diabetic patients may lead to reduction in the risk of HCC recurrence and improvement of overall survival.

Peer review

This is an important study in which the effect of inadequate maintenance of blood glucose in diabetes has been shown as a significant risk factor for distant recurrence of hepatocellular carcinoma and poor survival after curative radiofrequency ablation therapy.

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BRIEF ARTICLE

Gastrointestinal tuberculosis is not associated with proton pump inhibitors: A retrospective cohort study

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Abstract

AIM: To evaluate the effect of proton pump inhibitors (PPIs) on the development of gastrointestinal tuberculosis.

METHODS: All patients who were more than 20 years old and who had received a prescription for PPIs among those who visited Seoul National University Hospital from January 1, 2005 to December 31, 2009 were identified. Due to the low sensitivity of the microbiologic test and the nonspecific pathologic findings, the diagnosis of gastrointestinal tuberculosis was confirmed through the presence of active ulcerations and the responses to anti-tuberculosis medications. The patients were divided into two groups according to treatment duration (group 1: $\leqslant$ 3 mo; group 2: > 3 mo) and were followed up from the time they took the first prescription of PPIs until their last visit. Logistic regression analysis was used to calculate the relative risks (RR) and 95%CI, adjusting for covariates.

RESULTS: Among the 61 834 patients exposed to PPIs (50 534 in group 1; 11 300 in group 2), 21 patients were diagnosed with PPI-associated gastrointestinal tuberculosis during 124 274 person-years of follow-up. Of 21 patients, the 12 who revealed only scar changes in the colonoscopy were excluded from the statistical analyses. Of those who remained, 2 were excluded because they underwent gastrointestinal endoscopy within 4 wk of the first prescription for PPIs. Longer exposure to PPI was associated with a higher mean age (55.0 ± 14.5 in group 1 *vs* 58.2 ± 13.3 in group 2, $P < 0.001$) and a higher Charlson co-morbidity index (0.50 ± 0.93 in group 1 *vs* 0.77 ± 1.14 in group 2, $P < 0.001$). The true incidence of active gastrointestinal tuberculosis was 0.65 per 1000 person-years in group 1 and 0.03 per 1 000 person-years in group 2. Like the less-than-three-month PPI treatment period in group 1, the over-three-month PPI therapy period in group 2 was not associated with increased risk of acquiring gastrointestinal tuberculosis, after adjusting for age and co-morbidities, whereas the Charlson co-morbidity index was associated with increased risk of acquiring gastrointestinal tuberculosis based on the score [RR: (reference 1) in group 1 *vs* 1.518 in group 2; 95% CI: 1.040-2.216, $P = 0.03$].

CONCLUSION: Long-term PPI therapy does not seem to be associated with increased risk of acquiring gas-

trointestinal tuberculosis, but a higher Charlson comorbidity index is associated with such.

Key words: Proton pump inhibitor; Acid suppression; Tuberculosis; Gastrointestinal tuberculosis; Tuberculous colitis

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INTRODUCTION

Gut flora is closely related to human health and disease[1]. The intestinal microflora is assumed to be affected by a series of factors that determine the intraluminal environment: the pH in the gastrointestinal tract, oxygen tension, nutrient availability, colonic physiology, bacterial interference, *etc.*[2]. One of the major factors controlling the bacterial distribution in the gastrointestinal tract is the gastric acid barrier, which may be affected by the use of inhibitors of gastric acid secretion, gastrectomy, and dietary indiscretion and stress[3]. The establishment of enteric infection has been considered directly related to gastric acidity reduction[4,5]. Several recent studies associated proton pump inhibitors (PPIs) with a two- to three-fold increase in the risk of *Clostridium difficile* infection[6-8].

PPIs are currently the most powerful gastric acid suppressants and the drug of choice for the treatment of gastroesophageal reflux disease and peptic ulcer. PPIs are very powerful acid suppressants whose effects mean the percentage time intragastric pH > 4 may increase from 20% at baseline to over 60% within a week[9]. Today's life expectancy is longer than ever before, and the number of patients who take antiplatelet agents to prevent the onset of vascular diseases is increasing. Along with this, more and more patients are obliged to take prophylactic gastric acid suppressants, including PPIs, to prevent severe complications, such as peptic ulcer bleeding.

Tuberculosis is still an important health problem in many developing countries, including South Korea[10]. Unlike in developing countries, the disease used to be uncommon in developed countries, but it has re-emerged in the Western countries as a result of the acquired immunodeficiency syndrome (AIDS) epidemic therein as well as the influx of immigrants from developing countries[11-13]. Gastrectomy has been known to be a potential risk factor for tuberculosis for decades[14-16]. To these authors' knowledge, however, there has been no report about a possible association between PPI use and gastrointestinal tuberculosis. This study was thus conducted to evaluate the effect of PPIs on the development of gastrointestinal tuberculosis.

MATERIALS AND METHODS

Setting and design

This study is part of a hospital-based longitudinal cohort study entitled "Seoul National University Hospital (SNUH) PPI Safety Study," in which these authors analyzed the data regarding patients who visited SNUH and who were treated with PPI between January 1, 2005, and December 31, 2009. SNUH is a large urban tertiary care center in Seoul, South Korea. The hospital's institutional review board approved the study with a waiver of informed consent.

Data sources

Data were obtained from a clinical data warehouse fully synchronized with the electronic medical recording (EMR) system created as part of the usual care. SNUH Clinical Data Warehouse (CDW) contains all the information from each visit, not only routine clinical data such as the demographics, diagnosis, medication profiles, laboratory results, and lengths of stay of inpatients since 2001 but also the electronic charts since 2004.

Patients and case definitions

All patients who were at least 20 years old at their first visit and who ingested PPI on prescription during the five-year screening period were included in the study. According to a large retrospective study including 225 Korean patients, histological examination of the colonoscopic biopsy specimens revealed caseous necrosis in only 11.1% of the patients, and acid-fast bacilli (AFB) in 17.3% of the patients. *Mycobacterium tuberculosis* was isolated from the culture of biopsy specimens in 29.3% of the patients[17]. Even though granulomas were observed in 72.4% of the patients, granuloma is not a specific finding in intestinal tuberculosis. Due to the low sensitivity of the microbiologic test and the nonspecific pathologic findings, the diagnosis of active gastrointestinal tuberculosis was confirmed through both an endoscopic finding of active transverse ulcerations and a good response to anti-tuberculosis treatment, which was confirmed through a follow-up colonoscopy within three months from the initiation of the treatment. The definition of good therapeutic response was complete or near-complete healing of all the ulcerations in the follow-up colonoscopy.

The patients in whom colonoscopy revealed only scar changes were excluded from the statistical analysis for two reasons: (1) the exact temporal relationship between PPI ingestion and the development of gastrointestinal tuberculosis could not be verified; and (2) the diagnosis of tuberculosis could not be confirmed as there was no need for anti-tuberculosis treatment and due to the low sensitivity of the histological evaluation of the biopsy specimens. As tuberculosis is a chronic inflammatory disease, the patients who were diagnosed with gastrointestinal tuberculosis within four weeks of their first prescription for PPIs were also excluded from the statistical analyses.

The primary exposure of interest was receipt of PPIs. PPI

Table 1 Clinical information of the patients who were endoscopically diagnosed with suspicious tuberculosis

	Gender/ age	Group[1]	PPI duration before diagnosis (d)	Reason for endoscopy	Endoscopy findings	Pathology findings	Decision for analysis	Note
1	F/65	2	639	Diarrhea	Scar, cecum	No biopsy	Excluded	
2	F/73	1	844	Abdominal pain	Scar, cecum	No biopsy	Excluded	
3	F/65	2	1177	Routine check	Scar, cecum	No biopsy	Excluded	
4	F/60	1	415	Routine check	Scar, terminal ileum	No biopsy	Excluded	Small bowel resection due to tuberculosis 20 years ago
5	F/64	1	616	Anemia	Scar, descending colon	No biopsy	Excluded	
6	F/67	2	56	Abdominal pain	Scar, cecum	No biopsy	Excluded	
7	F/55	2	87	Routine check	Scar, cecum	No biopsy	Excluded	
8	F/82	2	523	Constipation	Scar, cecum	No biopsy	Excluded	
9	F/58	1	65	Lower abdominal pain	Scar, cecum	No biopsy	Excluded	
10	F/55	1	612	Routine check	Scar, cecum	No biopsy	Excluded	
11	M/52	1	894	Blood-tinged stool	Scar, cecum	No biopsy	Excluded	
12	F/61	2	29	Bloating	Scar, cecum	No biopsy	Excluded	
13	F/72	1	125	Hematochezia	Ulcers, cecum	Chronic active colitis	Included	
14	M/44	1	28	Anemia	Ulcers, cecum/ transverse colon	Non-caseating granuloma, negative PCR[2]	Included	
15	F/63	2	55	Epigastric pain	Ulcer, gastric cardia	Non-caseating granuloma, positive PCR[2]	Included	Palpable right supraclavicular lymph node
16	M/49	1	7	Melena	Ulcers, terminal ileum	Chronic active ileitis	Excluded	
17	M/41	1	574	Loose stool	Ulcers, cecum	Non-caseating granuloma, positive PCR[2]	Included	
18	M/20	1	49	Lower abdominal pain	Ulcers, ileocecal valve	Chronic active colitis	Included	
19	F/66	1	1	Melena	Ulcers, terminal ileum	Chronic active ileitis	Excluded	
20	F/59	1	224	Lower abdominal pain	Ulcers, ileocecal valve	Chronic active colitis,	Included	
21	F/46	1	28	Epigastric pain	Ulcers, mid-esophagus	Non-caseating granuloma, positive PCR[2]	Included	

[1]Patients with PPI treatment for three months or less as group 1, and patients with more-than-three-month PPI treatment as group 2; [2]Polymerase chain reaction (PCR) for *Mycobacterium tuberculosis*. PPI: Proton pump inhibitor; M: Male; F: Female.

exposure was classified by overall dosing period before the first endoscopy showing suspicious gastrointestinal tuberculosis or the last prescription date in patients without gastrointestinal tuberculosis. The patients with less than three months of PPI treatment were defined as group 1, and those with three or more months of PPI treatment were defined as group 2. To normalize the different acid-suppressive capacities among the PPI regimens, omeprazole 20 mg, lansoprazole 30 mg, rabeprazole 20 mg, pantoprazole 40 mg, and esomeprazole 40 mg were defined as standard daily doses of PPI. To calculate the adjusted dosing period, the period of the half-dose regimen was multiplied by 0.5, and the period for the double-dose regimen was multiplied by 2.

Statistical analysis

The covariates that may influence the risk of acquiring gastrointestinal tuberculosis and those that may influence the exposure to PPIs were included in the analysis. These variables were age, sex, and co-morbidities. The co-morbidities were determined from the registered diagnosis by the attending physician using the International Classification of Diseases 10th Revision code. Quan's algorithm was used to define the 17 Charlson co-morbidities, and the Charlson index was calculated[18,19].

Unadjusted comparisons were performed using the *t*-test, ANOVA test, Mann-Whitney test, χ^2 test, or Fisher exact test, as appropriate. Logistic regression modeling was used to estimate the relative risk of acquiring gastrointestinal tuberculosis in multivariate analyses. Statistical analysis was performed using the SAS software, version 9.2 (SAS Institute Inc., Cary, North Carolina).

RESULTS

Among the patients who visited SNUH during the five-year study period, 61 834 patients received PPIs and 7 gastrointestinal tuberculosis cases were identified. After excluding the patients who were diagnosed with suspicious gastrointestinal tuberculosis through endoscopy prior to the first prescription of PPIs, a total of 21 patients were screened. Of these, 12 patients who revealed only scar changes in colonoscopy were excluded from the statistical analyses. Of those who remained, 2 were excluded because they underwent gastrointestinal endoscopy within 4 wk of the first prescription of PPIs (Table

Table 2 Summary of descriptive statistical analysis *n* (%)

	Group 1	Group 2
Patients (*n*)	50 534	11 300
Cases	6 (0.012)	1 (0.009)
Esophageal tuberculosis	1	0
Gastric tuberculosis	0	1
Colonic tuberculosis	5	0
Total follow-up (person-mo)	1 102 947	388 345
Average follow-up (mo)	21.8	34.4
Incidence per 1000 person/yr	0.65	0.03

1). Anti-tuberculosis medications (isoniazid, rifampicin, ethambutol, and pyrazinamide) were prescribed to the finally selected 7 patients. Follow-up endoscopy was performed 2 or 3 mo after the start of the anti-tuberculosis treatment, and revealed complete healing or almost-healed ulcerations in all 7 patients.

The true incidence of gastrointestinal tuberculosis was 0.65 per 1000 person-years in group 1 and 0.03 per 1000 person-years in group 2 (Table 2). The characteristics of each group are shown in Table 3. The mean age (± SD) was 55.0 ± 14.5 years in group 1 and 58.2 ± 13.3 years in group 2. Longer exposure to PPI was associated with a higher Charlson co-morbidity index and a higher age (Table 3).

Table 4 presents the demographic and clinical characteristics of the active-gastrointestinal-tuberculosis and non-tuberculosis groups. Due to the small number of patients in the active-tuberculosis group, there was no significant difference between the two groups.

Table 5 shows the results of the multivariable analysis using logistic regression. A longer PPI treatment period (over three months) was not associated with increased risk of acquiring active gastrointestinal tuberculosis. The Charlson index was associated with significantly increased risk of acquiring active gastrointestinal tuberculosis by over 50% per score 1.

Table 3 Demographic and clinical characteristics of each group (mean ± SD)

	Group 1	Group 2	*P*-value
No. of patients	50 534	11 300	
Age, yr	54.96 ± 14.50	58.18 ± 13.31	< 0.001
Gender, male (%)	52.5	46.7	< 0.001
PPI duration, d	25.17 ± 21.51	285.78 ± 437.02	< 0.001
Co-morbidities, *n* (%)			
AIDS	75 (0.15)	6 (0.05)	0.011
Cerebrovascular disease	3655 (7.23)	1201 (10.63)	< 0.001
Congestive heart failure	196 (0.39)	71 (0.63)	< 0.001
Chronic pulmonary disease	184 (0.36)	64 (0.57)	0.002
Dementia	332 (0.66)	159 (1.41)	< 0.001
DM	4438 (8.78)	1600 (14.16)	< 0.001
DM without chronic complication	4280 (8.47)	1532 (13.56)	< 0.001
DM with chronic complication	158 (0.31)	68 (0.60)	< 0.001
Liver disease, mild	3182 (6.30)	1475 (13.05)	< 0.001
Hemiplegia or paraplegia	57 (0.11)	8 (0.07)	0.213
Liver disease, moderate or severe	488 (0.97)	205 (1.81)	< 0.001
Any malignancy	62 (0.12)	13 (0.12)	0.833
Metastatic solid tumor	310 (0.61)	69 (0.61)	0.972
Myocardial infarction	598 (1.18)	121 (1.07)	0.313
Peripheral vascular disease	368 (0.73)	114 (1.01)	0.002
Peptic ulcer	7011 (13.88)	1569 (13.88)	0.977
Rheumatologic disease	284 (0.56)	421 (3.73)	< 0.001
Renal disease	498 (0.99)	342 (3.03)	< 0.001
Charlson index	0.50 ± 0.93	0.77 ± 1.14	< 0.001
Score = 0 *n* (%)	33297 (65.89)	6167 (54.58)	< 0.001
Score = 1 *n* (%)	15536 (30.75)	4363 (38.61)	
Score ⩾ 3 *n* (%)	1699 (3.36)	770 (6.81)	
History of admission (%)	29138 (57.66)	6692 (59.22)	0.002
History of ICU admission (%)	1668 (3.30)	352 (3.12)	0.315

PPI: Proton pump inhibitor; AIDS: Acquired immune deficiency syndrome; ICU: Intensive care unit; DM: Diabetes mellitus.

DISCUSSION

This cohort study was conducted not only to evaluate the possible role of more-than-three-month PPI treatment but also to calculate the incidence rate of, and to find the risk factors for, acquiring active gastrointestinal tuberculosis in all the at-least-20-year-old patients who visited SNUH and who were treated with PPI between January 1, 2005 and December 31, 2009. As a result, more-than-three-month PPI treatment was found not to be associated with increased risk of acquiring active gastrointestinal tuberculosis. The annual incidence rate of tuberculosis was reported to be 97 per 100 000 in 2010 in the South Korean general population[10]. The calculated incidence rate of active gastrointestinal tuberculosis seems to be much lower in PPI-treated patients in the present study (Table 2), even considering the reportedly small proportion of gastrointestinal tuberculosis in the whole tuberculosis population[20].

A diagnosis of gastrointestinal tuberculosis can be confirmed if a characteristic caseous granuloma, positive smear of AFB, or positive culture of mycobacterium is observed in the biopsy specimen[12]. Compared to pulmonary tuberculosis, however, there is a relatively small absolute number of AFB in gastrointestinal tuberculosis[21], and caseous granuloma is infrequently observed in patients with an early-stage disease or who have been treated with anti-tuberculosis medications[22]. Many studies have been performed to assess the diagnostic accuracy of such histological markers, and there have been a number of reports showing granuloma in 41%-48%[23-25], caseous granuloma in 8%-18%[24-26], positive smear of AFB in 0%-100%[24,25,27,28], and positive culture of AFB in 0%-69%[24-26,28] of gastrointestinal-tuberculosis patients. As there have been many reports showing variable results, it seems impossible to set universal standards for diagnosing gastrointestinal tuberculosis. There are adjunct diagnostic modalities, such as polymerase chain reaction (PCR) for *Mycobacterium tuberculosis*, and endoscopic findings. Although PCR is a method that shows over 50% diagnostic sensitivity, its substantial false positivity limits its role to that of an adjunctive test for the diagnosis of gastrointestinal tuberculosis[29]. In the present study, both non-caseating granuloma and positive PCR were observed in two patients with gastroesophageal

Table 4 Demographic and clinical characteristics of the active-tuberculosis and non-tuberculosis groups (mean ± SD)

	Active tuberculosis	Non-tuberculosis[1]
No. of patients	7	61 790
Age, yr	50.6 ± 17.7	55.6 ± 14.3
Gender, male (%)	3 (43)	31 803 (51)
PPI duration	47.7 ± 97.9	72.8 ± 213.1
Charlson index	1.29 ± 2.36	0.55 ± 0.97
Score = 0 *n* (%)	5 (71)	39 459 (64)
Score = 1, 2 *n* (%)	0 (0)	19 899 (32)
Score ⩾ 3 *n* (%)	2 (29)	2467 (4)
History of admission	6 (86)	35 824 (58)

[1]Patients without gastrointestinal tuberculosis irrespective of the temporal relationship with proton pump inhibitor treatment or disease activity. PPI: Proton pump inhibitor.

Table 5 Results of the multivariable analysis using logistic regression

Factor	Relative risk (95%CI)	*P*-value
PPI exposure		
Group 1	Reference 1	
Group 2	0.697 (0.083-5.891)	0.74
Age, increase by year	0.972 (0.923-1.023)	0.27
Charlson index, increase by score 1	1.518 (1.040-2.216)	0.03
Sex	1.697 (0.374-7.690)	0.49
History of admission	4.317 (0.501-37.220)	0.18

PPI: Proton pump inhibitor.

tuberculosis, but non-caseating granuloma was observed in only 2 of the 7 patients with intestinal tuberculosis, and PCR was positive in 1 of the 2 patients who showed granuloma.

The endoscopic findings of gastrointestinal tuberculosis are often nonspecific[12,30]. Intestinal tuberculosis and Crohn's disease are chronic inflammatory bowel disorders that are difficult to differentiate from each other[31,32]. A study on colonoscopic findings reported that four parameters (involvement of fewer than 4 segments, a patulous ileocecal valve, transverse ulcers, and scars or pseudopolyps) were more frequently observed in intestinal tuberculosis patients than in Crohn's disease patients. Four parameters (anorectal lesions, longitudinal ulcers, aphthous ulcers, and cobblestone appearance) were significantly more common in Crohn's disease patients than in intestinal tuberculosis patients. A systematic analysis of the 8 parameters of colonoscopy was very useful in the differential diagnosis as it could differentiate between intestinal tuberculosis and Crohn's disease with 87.5% accuracy[33]. In the endemic areas of tuberculosis, it seems reasonable to prescribe anti-tuberculosis medications for patients with endoscopic findings favoring intestinal tuberculosis even if there is no specific histological finding from the biopsy specimens[12]. In the present study, anti-tuberculosis medications were also prescribed for 5 patients with only nonspecific chronic inflammations found through biopsy. Intestinal tuberculosis was confirmed in all 5 patients through follow-up colonoscopy, which revealed good therapeutic responses as well as symptom relief.

This study has a number of strengths. First, to these authors' knowledge, this study is the first study that evaluated PPI's role in the development of gastrointestinal tuberculosis. Second, all the data in this study were extracted from the SNUH CDW system that is fully synchronized with the EMR system and is optimized for research. Using the SNUH CDW system, 61 834 patients among the over one million patients who visited SNUH during the 5-year study period were rapidly screened, and the patients with gastrointestinal tuberculosis were sensitively sought out *via* browsing endoscopy and pathology reports. Most of all, compared to cohort studies using a public database, through which only the cumulative incidence rate could be estimated, it is notable that the true incidence rate of gastrointestinal tuberculosis in PPI-treated patients was calculated. The calculated incidence rate was as low as 0.65 per 1000 person-years in group 1 and 0.03 per 1000 person-years in group 2, and anti-tuberculosis medications were shown to be effective in all the patients with active gastrointestinal tuberculosis. Therefore, the risk of acquiring gastrointestinal tuberculosis does not seem to be clinically significant in PPI-treated patients.

This study, however, has several key limitations. First, the study was performed with patients from a single urbanized tertiary care hospital. In general, a hospital cohort is vulnerable to selection bias, and the results have poor generalizability. In South Korea, a single compulsory medical insurance takes effect. Due to the open medical delivery system, all the patients are practically free to visit tertiary care hospitals. In this study, 64% of the patients fall under Charlson index 0. Therefore, their possible difference from primary care patients in the aspect of clinical severity does not seem substantial. Another limitation of a hospital cohort is its dynamic nature, which enables the members to easily join or drop out. Fortunately, even if this study was performed in PPI-treated patients during a 5-year period, the patients could be followed up for 21.8 mo in group 1 and for 34.4 mo in group 2. Therefore, the adherence of the patients in this study seems to have been good.

The second limitation is the potentially different diagnostic sensitivity. This study is an observational study. Therefore, the patients did not necessarily undergo gastrointestinal endoscopy. The implementation of endoscopy was determined according to the patient's symptoms/signs, patient's will, and doctor's decision. There might have been differences in these factors between group 1 and group 2. In this study, most of the 12 patients with only ileo-colonic scars were females and underwent colonoscopy due to mild symptoms or as a routine check. Half of them belonged to group 2, showing a relatively higher incidence in group 2 compared to 1 in the patients with active gastrointestinal tuberculosis (Table 1). Presumably, this may be due to the more frequent requests for routine check-up from or the

stricter adherence to the hospital of female patients than male patients. In the 9 patients diagnosed with active gastrointestinal tuberculosis, however, there were nearly equal numbers of male and female patients. Endoscopy was performed to assess bloody diarrhea, anemia, and severe pain, which seemed to be symptoms suggesting active gastrointestinal tuberculosis (Table 1). In this study, statistical analysis was performed in the patients with active gastrointestinal tuberculosis, which could suggest that the results of this study were not highly biased by the patients' will or adherence.

In conclusion, long-term PPI therapy does not seem to be associated with increased risk of acquiring gastrointestinal tuberculosis whereas a higher Charlson comorbidity index is associated with such. These results, however, may not exempt further monitoring due to the small case number.

COMMENTS

Background

Proton pump inhibitors (PPIs) are currently the most powerful gastric acid suppressants. Several studies have associated acid suppression with increased risk of acquiring gastrointestinal infectious diseases, such as *Clostridium difficile* infection.

Research frontiers

Gastrectomy has been known to be a potential risk factor for tuberculosis, and gastric-acid suppression may play a role in its pathogenesis. There has been no report about a possible association between PPI use and gastrointestinal tuberculosis.

Innovations and breakthroughs

Seoul National University Hospital Clinical Data Warehouse is a database that is fully synchronized with an electronic medical recording system. Using the high-quality dataset, gastrointestinal-tuberculosis patients were efficiently screened, and the true incidence and relative risk of longer PPI treatment were calculated.

Applications

Long-term PPI therapy does not seem to be associated with increased risk of acquiring gastrointestinal tuberculosis.

Peer review

The response to therapy must be evaluated very strictly as this was adopted as a diagnostic criterion.

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BRIEF ARTICLE

Clinical outcomes and predictive factors in oral corticosteroid-refractory active ulcerative colitis

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Abstract

AIM: To evaluate the clinical outcomes and prognostic factors after intravenous corticosteroids following oral corticosteroid failure in active ulcerative colitis patients.

METHODS: Consecutive patients with moderate to severe ulcerative colitis who had been treated with a course of intravenous corticosteroids after oral corticosteroid therapy failure between January 1996 and July 2010 were recruited at Severance Hospital, Seoul, South Korea. The disease activity was measured by the Mayo score, which consists of stool frequency, rectal bleeding, mucosal appearance at flexible sigmoidoscopy, and Physician Global Assessment. We retrospectively evaluated clinical outcomes at two weeks, one month, three months, and one year after the initiation of intravenous corticosteroid therapy. Two weeks outcomes were classified as responders or non-responders. One month, three month and one year outcomes were classified into prolonged response, steroid dependency, and refractoriness.

RESULTS: Our study included a total of 67 eligible patients. At two weeks, 56 (83.6%) patients responded to intravenous corticosteroids. At one month, complete remission was documented in 18 (32.1%) patients and partial remission in 26 (46.4%). Eleven patients (19.7%) were refractory to the treatment. At three months and one year, we found 37 (67.3%) and 25 (46.3%) patients in prolonged response, ten (18.2%) and 23 (42.6%) patients in corticosteroid dependency, 8 (14.5%) and 6 (11.1%) patients with no response, respectively. Total 9 patients were underwent elective proctocolectomy within 1 year. The duration of oral corticosteroid therapy (> 14 d *vs* ⩽ 14 d, $P = 0.049$) and lower hemoglobin level (⩽ 11.0 mg/dL *vs* >11.0 mg/dL, $P = 0.02$) were found to be poor prognostic factors for response at two weeks. For one year outcome, univariate analysis revealed that only a partial Mayo score (⩾ 6 *vs* <6, $P = 0.057$) was found to be associated with a poor response.

CONCLUSION: The duration of oral corticosteroid therapy and lower hemoglobin level were strongly associated with poor outcome.

Key words: Clinical outcome; Prognosis; Corticosteroid; Ulcerative colitis

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INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory disease of the colon with unknown etiology and is characterized by a typical natural course with recurrent flares of mucosal inflammation. About 15% of patients with UC have been reported to have an overall chance of acute exacerbation and require admission to hospital and treatment with systemic corticosteroids[1,2]. Systemic corticosteroids remain as the gold standard treatment of acute moderate to severe UC. However, about 15%-57% of UC patients remain steroid-dependent or refractory even after steroid treatment[3]. Before the mid-1990s, proctocolectomy was the only treatment in steroid-unresponsive UC patients. Recently, intensive drug therapies have been shown to be effective in reducing and delaying the need for colectomy[4,5].

For UC patients who fail to improve with a maximal dosage of 5-aminosalicylic acids, oral steroid therapy should be considered. Moreover, for those who also do not respond to orally administered steroids, hospital admission is usually required for intensive intravenous treatment[6,7]. However, to the best of our knowledge, there has been no published study describing the clinical course of patients with acute attack of UC who were treated first with oral corticosteroids and subsequently with intravenous corticosteroids due to oral steroid failure. Before demonstrating the efficacy of second line drugs, the response and the clinical outcomes of intravenous corticosteroid therapy after oral administration need to be clarified. In addition, identification of early predictors of intravenous corticosteroid responsiveness could facilitate appropriate patient selection and well-timed administration of second line therapies in such UC patients. Clinically, earlier detection of response is important to help estimate risks, benefits, and duration of treatment, as this will enable the use of alternative drugs before complications of long term use of systemic corticosteroids can develop. However, data on predictive response factors of intravenous corticosteroids following oral corticosteroid therapy are lacking.

Here we sought to identify predictive clinical or biological factors associated with intravenous corticosteroid responsiveness in UC patients that initially did not respond to oral corticosteroids, as well as two week, one month, three month, and one year clinical outcomes of such treated patients with acute attack of UC.

MATERIALS AND METHODS

Patients

The clinical records of patients with active UC who were treated with intravenous corticosteroids immediately after treatment failure of oral corticosteroids at the Yonsei University College of Medicine, Seoul, South Korea between January 1996 and July 2010 were retrospectively evaluated. The diagnosis of ulcerative colitis was based on the accepted clinical, endoscopic, and histopathological criteria[8]. The criteria for eligibility were male or female patients with a diagnosis of UC followed regularly for at least 1 year. The exclusion criteria were patients with a history of corticosteroid therapy at other hospitals, corticosteroid use for diseases other than UC and a follow-up duration of less than 1 year. All enrolled patients were initiated intravenous corticosteroid therapy after admission after failure of oral steroid therapy which was done at outpatient clinic.

Intravenous corticosteroid therapy was initiated with intravenous administration of 100 mg of hydrocortisone every eight hours. Intravenous corticosteroid therapy was continued for 1-2 wk, with the treatment duration depending on the individual patient conditions, followed by gradual tapering of corticosteroids. After clinical improvement of UC, the dose of intravenous hydrocortisone was reduced to 200 mg daily. If the patients had no clinical exacerbation of UC, they were administered 30 mg/d of oral corticosteroid therapy before discharge. Our oral corticosteroid tapering policy was to reduce prednisolone by 5 or 10 mg weekly for patients with improved clinical symptoms but to sustain the current dose of prednisolone for one week for patients with lasting clinical symptoms[9,10]. All patients who were concomitantly taking sulfasalazine (2-4 g/d) or mesalamine (1.5-4 g/d) at the time of flare up continued therapy. Patients who took immuomodulators at the time of acute flare up also maintained their initial immuomodulator therapy while taking corticosteroids.

Our study is a retrospective study from a prospectively collected database. The data are stored in a form of Assess file as well as paper form. After then, the questionnaire including Mayo or partial Mayo scores including Physician Global Assessment (PGA) is updated every visit of the patient to outpatient clinics.

The disease activity was measured by the Mayo score, which consists of stool frequency, rectal bleeding, mucosal appearance at flexible sigmoidoscopy, and PGA[11,12]. Each component was scored 0 to 3 points, and the total score ranged from 0 to 12 points. However, a measurement of this score necessarily requires invasive flexible sigmoidoscopy, which limits repeated measurement. Therefore, most actual disease activity was measured on a nine-point partial Mayo score, which excluded the mucosal appearance at endoscopy[13]. At the initiation of oral corticosteroid therapy, demographic data including age, age at diagnosis, duration, gender, number of acute attacks, extent of disease, concomitant medications, duration of oral corticosteroid therapy, Mayo score, and partial Mayo score were collected. Partial Mayo score and laboratory parameters were also recorded at two weeks, one month, three months, and one year after the time of initiation of intravenous corticosteroid therapy. This study was approved by the institutional board of Severance Hospital.

Definitions

Clinical outcome was measured at two weeks, one month,

three months, and one year after the initiation of intravenous corticosteroids. The classification of response to intravenous corticosteroid therapy was adopted from previous studies with minor modification[3,11,14,15]. Patients at two weeks were classified as responders or non-responders. Non-responder of intravenous therapy after oral corticosteroid therapy was defined as persistent active disease despite administration of intravenous corticosteroids over two weeks, death due to UC attack before day 14, proctocolectomy before day 14, or secondary alternative drug use such as cyclosporine and infliximab before day 14. Proctocolecctomy was performed when the patient had intractable bloody diarrhea, a continued PGA score of 3, severe anemia, persistent abdominal pain, or severe malnutrition despite intensive medical treatment. One month outcomes were classified as complete remission, partial remission, or refractoriness. Complete remission was defined as a stool frequency ≤ 2/d with no rectal bleeding, stool urgency, fever, or any other systemic symptoms, and a PGA score of 0. Partial remission was defined as stool frequency ≤ 4/d or ≤ 50% of initial stool frequency with regression of other clinical symptoms and a PGA score of 1 or 2. Refractoriness was defined as persistent active status despite administration of prednisolone up to 30 mg/d or the equivalent dose over the period of four weeks[14]. Three month outcomes were classified into prolonged response, steroid dependency, and refractoriness. Prolonged response was defined as sustained complete or partial remission during the planned dose reduction or after the completion of corticosteroids[3]. Steroid dependency was defined as need for the same corticosteroid dose for more than two weeks despite clinical improvement, requiring an increased dose, or restarting corticosteroid therapy within two weeks because of exacerbation of symptoms[11,14]. Refractoriness was defined as no improvement of clinical symptoms despite continued corticosteroid use. Similarly, outcomes at one year were subdivided into three groups. Prolonged response was characterized by two conditions: maintaining complete or partial remission after discontinuation of corticosteroid therapy and requiring the same dose for more than two weeks or an increasing dose of corticosteroids only in the first three months. Steroid dependency was defined as restart of corticosteroid therapy due to recurrent flare-up of UC after the first three months or being unable to reduce prednisolone to 10 mg/day within three months. Non-response was defined in the same way as for the intermediate outcomes.

Statistical analysis

Continuous variables were presented as the mean ± SD or median (range) and were compared using two-sample *t* tests. Categorical variables were compared by χ^2 tests or Fisher's exact test. Logistic regression analysis was performed to identify predictive variables of clinical outcomes. *P* values less than 0.05 were considered statistically significant. All the statistical analyses were performed using the statistical software package SPSS 12.0 for Windows (SPSS Inc., Chicago, IL).

Table 1 Baseline characteristics of patients with active ulcerative colitis receiving intravenous corticosteroids after oral corticosteroid therapy failure

	Total patients (*n* = 67)
Gender (male/female)	39/28 (58.2/41.8)
Age (yr)	35 (13-78)
Age at diagnosis (yr)	31 (11-78)
Disease duration (mo)	24 (0-132)
Disease extent	
Proctitis	5 (7.5)
Left-sided colitis	21 (31.3)
Extensive colitis	41 (61.2)
First attack of UC	32 (47.8)
Number of previous flares	2 (1-4)
Initial disease activity	
Full Mayo score	9 (5-10)
Partial Mayo score	6 (3-7)
Initial prednisolone dose (mg)	
≥ 30 and < 40	35 (52.2)
≥ 40	32 (47.8)
Duration of oral corticosteroid use (d)	13 (3-50)
Maintenance before flare up	
None/salicylates/azathioprine	6/47/14
Concomitant medications	
Salicylates	53
Azathioprine	14
CRP (mg/dL)	10.9 (0.1-153.0)
ESR (mm/h)	40.0 (3.0-120.0)
Hemoglobin (mg/dL)	11.7 (6.4-16.5)
Albumin (mg/dL)	3.6 (2.3-5.0)

Data are expressed as absolute numbers (percentage) or median. UC: Ulcerative colitis; CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate.

RESULTS

Patient characteristics

A total of 67 patients, 39 men and 28 women, with active UC were included in this study (Table 1). The median age at diagnosis was 31 years (range: 11-78 years), and median disease duration at the time of oral corticosteroid therapy was 24 mo (range: 0-132 mo). The median length of oral corticosteroid use before intravenous therapy was 13 d (range: 3-50 d). Forty-one (61.2%) patients had extensive disease, and 32 (47.8%) had their first attack of active UC.

Clinical outcomes after intravenous corticosteroid therapy

Clinical outcomes of patients were organized in a flow chart (Figure 1). At two weeks after treatment, 56 (83.6%) patients responded to intravenous systemic corticosteroids. Seven of the 11 non-responders underwent proctocolectomy before day 14. Three patients were treated with an intravenous tumor necrosis factor-α blocker, and one patient was treated with intravenous cyclosporine. At one month after treatment, 18 (32.1%) patients were in complete remission, 26 (46.4%) in partial remission, and 12 (21.5%) had no response. Collectively, 21.5% of

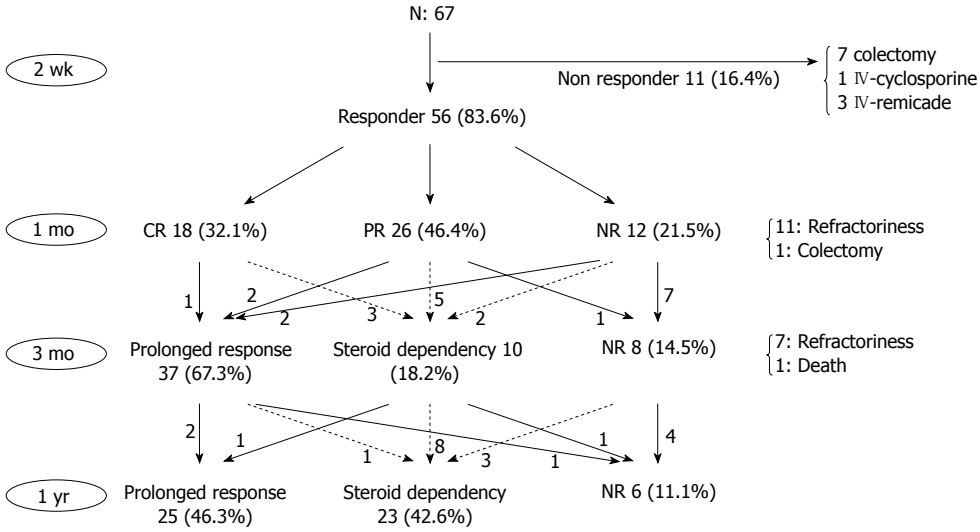

Figure 1 Clinical outcomes of patients with active ulcerative colitis treated with intravenous corticosteroids in oral corticosteroid refractory patients. CR: Complete remission; PR: Partial remission; NR: Non-response.

patients showed treatment failure to systemic corticosteroid therapy, and the other 78.5% of patients showed a partial or complete response to intravenous corticosteroid therapy. At three months, 37 patients had prolonged response, ten had steroid dependency, and seven showed no response. Of the 11 patients who were refractory to corticosteroid therapy at one month, two were in partial remission, two were steroid-dependent, seven had persistent refractoriness at three months, and one patient, a 59-year-old man who underwent proctocolectomy, died of pneumonia and sepsis. At one year, 25 of the 67 patients (37.3%) were categorized with prolonged response, 23 as steroid-dependent (34.3%), and six as non-responders (9.0%).

In our study, cytomegalovirus infection was detected in 5 patients. All were diagnosed by histologic examinations and were treated with ganciclovir. Of these, one patient underwent proctocolectomy within 14 d after the treatment. The rest of them responded to ganciclovir treatment. Finally, three patients were in partial remission and one patient was steroid dependent at one year.

Predictive factors for favorable outcomes

We performed univariate and multivariate analyses to detect clinical or laboratory factors capable of predicting poor outcomes of intravenous corticosteroid therapy after oral corticosteroid therapy failure. For the evaluation of intravenous corticosteroids at two weeks, we divided all patients into two groups: responders and non-responders who experienced death, proctocolectomy, or secondary medical treatment before two weeks. Patients were also divided into good responders and poor responders at one month, three months, and at one year after the corticosteroids therapy: good responders were those who showed a prolonged response, and poor responders were steroid-dependent or non-responsive. Multivariate analysis was carried out using the factors that were found to be statistically significant by univariate analysis.

For two weeks outcomes, univariate analysis of predictors for non-responders showed that disease duration (> 24 mo), duration of oral corticosteroids use (> 14 days), and lower hemoglobin level (≤ 11 mg/dL) were associated with poor prognosis. According to multivariate analysis, the duration of oral corticosteroids use (> 14 d *vs* ≤ 14 d, $P = 0.049$) and lower hemoglobin level (≤ 11.0 mg/dL *vs* > 11.0 mg/dL, $P = 0.02$) remained predictive factors for non-responders (Table 2). No predictive factors for poor responders at one month or three months were identified (Tables 3 and 4). For one year outcome, univariate analysis revealed that only a partial Mayo score (≥ 6 *vs* < 6, $P = 0.057$) was found to be associated with a poor response (Table 5).

DISCUSSION

We analyzed the clinical outcomes and identified predictive factors associated with corticosteroid responsiveness of patients who had an acute attack of UC and had been administered intravenous corticosteroids after a previous course of systemic oral corticosteroid therapy. There have been many previous studies that have reported the predictors of clinical response to systemic corticosteroid treatment in moderate to severe active UC patients[16-19]. However, there are few reports of outcomes of a conservative approach of intravenous steroid treatment in moderate to severe active UC patients after oral corticosteroid therapy failure.

In our study, intravenous corticosteroid therapy following a failure after oral corticosteroid therapy was successful at inducing response at two weeks of treatment in 83.6% of patients. This result implies that intravenous corticosteroids could be administered when a lack of response to oral corticosteroid therapy is shown. This

Table 2 Comparison of clinical factors in immediate outcomes of the two groups of active ulcerative colitis patients receiving intravenous corticosteroids after oral corticosteroid therapy failure

	Responders (n = 56)	Non-responders[1] (n = 11)	P-value		Odds ratio (95%CI)
			Univariate	Multivariate	
Gender (M/F)	32/24 (57.1/42.9)	7/4 (63.6/36.4)	0.690		
Age (yr)	37 (13-78)	39 (29-50)	0.458		
Disease duration (mo)					
≤ 24	34 (60.7)	3 (27.3)	0.041	0.123	3.38 (0.72-15.88)
> 24	22 (39.3)	8 (72.7)			
First attack of UC	26 (46.4)	6 (54.5)	0.273		
Disease extent			0.884		
Proctitis	4 (7.1)	1 (9.1)			
Left-sided colitis	17 (30.4)	4 (36.4)			
Extensive colitis	35 (62.5)	6 (54.5)			
Disease activity					
Full Mayo score (< 9/≥ 9)	27/24 (52.9/47.1)	3/8 (27.3/72.7)	0.122		
Partial Mayo score (< 6/≥ 6)	14/42 (25/75)	1/10 (9.1/90.9)	0.227		
Initial prednisolone dose (mg)			0.622		
≥ 30 and < 40	29 (51.8)	6 (54.5)			
≥ 40	27 (48.2)	5 (45.5)			
Duration of oral corticosteroid use (d)					
≤ 14	33 (58.9)	3 (27.3)	0.054	0.049	4.9 (1.01-23.81)
> 14	23 (41.1)	8 (72.7)			
Concomitant medications			0.809		
Salicylates	44 (78.6)	9 (81.8)			
Azathioprine	12 (21.4)	2 (18.2)			
CRP (mg/dL)					
≤ 8	37 (66.1)	11 (84.6)	0.303		
> 8	19 (33.9)	2 (15.4)			
ESR (mm/h)	40 (3-120)	40 (12-83)	0.999		
Hemoglobin (mg/dL)					
≤ 11	18 (32.1)	8 (72.7)	0.012	0.02	0.16 (0.03-0.75)
> 11	38 (67.9)	3 (27.3)			
Albumin (mg/dL)	3.63 (2.3-5.0)	3.56 (2.8-4.8)	0.331		

[1]Non-responder group included patients that had secondary alternative drug use, proctocolectomy and death before day 14. M/F: Male/female; UC: Ulcerative colitis; CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate.

result was partly explained by previous pharmacokinetic studies, which demonstrated delayed prednisolone absorption after an oral dose in patients with acute colitis as compared to healthy controls and higher plasma levels after intravenous steroid administration[20,21]. In addition, intravenous steroid administration in the hospital ensures good treatment compliance.

The rate of failure of intravenous corticosteroid therapy in moderate to severe UC attacks was quite high, accounting for 30%-40% in recent studies investigating Western populations[16-19,22,23]. Moreover, Meyers *et al*[24] showed a better clinical improvement of intravenous corticotrophin therapy for acute UC in steroid-naïve patients than in patients who had already received steroid treatment. Considering that our enrolled patients were an oral corticosteroid failure group and approximately half of them were non steroid-naïve patients, our results at two weeks showed a much higher response rate to intravenous corticosteroid therapy compared with previous studies[16-19,22,23]. Moreover, In our study, 13.4% of patients underwent proctocolectomy or experienced death within one year, while the incidence of cumulative proctocolectomy was reported to be about one-fifth that of exacerbation of UC[25-27]. This difference in the results of steroid treatment reaffirms the previous report that UC patients in the Korean population have a lower cumulative probability of proctocolectomy compared to those in Western countries[28,29]. Taken together, these results suggest that Korean patients with UC might have a more favorable prognosis compared to Western counterparts. In order to investigate the clinical application of our results in the treatment of UC patients, further prospective studies are warranted.

One month after the initiation of intravenous corticosteroid therapy, 78.5% of patients (32.1% with complete response and 46.4% with partial response) showed clinical improvement, whereas 42.6% were dependent on steroids at one year. It has been previously reported that 22% of patients in a western study became steroid-dependent[3]. Recently, Yoon *et al*[10] reported that a steroid-naïve Korean UC patient showed a good response to steroid treatment, while 40% of UC patients eventually became steroid-dependent or refractory. Our study showed that the responses to intravenous corticosteroids following a failure after oral corticosteroid treatment were similarly favorable in the short term period. However, more than half of patients eventually became steroid-dependent or refractory in the long term.

Table 3 Comparison of clinical factors in one month outcomes of the two groups of active ulcerative colitis patients receiving intravenous corticosteroids after oral corticosteroid therapy failure

	Good responders[1] (*n* = 44)	Poor responders[2] (*n* = 12)	*P*-value univariate
Gender (M/F)	24/20 (54.5/45.5)	8/4 (66.7/33.3)	0.956
Age (yr)	37 (11-78)	38 (29-59)	0.342
Disease duration (mo)			
≤ 24	25 (56.8)	9 (75)	0.253
> 24	19 (43.2)	3 (3)	
First attack of UC	18 (40.9)	8 (66.7)	0.113
Disease extent			0.943
Proctitis	3 (6.8)	1 (8.3)	
Left-sided colitis	13 (29.5)	4 (33.3)	
Extensive colitis	28 (63.6)	7 (58.3)	
Disease activity			
Full Mayo score (< 9/≥ 9)	23/19 (54.8/45.2)	4/5 (44.4/55.6)	0.574
Partial Mayo score (< 6/≥ 6)	12/32 (27.3/72.7)	2/10 (16.7/83.3)	0.452
Initial prednisolone dose (mg)			0.429
≥ 30 and < 40	24 (54.5)	5 (41.7)	
≥ 40	20 (45.5)	7 (58.3)	
Duration of oral corticosteroid use (d)			
≤ 14	26 (59.1)	7 (58.3)	0.962
> 14	18 (40.9)	5 (41.7)	
Concomitant medications			0.212
Salicylates	33 (75)	11 (91.7)	
Azathioprine	11 (25)	1 (8.3)	
CRP (mg/dL)			
≤ 8	28 (63.6)	9 (75)	0.461
> 8	16 (36.4)	3 (25)	
ESR (mm/h)	40.7 (3-120)	37.2 (12-83)	0.464
Hemoglobin (mg/dL)			
≤ 11	15 (34.1)	3 (25)	0.550
> 11	29 (65.9)	9 (75)	
Albumin (mg/dL)	3.6 (2.3-5.0)	3.45 (2.6-4.8)	0.295

[1]Good responders included patients with prolonged response that maintained complete remission or partial remission after completion of corticosteroid therapy; [2]Poor responders included patients with steroid dependence and non-response. M/F: Male/female; UC: Ulcerative colitis; CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate.

Table 4 Comparison of clinical factors in three month outcomes of the two groups of active ulcerative colitis patients receiving intravenous corticosteroids after oral corticosteroid therapy failure

	Good responders[1] (*n* = 37)	Poor responders[2] (*n* = 18)	*P*-value univariate
Gender (M/F)	21/16 (56.8/43.2)	10/8 (55.6/44.4)	0.933
Age (yr)	35 (13-78)	41 (20-62)	0.146
Disease duration (mo)			
≤ 24	22 (59.5)	12 (66.7)	0.606
> 24	15 (40.5)	6 (33.3)	
First attack of UC	15 (40.5)	10 (55.6)	0.294
Disease extent			0.317
Proctitis	3 (8.1)	1 (5.6)	
Left-sided colitis	9 (24.3)	8 (44.4)	
Extensive colitis	25 (67.6)	9 (50)	
Disease activity			
Full Mayo score (< 9/≥ 9)	20/14 (58.8/41.2)	7/9 (43.8/56.3)	0.318
Partial Mayo score (< 6/≥ 6)	11/26 (29.7/70.3)	3/15 (16.7/83.3)	0.297
Initial prednisolone dose (mg)			0.925
≥ 30 and < 40	19 (51.4)	9 (50)	
≥ 40	18 (48.6)	9 (50)	
Duration of oral corticosteroid use (d)			0.639
≤ 14	23 (62.2)	10 (55.6)	
> 14	14 (37.8)	8 (44.4)	
Concomitant medications			0.180
Salicylates	27 (73)	16 (88.9)	
Azathioprine	10 (27)	2 (11.1)	
CRP (mg/dL)			
≤ 8	24 (64.9)	12 (66.7)	0.895
> 8	13 (35.1)	6 (33.3)	
ESR (mm/h)	36.9 (3-120)	47.6 (6-120)	0.251
Hemoglobin (mg/dL)			
≤ 11	14 (37.8)	4 (22.2)	0.247
> 11	23 (62.2)	14 (77.8)	
Albumin (mg/dL)	3.73 (2.7-5.0)	3.44 (2.3-4.6)	0.082

[1]Good responders included patients with prolonged response that maintained complete remission or partial remission after completion of corticosteroid therapy; [2]Poor responders included patients with steroid dependence and non-response. M/F: Male/female; UC: Ulcerative colitis; CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate.

Reports evaluating predictive factors for poor clinical outcomes have shown different and controversial results. Some found no predictive factors[15,24,30] while others showed several factors related to clinical outcomes[16-19,22,23]. In our study, the UC duration, the duration of oral corticosteroid use, and lower hemoglobin level were associated with failure of steroid treatment on univariate analysis. Of these variables, only duration of oral corticosteroid therapy and lower hemoglobin level were found to be independently associated with failure of corticosteroid therapy on multivariate analysis. Knowledge of the clinical factors associated with non-response to intravenous corticosteroid therapy after oral therapy may be useful in clinical decision-making. Our study was such an attempt to explain this situation. Our results showing the relationships between simple clinical factors, such as lower hemoglobin or duration of oral steroid use, and intravenous treatment outcome might help in predicting poor responders. Lower hemoglobin level as a risk factor for poor response in our study is in accordance with an earlier reports, which reflects that initial severity of disease might be a significant predictor of poor clinical outcome after steroid treatment[19,31,32]. Also, anemia is a common and important complication of IBD with a prevalence rate ranging from 8.8% to 66.6% in UC patients[33,34]. The quality of life, an ability of work, and cognitive function can be impaired because of anemia in IBD patients[35,36]. Impaired quality of life by anemia in UC patients could influence patient well-being sense and PGA. For this reason, lower hemoglobin level could be a risk factor for poor response in our study. However, none of the previous studies have evaluated duration of oral corticosteroid use before intravenous corticosteroids to predict outcome of corticosteroid therapy in patients with UC. We found that the duration of oral corticosteroid administration was an independent predictor of non-response to intravenous corticosteroid therapy on multivariate analysis in patients with acute exacerbation

Table 5 Comparison of clinical factors in one year outcomes of the two groups of active ulcerative colitis patients receiving intravenous corticosteroids after oral corticosteroid therapy failure

	Good responders[1] (*n* = 25)	Poor responders[2] (*n* = 29)	*P*-value univariate
Gender (M/F)	15/10 (60/40)	16/13 (55/45)	0.721
Age (yr)	35 (16-78)	39 (13-64)	0.445
Disease duration (mo)	30 (0-84)	29 (0-120)	0.325
≤ 24	14 (56)	20 (69)	
> 24	11 (44)	9 (31)	
First attack of UC	9 (36)	15 (51.7)	0.248
Disease extent			0.304
Proctitis	1 (4)	3 (10.3)	
Left-sided colitis	6 (24)	11 (37.9)	
Extensive colitis	18 (72)	15 (51.7)	
Disease activity			
Full Mayo score (< 9/≥ 9)	15/9 (62.5/37.5)	11/14 (44/56)	0.195
Partial Mayo score (< 6/≥ 6)	9/16 (36/64)	4/25 (13.8/86.2)	0.057
Initial prednisolone dose (mg)			0.785
≥ 30 and < 40	13 (52)	14 (48.3)	
≥ 40	12 (48)	15 (51.7)	
Duration of oral corticosteroid use (d)			0.337
≤ 14	17 (68)	16 (55.2)	
> 14	8 (32)	13 (44.8)	
Concomitant medications			0.771
Salicylates	19 (76)	23 (79.3)	
Azathioprine	6 (24)	6 (20.7)	
CRP (mg/dL)			
≤ 8	16 (64%)	19 (65.5)	0.907
> 8	9 (36%)	10 (34.5)	
ESR (mm/h)	34.1 (4-81)	46.3 (3-120)	0.171
Hemoglobin (mg/dL)			
≤ 11	9 (36)	8 (27.6)	0.507
> 11	16 (64)	21 (72.4)	
Albumin (mg/dL)	3.8 (2.7-4.7)	3.57 (2.3-5.0)	0.230

[1]Good responders included patients with prolonged response that maintained complete remission or partial remission after corticosteroid therapy had finished; [2]Poor responders included patients with steroid dependence and non-response. M/F: Male/female; UC: Ulcerative colitis; CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate.

of UC. Patients with UC with more severe disease activity achieved remission less often than who tended to achieve remission earlier[37]. The duration of oral corticosteroid therapy was likely to reflect the severity nature of UC in these non-responding patients. Additionally, prolonged oral corticosteroid therapy might be associated with mortality and morbidity such as infection and sepsis in UC patients undergoing surgery. With regard to the duration of intravenous corticosteroid treatment, the limit of 7-10 d for certifying the criteria of steroid resistance was based on historical series, which showed that the median time of remission of UC was 7.5 d and that prolonged treatment beyond 10 d did not increase the remission rate[37]. In contrast with this point of view, a large retrospective study of single experienced hospital was in favor of more conservative approach, which entered into remission within the 21 d of treatment[38]. Therefore it is difficult to define resistance to corticosteroid which day after treatment is used as a limit marker. None of the previous studies have shown duration of oral corticosteroid administration was an independent predictor of non-response to intravenous corticosteroid therapy. This result demonstrates that optimal timing of intravenous corticosteroids after oral therapy was an important clinical factor of medical treatment response. In other words, use of oral corticosteroids for more than two weeks appears to be less effective in terms of clinical outcome. Additionally, long term oral corticosteroids use could lead to worse clinical outcomes and complications. An extended pre-operative use of steroids might increase the risk of surgical complications[39,40]. UC patients undergoing an elective surgery have been shown to be at an increased risk of postoperative infectious complications in patients treated with corticosteroids[40]. In our study, a total of 9 patients underwent elective proctocolectomy within 1 year. Among of them, one patient died of pneumonia and sepsis after proctocolectomy. Therefore, in the management of UC, the optimal timing of administration of intravenous corticosteroids after oral corticosteroid failure should be determined within two weeks after oral corticosteroid therapy. However, the optimal time limit with oral corticosteroid therapy in the face of response has not been clearly defined by randomized controlled trials. Therefore, large scaled, prospective studies are needed to confirm this result.

Yoon *et al*[10] reported that partial Mayo score was a predictive factor of steroid dependency in steroid-naïve patients with UC. In our study, we also showed that initial higher partial Mayo score might be associated with long term poor prognosis of corticosteroid therapy. Considered overall, the partial Mayo scores could help to predict long term potential corticosteroid dependency or refractoriness in active UC patients who are treated with systemic corticosteroid therapy.

Our study on clinical outcomes and factors for response prediction after intravenous therapy after oral corticosteroids failure in active UC patient has clear clinical significance. There have been no previously published studies describing the clinical course of patients with acute attack of UC who were treated first with oral corticosteroids and subsequently intravenous corticosteroids. This study may provide valuable clinical data that can be used in the management of UC patients receiving oral corticosteroids therapy. Such studies could also be used to suggest optimal timing for administration of intravenous corticosteroids in UC patients who are treated with oral corticosteroids. Moreover, our enrolled UC patients possessed the quality of comparatively homogenous corticosteroid therapy indication and same dose of corticosteroids. These facts might be advantageous in terms of eliminating confounding factors.

There were several limitations in our study. Our study was a retrospective study in a single tertiary hospital. Mucosal healing has emerged as an important treatment goal in UC because evidence is accumulating that it can alter the clinical course of UC. However, evidence of cortico-

steroid's ability to promote mucosal healing is limited. A considerable portion of a period in this study was at moment before mucosal healing has emerged as an emerging parameter in UC. Then we could not evaluate the mucosal healing as a parameter of clinical outcomes in this study. Moreover, our study was not a placebo-controlled comparative study. Finally, the sample size was relatively small.

In conclusion, our study showed that most Korean patients with active UC responded well to intravenous corticosteroid therapy after oral corticosteroid therapy failure. However, a considerable number of patients turned out to be refractory to or dependent on this therapy. The duration of oral corticosteroid therapy and lower hemoglobin level were strongly associated with poor outcome. Further prospective studies are warranted to confirm these results and to determine the optimal timing and dose of corticosteroids.

COMMENTS

Background

Ulcerative colitis (UC) is a chronic inflammatory disease of the colon with unknown etiology and is characterized by a typical natural course with recurrent flares of mucosal inflammation. Systemic corticosteroids remain as the gold standard treatment of acute moderate to severe UC. For those who also do not respond to orally administered steroids, hospital admission is usually required for intensive intravenous treatment. However, there has been no published study describing the clinical course of patients with acute attack of UC who were treated first with oral corticosteroids and subsequently with intravenous corticosteroids due to oral steroid failure.

Research frontiers

This study showed that the duration of oral corticosteroids use (> 14 d *vs* ⩽ 14 d, $P = 0.049$) and lower hemoglobin level (⩽ 11.0 mg/dL *vs* > 11.0 mg/dL, $P = 0.02$) was predictive factors for non-responders. Lower hemoglobin level and duration of corticosteroids may be useful in clinical decision-making as the clinical factors associated with non-response to intravenous corticosteroid therapy after oral therapy.

Innovations and breakthroughs

These results showing the relationships between lower hemoglobin or duration of oral steroid use, and intravenous treatment outcome might help in predicting poor responders. Low hemoglobin level as a risk factor for poor response is in accordance with an earlier report which reflects that initial severity of disease might be a significant predictor of poor clinical outcome. However, none of the previous studies have evaluated duration of oral corticosteroid use before intravenous corticosteroids to predict outcome of corticosteroid therapy in patients with UC. This result demonstrates that optimal timing of intravenous corticosteroids after oral therapy was an important clinical factor of medical treatment response.

Peer review

This paper is well written and the authors highlight the limitation of the study appropriately in their discussion: Retrospective design, not strictly comparative in terms of therapies, and small numbers. Nonetheless, there is an important observation in terms of better management of patients with UC. Some of the findings have been previously reported (low hemoglobin as a risk factor for refractoriness) but it is valuable to see the principles applied to a different population. The comparative data is compelling and statistically significant using appropriate methods. Duration of oral steroid administration may be a very useful predictor of outcome in these cases. The authors are right to emphasize the need for further study in this area.

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BRIEF ARTICLE

Dual probiotic strains suppress high fructose-induced metabolic syndrome

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Abstract

AIM: To investigate the effect of novel probiotics on the clinical characteristics of high-fructose induced metabolic syndrome.

METHODS: Male Wistar rats aged 4 wk were fed a 70% w/w high-fructose diet (n = 27) or chow diet (n = 9) for 3 wk to induce metabolic syndrome, the rats were then randomized into groups and administered probiotic [*Lactobacillus curvatus* (*L. curvatus*) HY7601 and *Lactobacillus plantarum* (*L. plantarum*) KY1032] at 10^9 cfu/d or 10^{10} cfu/d or placebo by oral gavage for 3 wk. Food intake and body weight were measured once a week. After 6 wk, the rats were fasted for 12 h, then anesthetized with diethyl ether and sacrificed. Blood samples were taken from the inferior vena cava for plasma analysis of glucose, insulin, C-peptide, total-cholesterol, triglycerides and thiobarbituric acid-reacting substances. Real-time polymerase chain reaction was performed using mouse-specific Taqman probe sets to assess genes related to fatty acid β-oxidation, lipogenesis and cholesterol metabolism in the liver. Target gene expression was normalized to the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase.

RESULTS: Rodents fed a high-fructose diet developed clinical characteristics of the metabolic syndrome including increased plasma glucose, insulin, triglycerides, total cholesterol and oxidative stress levels, as well as increased liver mass and liver lipids compared to chow fed controls. Probiotic treatment (*L. curvatus* HY7601 and *L. plantarum* KY1032) at high (10^{10} cfu/d) or low dosage (10^9 cfu/d) lowered plasma glucose, insulin, triglycerides and oxidative stress levels. Only high-dose probiotic treatment reduced liver mass and liver cholesterol. Probiotic treatment reduced lipogenesis *via* down-regulation of SREBP1, FAS and SCD1 mRNA levels and increased β-oxidation *via* up-regulation of PPARα and CPT2 mRNA levels.

CONCLUSION: Probiotic *L. curvatus* HY7601 and *L. plantarum* KY1032 combined suppressed the clinical characteristics of high-fructose-induced metabolic syndrome, therefore, may provide a natural alternative for the treatment of diet-induced metabolic syndrome.

Key words: Dyslipidemia; Fasting glucose; Gut microbiota; High-fructose diet; Inflammation; Insulin resistance; Lactobacillus; Metabolic syndrome; Oxidative stress; Probiotic

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INTRODUCTION

Metabolic syndrome is a rapidly growing worldwide pandemic, which is associated with a greater risk of multiple chronic pathologies including cardiovascular disease and Type 2 diabetes. Metabolic syndrome is characterised by a cluster of metabolic abnormalities including insulin resistance, elevated fasting glucose, elevated plasma triglycerides, elevated blood pressure, low-grade inflammation, abdominal obesity and reduced high-density lipoprotein (HDL)-cholesterol[1,2]. There is no universal cause of metabolic syndrome, although major underlying risk factors include abdominal obesity, physical inactivity or diabetogenic diets[1].

Diabetogenic diets with high fructose content have been strongly implicated in the development of metabolic syndrome, cardiovascular disease and Type 2 diabetes[3]. Food manufacturers are increasingly using fructose corn syrup to increase sweetness, as well as the palatability of food and beverages. However, there is growing experimental evidence that excessive fructose intake can lead to metabolic abnormalities, including insulin resistance, dyslipidemia as well as abdominal adiposity in both animals and humans[4]. Animals fed a high-fructose diet develop clinical characteristics of metabolic syndrome, therefore, high-fructose fed animals are particularly useful for assessing potential therapeutic interventions against metabolic syndrome[5]. Excess dietary fructose can be converted to triglycerides through *de novo* lipogenesis, resulting in increased lipid accumulation in the liver and elevated blood lipid levels. Over a period of time prolonged high intracellular and systemic lipid levels can cause increased oxidative stress and inflammation, both of which can trigger insulin resistance, leading to increased blood glucose levels. Due to the lack of effective drugs to treat metabolic syndrome, there is growing interest in natural therapeutics to prevent or manage metabolic syndrome.

Over the past five years, probiotics have rapidly emerged as natural therapeutics with potential to target key risk factors associated with metabolic syndrome[6,7]. Probiotics consist of single or multiple live bacterial species, which may directly or indirectly modulate gut microbial activity and improve host health. The human gut harbours between 10^{14} bacterial species collectively forming the gut microbiota[8]. Gut microbial communities are proposed to provide the host with the ability to harvest otherwise inaccessible energy from the diet[9-11] and modulate host genes associated with energy storage in adipose tissue[2,12]. Probiotics have been widely assessed *in-vivo* in diet-induced obesity models[13-17], however, different probiotic species even from the same family can exert variable effects on lipid accumulation and obesity[18], therefore, it remains essential to assess the effectiveness of probiotic strains in different animal disease models *in-vivo*. Probiotic yogurt containing multiple *Lactobacillus* strains has been reported to alleviate fasting blood glucose, plasma insulin and triglyceride in high-fructose fed rats[19]. However, few other studies have considered the impact of probiotics on high-fructose diet-induced metabolic syndrome. To our knowledge, the dose-dependent and metabolic effects of probiotic treatment in high-fructose-induced metabolic syndrome remain to be established.

The aim of this study was to assess the dose-dependent effects of a probiotic consisting of *Lactobacillus curvatus* (*L. curvatus*) HY7601 and *Lactobacillus plantarum* (*L. plantarum*) KY1032 on hyperlipidemia, hyperglycemia, insulin resistance, oxidative stress and hepatic metabolism related gene expression in high-fructose fed rats with metabolic syndrome. We hypothesized that probiotic treatment may protect against dysregulated metabolism induced by a high-fructose diet in a dose-dependent manner.

MATERIALS AND METHODS

Animals, diets and experimental design

Male Wistar rats (n = 36) aged 4 wk were purchased from Jackson Laboratories (Bar Harbor, United States). All rats were individually housed under a constant temperature and humidity (22 ± 1 ℃, 55% ± 10%) with a 12 h light/12 h dark cycle. The experimental design consisted of a pretreatment phase (0-3 wk) and a treatment phase (3-6 wk). During the pretreatment phase, male Wistar rats were fed a 70% w/w high-fructose diet (n = 27) to induce metabolic abnormalities or a chow diet (n = 9) for 3 wk. The composition of the high-fructose diet was formulated according to Table 1. During the treatment phase, the placebo (HF) group (n = 9), low dose probiotic (LP) group (n = 9) and high dose probiotic (HP) group (n = 9) were fed the same high-fructose diet with placebo, 10^9 cfu probiotics or 10^{10} cfu probiotics, respectively, administered orally each day for a further 3 wk. The chow control group was fed the same chow diet with placebo administered orally each day for a further 3 wk. Freeze-dried *Lactobacillus* strains were produced by Culture Systems Inc. (United States), and packed with lactose according to Table 2. Each pack was resuspended in 500 μL distilled water prior to administration. Food intake and body weight were measured once a week. Before sacrifice, rats were fasted for 12 h and anesthetized with diethyl ether. Blood samples were taken from the inferior vena cava for plasma analysis. Epididymal adipose tissue and liver tissue were removed, rinsed with phosphate buffered saline, weighed and immediately frozen at -70 ℃. The experimental design was approved by the Ethics Committee of Korea Yakult Company Limited R and D Center.

Blood analysis

Plasma glucose, insulin and C-peptide concentrations

Table 1 Composition of high fructose diet

Ingredient	High fructose diet (g)
Casein	200.0
L-cystine	3.0
Fructose	700.0
Cellulose powder	50.0
Corn oil	25.0
Lard	20.0
Mineral Mix S10026	10.0
DiCalcium Phosphate	13.0
Calcium Carbonate	5.5
Potassium Citrate	16.5
Vitamin Mix V10001	10.0
Choline Bitartrate	2.0
Total	1000.0

Table 2 The composition of each supplement pack

	Placebo (mg)	10^9 probiotics (mg)	10^{10} probiotics (mg)
Lactobacillus curvatus HY7601	-	5.0 (5×10^8)	50.0 (5×10^9)
Lactobacillus plantarum KY1032	-	2.5 (5×10^8)	25.0 (5×10^9)
Lactose	100.0	92.5	25.0
Total	100.0	100.0	100.0

Freeze-dried *Lactobacillus curvatus* HY7601 and *Lactobacillus plantarum* KY1032 concentration was 1×10^8 and 2×10^8 cfu/mg, respectively.

were determined using the glucose assay kit (Cayman, United States), insulin enzyme-linked immunosorbent assay (ELISA) kit (Millipore, United States) and rat C-peptide ELISA kit (EIAab, China), respectively, according to the manufacturer's instructions. Insulin resistance was assessed based on homeostasis model assessment of insulin resistance (HOMA-IR), calculated as the product of fasting plasma glucose (FPG) and insulin (FPI), divided by a constant[20]. The equation was [FPG (mg/dL) × FPI (μU/mL)]/2430. Plasma total-cholesterol and triglyceride concentrations were determined using commercial kits (AsanPharm, South Korea). Plasma thiobarbituric acid-reacting substances (TBARS) were measured to assess oxidative stress as described previously[21].

Hepatic lipid profile analysis

Hepatic lipids were extracted as previously reported[22]. The dried lipid residues were dissolved in 1 mL of isopropanol for the triglyceride and cholesterol assays. Hepatic triglyceride and cholesterol concentrations were measured using the same commercial kits (AsanPharm, South Korea) used for the plasma analysis.

RT-qPCR

Total RNA was extracted from liver (15 mg) tissue using an RNAqueous kit (Ambion, United States). DNA was removed with a Turbo DNA-free kit (Ambion, United States). RNA integrity was verified and RNA quantified using a GeneQuant Pro spectrophotometer (GE Healthcare, United States). Total RNA (2 μg) was reverse-transcribed into cDNA with a high-capacity RNA-to-cDNA kit (Applied Biosystems Inc., United States). Then cDNA was amplified on a 7500 Real Time PCR System (Applied Biosystems Inc., United States) using mouse-specific Taqman probe sets (Table 3) under the following conditions: 95 ℃ for 10 min, followed by 40 cycles at 95 ℃ for 15 s, and 60 ℃ for 1 min. Target gene expression was normalized to the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (catalog number Rn01775763_g1). Data was analyzed using the ABI 7500 System Sequence Detection software (Applied Biosystems Inc., United States) and presented as mean ± SE.

Table 3 Catalog numbers of Taqman probes

Taqman probe	Catalog number
Peroxisome proliferative activated receptor a	Rn00566193_m1
Carnitine palmitoyl transferase 1	Rn00580702_m1
Carnitine palmitoyl transferase 2	Rn00563995_m1
Acyl-CoA oxidase 1	Rn01460628_m1
Sterol regulatory element binding protein-1	Rn01495769_m1
Fatty acid synthase	Rn00569117_m1
Stearoyl-CoA desaturase 1	Rn00594894_g1
Cholesterol 7 alpha-hydroxylase	Rn00564065_m1
Low density lipoprotein receptor	Rn00598442_m1

Statistical analysis

All data were presented as mean ± SE. Statistical analysis was performed using SPSS software (SPSS Inc., United States). Data were analyzed by one way analysis of variance, and the differences between experimental groups were evaluated using Duncan's multiple range test at the $P < 0.05$ level. Significant differences between chow control and HF control groups were determined using unpaired Student's *t*-test. Significant differences between week 3 and week 6 in each parameter were determined using the paired Student's *t*-test, and the values were considered statistically significant when $P < 0.05$.

RESULTS

Effects of probiotic treatment on food intake, body weight and tissue mass

Metabolic syndrome was induced in rodents by feeding them a high-fructose diet over 3 wk, while a control group was fed a chow diet. Rodents with metabolic syndrome were then randomized into three treatment groups and fed a high-fructose diet for a further 3 wk, with daily treatment of either probiotic (HP) at a dose of 1×10^{10} cfu/d, probiotic (LP) at a dose of 1×10^9 cfu/d or placebo administered by oral gavage. The chow control group were fed the same diet for the same period and administered placebo by oral gavage. Average food intake was suppressed in the high-fructose diet fed rodents compared to the chow fed controls (Figure 1A). However, low or high dose probiotic treatment had no significant effect on food intake (Figure 1A). Body weight gain and epididymal fat mass were not significantly affected by high-fructose feeding or probiotic treatment regardless of dosage (Figure 1B and C). Importantly, average liver

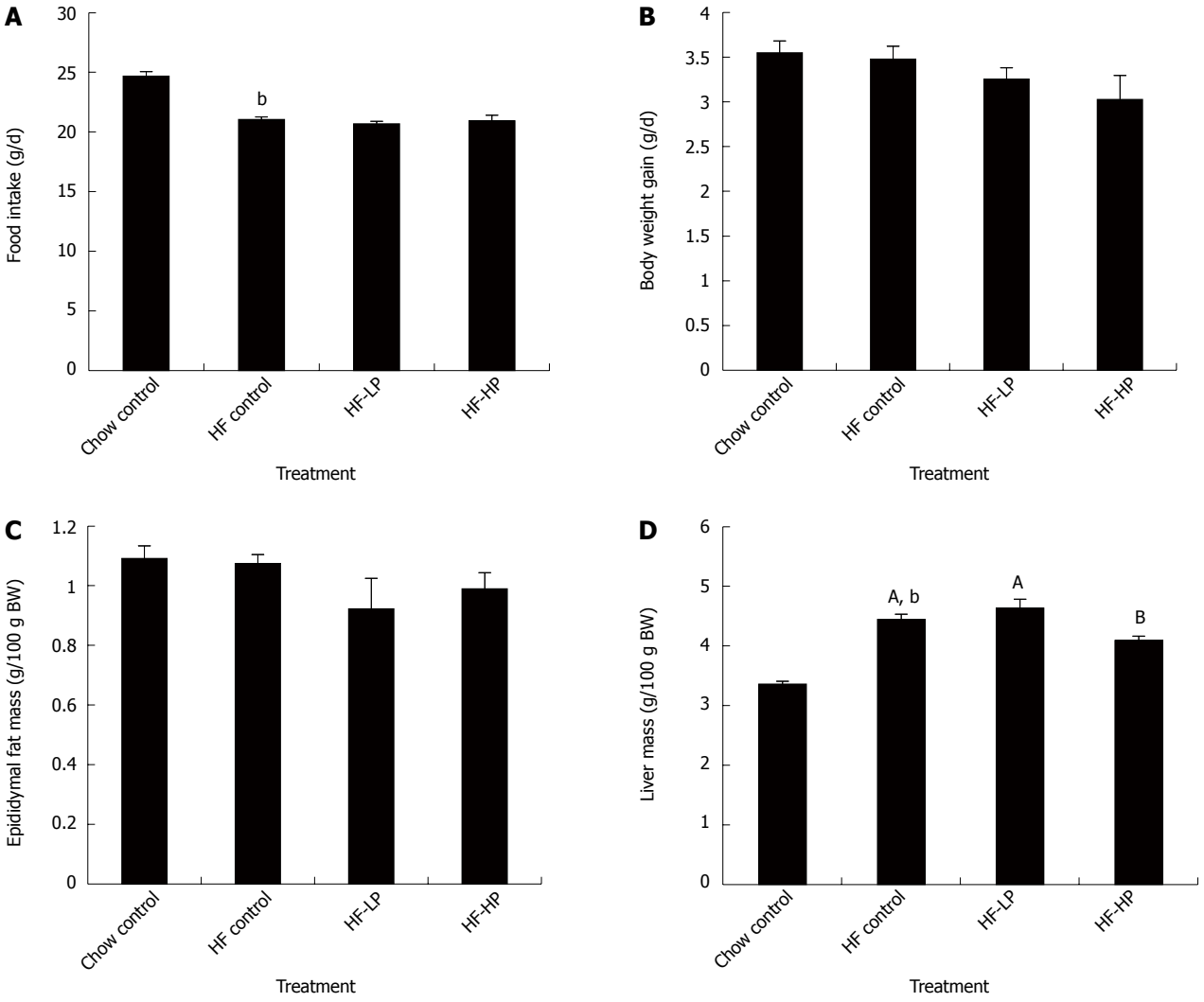

Figure 1 Effects of probiotic treatment on food intake (A), body weight gain (B), epididymal fat mass (C) and liver mass (D) in high-fructose diet-fed rats. Results are expressed as mean ± SE. $^{b}P < 0.01$ *vs* chow control by unpaired Student's *t*-test; ^{AB}Bars with different capital letters are significantly different at $P < 0.05$ by Duncan's multiple range tests. HP: High dose probiotic; HF: High fructose diet; LP: Low dose probiotic; BW: Body weight.

mass, which was significantly increased by high-fructose diet, was significantly lower following HP treatment by 10% ($P < 0.01$) compared to high-fructose fed controls (Figure 1D).

Effects of probiotic treatment on dyslipidemia

Hypertriglyceridemia was effectively induced by a high-fructose diet within 3 wk (163.91 ± 6.50 mg/dL *vs* 49.26 ± 3.99 mg/dL, HF and chow group, respectively). However, subsequently 3-wk of probiotic treatment significantly reduced average plasma triglyceride levels by 46% compared to placebo treatment in the high-fructose fed rodents (Figure 2A). The probiotic treatment had no influence on either total cholesterol or HDL cholesterol levels, which were increased by high-fructose diet intake (Figure 2B and C).

Effects of probiotic treatment on hyperglycemia, insulin resistance and oxidative stress

The average plasma glucose levels of the high-fructose fed rats were significantly higher than that of the chow fed controls at week 6, but were effectively reduced following LP and HP treatment by 24% ($P < 0.05$) and 14% ($P < 0.05$), respectively, (Figure 3A). The average plasma insulin levels of the high-fructose fed rats were significantly higher at both weeks 3 and 6 compared to the chow fed controls. However, after HP treatment plasma, insulin levels were substantially lower (31%, $P < 0.05$) compared to the high-fructose fed rats. Moreover, the plasma insulin levels following LP and HP treatment were reduced by 33% ($P < 0.01$) and 29% ($P < 0.05$), respectively, compared to before treatment (Figure 3B). The HOMA-IR, a representative index of insulin resistance, was significantly higher in the high-fructose fed rats compared to the chow fed controls at week 6, but was significantly reduced by LP (35%) and HP (34%) treatment (Figure 3C). Furthermore, HOMA-IR following LP treatment was 25% ($P < 0.05$) lower compared to before treatment (Figure 3C). Plasma C-peptide is another biomarker associated with insulin resistance.

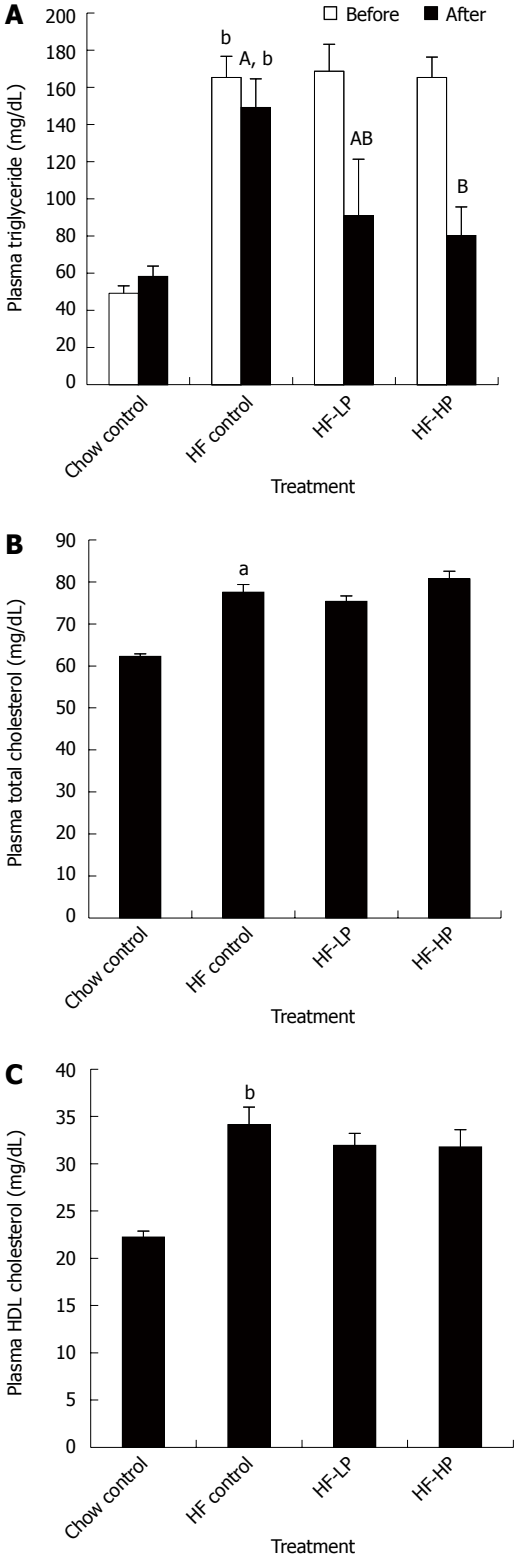

Figure 2 Effects of probiotic treatment on plasma triglyceride (A), plasma total cholesterol (B) and plasma high-density lipoprotein cholesterol (C) in high-fructose diet-fed rats. Results are expressed as mean ± SE. $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* chow control by unpaired Student's *t*-test; ^{AB}Bars with different capital letters are significantly different at $P < 0.05$ by Duncan's multiple range tests. HF: High fructose diet; HP: High dose probiotic; LP: Low dose probiotic; HDL: High-density lipoprotein.

Plasma C-peptide level was 24% ($P < 0.05$) lower following HP treatment compared to placebo treatment in high-fructose fed rats (Figure 3D). Moreover, plasma C-peptide levels after HP treatment were 28% ($P < 0.05$) lower compared to before treatment (Figure 3D). In addition, probiotic treatment of either LP or HP decreased plasma TBARS levels by 37% ($P < 0.01$) and 50% ($P < 0.001$), respectively (Figure 3E).

Effects of probiotic treatment on hepatic lipid content and gene expression

Both hepatic triglyceride and cholesterol levels in the high-fructose fed rats were significantly higher than those in the chow fed controls. Probiotic treatment with either LP or HP tended to reduce hepatic triglyceride levels (Figure 4A), and HP significantly reduced (27%, $P < 0.05$) hepatic cholesterol levels compared to the high-fructose fed controls (Figure 4B).

In order to determine the mechanisms underlying the probiotic effect on hepatic lipid homeostasis, we examined the mRNA levels of hepatic genes associated with lipid metabolism (Figure 5A-C). High-fructose diet intake altered gene expression compared to chow diet intake. The changes in gene expression in the high-fructose group indicated decreases in fatty acid β-oxidation (PPARα, CPT1, CPT2 and ACOX1), cholesterol uptake (LDLR) and bile acid synthesis (CYP7A1), and increases in fatty acid synthesis (SREBP1, FAS, SCD1). Importantly, HP treatment significantly reversed high-fructose-induced gene expression changes including up-regulation of PPARα (+76%), CPT2 (+66%) and CYP7A1 (+71%), and down-regulation of SREBP1 (-30%), FAS (-54%) and SCD1 (-23%), although LP treatment did not cause any significant changes in hepatic lipid metabolism gene expression.

DISCUSSION

Recent studies indicate that the gut microbiota plays an important role in host lipid and glucose metabolism. Therefore, therapeutic probiotics which can manipulate the gut microbiota may also prevent some of the risk factors underlying the development of metabolic syndrome including dyslipidemia, elevated fasting glucose levels and insulin resistance[23]. In the present study, we used a novel probiotic consisting of *L. curvatus* HY7601 and *L. plantarum* KY1302 isolated from Korean fermented cabbage. We showed that probiotic administered to high-fructose fed mice reversed the risk factors underlying the metabolic syndrome. Previous evidence from high-fructose diet-fed rat studies indicated that a probiotic-cultured yogurt called Dahi can also improve metabolic abnormalities[19,24]. Importantly, in the present study, we established the dose-dependent effects of a novel combination of probiotic strains on metabolic syndrome.

High-fructose intake is reported to promote lipogenesis and suppress glucose intake[25-27]. Consistent with other studies of high-fructose fed animals, hepatic gene expression analysis indicated that lipogenesis was increased *via*

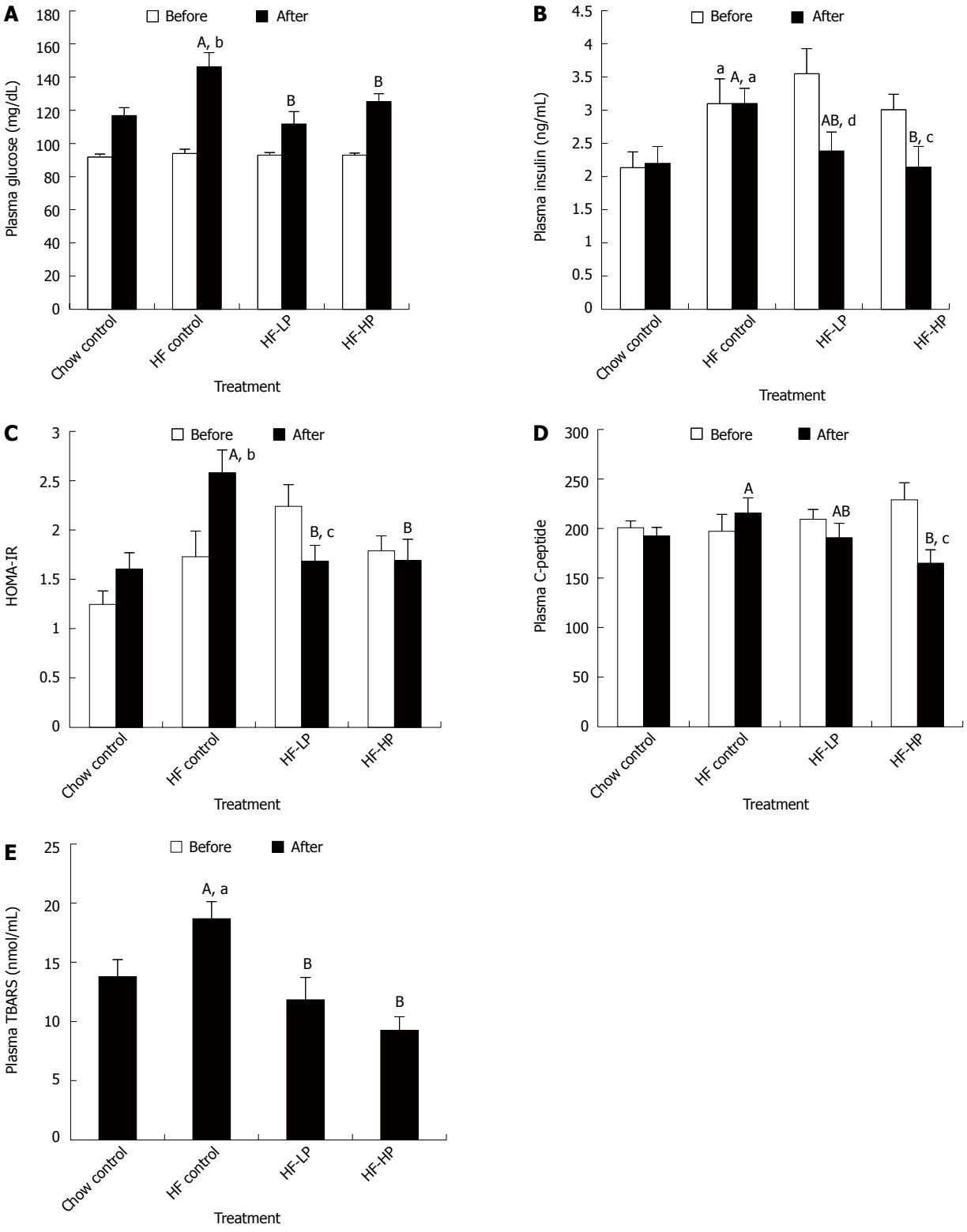

Figure 3 Effects of probiotic treatment on plasma glucose, plasma insulin, insulin resistance, plasma C-peptide and plasma thiobarbituric acid-reacting substances in high-fructose diet-fed rats. Results are expressed as mean ± SE. [a]*P* < 0.05 and [b]*P* < 0.01 HF control *vs* chow control by unpaired Student's *t*-test; [c]*P* < 0.05 and [d]*P* < 0.01 week 6 *vs* week 3 by paired Student's *t*-test; [AB]Bars with different capital letters are significantly different at *P* < 0.05 by Duncan's multiple range tests. HF: High fructose diet; LP: Low dose probiotic; HP: High dose probiotic; TBARS: Thiobarbituric acid-reacting substances.

upregulation of SREBP1, FAS and SCD1, conversely β-oxidation was decreased *via* downregulation of PPARα and PPARα-regulated CPT1, CPT2 and ACOX1 expression[25-27]. Increased lipogenesis and decreased β-oxidation lead to excess accumulation of cellular lipids, as shown by the liver enlargement and hypertriglyceridemia in the high-fructose fed rats in the present study. Hypertriglyceridemia is known to be an important predictor of car-

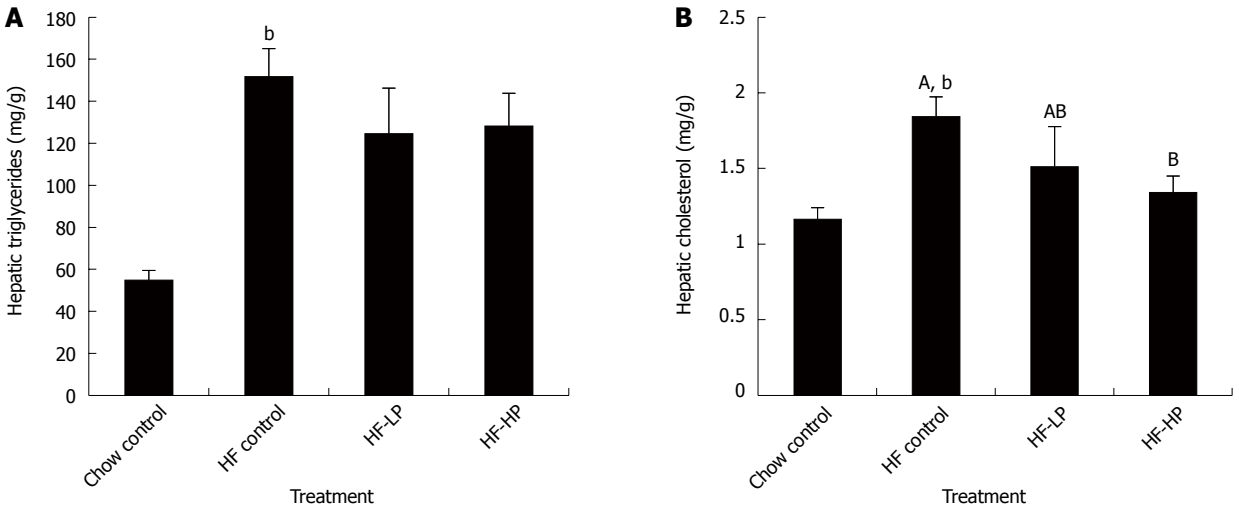

Figure 4 Effects of probiotic treatment on hepatic triglyceride and hepatic total cholesterol in high-fructose diet-fed rats. Results are expressed as mean ± SE. $^{b}P < 0.01$ HF control *vs* chow control by unpaired Student's *t*-test; ^{AB}Bars with different capital letters are significantly different at $P < 0.05$ by Duncan's multiple range tests. HF: High fructose diet; LP: Low dose probiotic; HP: High dose probiotic.

diovascular disease mortality in subjects with diabetes or impaired glucose tolerance[28], therefore, reducing plasma triglyceride levels may improve long-term health.

Previous studies have shown that probiotics can alter the gut microbiota[29], and direct gut microbiota manipulations in germ-free mice significantly affect host lipid metabolism[2,12]. Here we showed that 10^{10} cfu/d probiotic treatment led to upregulated PPARα and CPT2 expression reflecting activation of β-oxidation, and downregulated SREBP1, FAS and SCD1 expression reflecting suppression of lipogenesis. Moreover, these probiotics induced transcriptional changes which resulted in a significant reduction in liver mass and plasma triglyceride levels. These findings are consistent with a previous report which showed that *L. plantarum* KY1032 inhibits lipid droplet accumulation during adipocyte differentiation. In contrast to plasma triglyceride levels, hepatic triglyceride levels were only slightly reduced by probiotic treatment. We hypothesized that the probiotic-induced increase in hepatic β-oxidation related gene expression was partly to clear excess hepatic triglycerides generated through high-fructose-induced *de novo* lipogenesis.

Contrary to hepatic triglyceride levels, cholesterol levels were significantly reduced by probiotic treatment, whereas plasma cholesterol levels were unchanged. We assessed whether the reduction in hepatic cholesterol following probiotic treatment was due to altered hepatic cholesterol metabolism related gene expression. High-fructose diet suppressed CYP7A1 expression, which encodes cholesterol 7 α-hydroxylase, the rate-limiting enzyme involved in the formation of bile from cholesterol. However, 10^{10} cfu/d probiotic treatment upregulated CYP7A1 expression indicating increased bile synthesis activity. Some probiotics are also reported to increase bile salt hydrolase activity[30]. Hepatic LDLR expression remained unchanged, suggesting that probiotic treatment did not enhance uptake of plasma cholesterol into the liver, which is consistent with the absence of changes in plasma cholesterol levels. However, the effect of probiotics on liver cholesterol metabolism may be partly dependent on the diet regime used, because in high-fat or high-cholesterol diet fed animals, probiotics are reported to decrease plasma and hepatic cholesterol levels together.

The probiotic effect on host lipid levels may also contribute to the observed improvement in glucose homeostasis. Excess intracellular lipids can inhibit insulin signalling, hence reduce insulin-stimulated glucose uptake leading to hyperglycemia and hyperinsulinemia as shown in this study. We observed that probiotic treatment at 10^{9} or 10^{10} cfu/d significantly lowered plasma glucose, insulin and C-peptide concentrations, and reduced insulin resistance indicated by the reduced HOMA-IR index[20,31]. Excess fructose intake is also reported to promote lipid peroxidation and oxidative stress is implicated in the pathogenesis of insulin resistance[32,33]. In the present study, probiotic treatment at 10^{9} or 10^{10} cfu/d significantly reversed the oxidative stress present in the high-fructose fed rats with metabolic syndrome, indicated by lower plasma TBARS levels. Some *Lactobacillus* strains are also reported to possess anti-oxidative activity. For example, probiotics have been reported to reduce exercise-induced oxidative stress, *via* increases in anti-oxidative activity, which helps neutralize reactive oxygen species[34]. Furthermore, studies in human Type 2 diabetes show probiotic supplementation can increase superoxide dismutase and glutathione peroxidase activities[35], which are anti-oxidants that help protect against oxidative stress.

While evidence is growing on the effects of probiotic treatment on many chronic pathologies, some issues remain to be addressed. Probiotics are widely assumed to modulate the gut microbiota, and exert health benefits *via* direct modification of gut microbial communities[8]. Studies on diet-induced mice indicate that probiotics alter the gut microbiota[29], similar to our recent experience with the same probiotic used in this study (unpublished observations). However, in another study using germ-

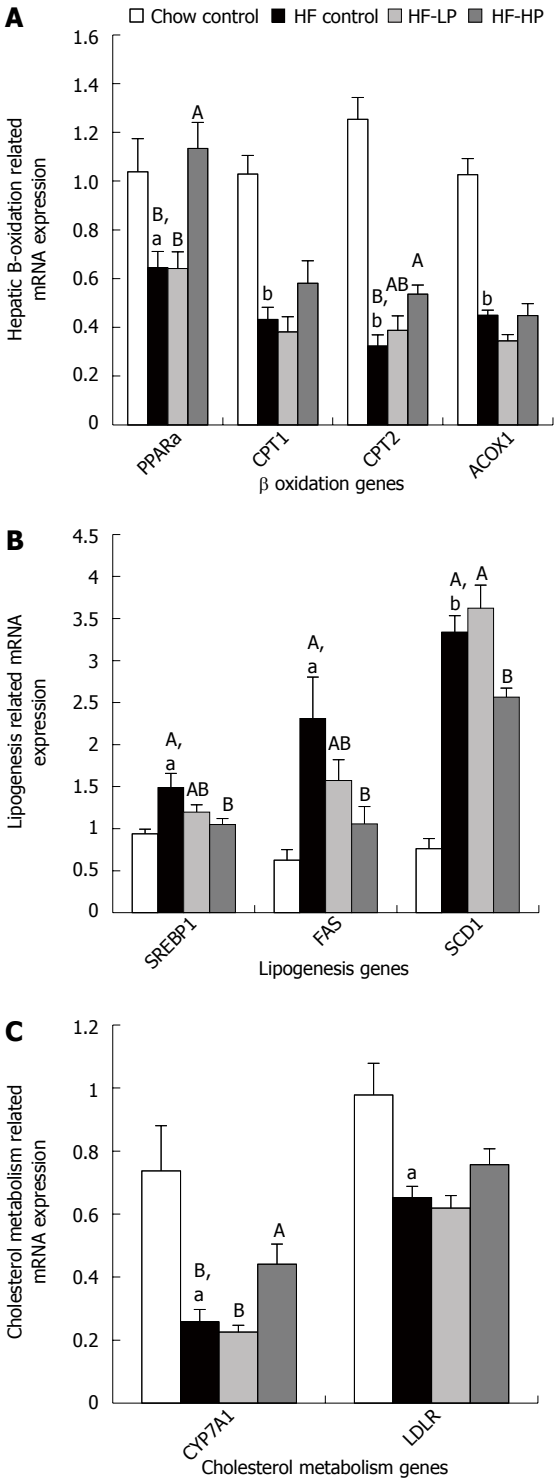

Figure 5 Effects of probiotic treatment on hepatic B-oxidation, lipogenesis and cholesterol metabolism related gene expression in high-fructose diet-fed rats. Results are expressed as mean ± SE. $^{a}P < 0.05$ and $^{b}P < 0.01$ HF control *vs* chow control by unpaired Student's *t*-test; ^{AB}Bars with different capital letters are significantly different at $P < 0.05$ by Duncan's multiple range tests. HF: High fructose diet; LP: Low dose probiotic; HP: High dose probiotic; FAS: Fatty acid synthase; SREBP1: Sterol regulatory element-binding protein-1; PPARα: Peroxisome proliferator-activated receptor α; CPT: Carnitine palmitoyltransferase; ACOX: Acyl-coenzyme A oxidase; SCD: Steaoryl-CoA desaturase; CYP7A1: Cholesterol 7α-hydroxylase gene; LDLR: Low-density lipoprotein receptor.

free mice transplanted with a small artificial gut microbial community, a multi-species probiotic failed to change the gut microbiota[36]. Whether multi-species probiotics are more effective than single-species probiotics is not clear, because few studies make this comparison. We used a two species probiotic in the present study, and based on our preliminary data the results suggest that combined species are more effective than either species alone. Finally, whether probiotics exert dose-dependent effects against metabolic syndrome has not been evaluated, nor has the minimum amount of live bacteria that is necessary for functional health benefits. Evidence from the present study indicates that probiotics containing 10^{10} cfu/d showed the greatest effectiveness against high-fructose-induced metabolic syndrome over a relatively short period, without any adverse effects. In contrast, probiotic treatment at 10^{9} cfu/d exerted minimal effects on hypertriglyceridemia, but effectively improved hyperglycaemia and insulin resistance. In the case of hepatic gene expression, 10^{9} cfu/d probiotic treatment had no modulatory effect on any of the genes tested, but probiotic treatment at 10^{10} cfu/d modulated lipogenesis and β-oxidation related genes.

In conclusion, probiotic *L. curvatus* HY7601 and *L. plantarum* KY1032 combined can suppress the clinical characteristics of high-fructose-induced metabolic syndrome, therefore, may provide a natural alternative for the treatment of diet-induced metabolic syndrome.

ACKNOWLEDGMENTS

Choi MS and McGregor RA declare no conflicts of interest. Park DY, Ahn YT and Huh CS are current or past employees of Korea Yakult Co., Ltd.

COMMENTS

Background

Metabolic syndrome is a growing health problem characterised by elevated blood sugar and blood lipid levels, insulin resistance, high blood pressure and abdominal obesity. Poor diet consisting of high sugar or high fat content is a major risk factor for metabolic syndrome. There is no universal treatment for metabolic syndrome. Probiotics found in fermented foods have emerged as a natural way of protecting against metabolic syndrome, but many probiotic strains have varying metabolic effects. Therefore, a major challenge for scientists is discovering probiotic strains, which may help protect against metabolic syndrome.

Research frontiers

Probiotic strains identified in kimchi, a traditional Korean fermented cabbage consumed regularly in South Asian countries, are reported to have various beneficial properties. Current research aims to determine whether these probiotic strains are effective in different animal models of disease including metabolic syndrome, Type 2 diabetes and obesity.

Innovations and breakthroughs

The authors showed that two probiotic strains, *Lactobacillus curvatus* (*L. curvatus*) HY7601 and *Lactobacillus plantarum* (*L. plantarum*) KY1032, can suppress metabolic abnormalities such as hypertriglyceridemia, hyperglycemia and insulin resistance in high-fructose-induced metabolic syndrome. These probiotic health benefits were associated with decreased lipogenesis and increased β-oxidation-related gene expression in the liver.

Applications

Probiotic with *L. curvatus* HY7601 and *L. plantarum* KY1032 may provide a natural supplement for the management of the underlying risk factors of metabolic syndrome.

Terminology

Probiotics consist of live micro-organisms which confer beneficial effects on host health. Hypertriglyceridemia is prolonged elevated triglyceride levels in blood. Hyperglycemia is prolonged elevated glucose levels in the blood. Insulin resistance is the inability of insulin to stimulate glucose uptake pathways in fat, skeletal muscle and the liver.

Peer review

This paper investigated the effect of novel probiotics on the clinical characteristics of high-fructose induced metabolic syndrome. The authors were suggesting a mechanism. One novel thing of this paper is that no one has looked at these specific microbes in this combination. It is well written.

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BRIEF ARTICLE

Clinical significance and risk factors of postembolization fever in patients with hepatocellular carcinoma

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Author contributions: Jun CH and Choi SK performed the majority of the study and wrote the manuscript; Cho SB, Park SY, and Park CH were involved in editing the manuscript; Ki HS, Lee GH, Park KJ were involved in data acquisition, analysis or interpretation; all authors were involved in revising and approving the final version for publication.

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Abstract

AIM: To investigate tumor response and survival in patients with postembolization fever (PEF) and to determine the risk factors for PEF.

METHODS: Four hundred forty-three hepatocellular carcinoma (HCC) patients who underwent the first session of transcatheter arterial chemoembolization (TACE) between January 2005 and December 2009 were analyzed retrospectively. PEF was defined as a body temperature greater than 38.0 ℃ that developed within 3 d of TACE without evidence of infection. The tumor progression-free interval was defined as the interval from the first TACE to the second TACE based on mRECIST criteria. Clinical staging was based on the American Joint Committee on Cancer tumor, node, metastases (TNM) classification of malignant tumors. All patients were admitted before their 1st TACE treatment, and blood samples were obtained from all patients before and after treatment. Clinicoradiological variables and host-related variables were compared between two groups: patients with PEF *vs* patients without PEF. Additionally, variables related to 20-mo mortality and tumor progression-free survival were analyzed.

RESULTS: The study population comprised 370 (85.4%) men and 73 (14.6%) women with a mean age of 62.29 ± 10.35 years. A total of 1836 TACE sessions were conducted in 443 patients, and each patient received between 1 and 27 (mean: 4.14 ± 3.57) TACE sessions. The mean follow-up duration was 22.23 ± 19.6 mo (range: 0-81 mo). PEF developed in 117 patients (26.41%) at the time of the first TACE session. PEF was not associated with 20-mo survival (P = 0.524) or computed tomography (CT) response (P = 0.413) in a univariate analysis. A univariate analysis further indicated that diffuse-type HCC (P = 0.021), large tumor size (≥ 5 cm) (P = 0.046), lipiodol dose (≥ 7 mL, P = 0.001), poor blood glucose control (P = 0.034), alanine aminotransferase (ALT) value after TACE (P = 0.004) and C-reactive protein (CRP) value after TACE (P = 0.036) served as possible risk factors correlated with PEF. The ALT value after TACE (P = 0.021) and lipiodol dose over 7 mL (P = 0.011) were independent risk factors for PEF in the multivariate analysis. For the 20-mo survival, poor blood sugar control (P < 0.001), portal vein thrombosis (P = 0.001), favorable CT response after TACE (P < 0.001), initial aspartate aminotransferase (P = 0.02), initial CRP (P = 0.042), tumor size (P < 0.001), TNM stage (P < 0.001) and lipiodol dose (P < 0.001) were possible risk factors in the univariate analysis. Tumor size (P = 0.03), poor blood sugar control (P = 0.043), and portal vein thrombosis (P = 0.031) were significant predictors of survival in the multivariate analysis. Furthermore, the tumor progression-free interval was closely associated with CRP > 1 mg/dL (P = 0.003), tumor size > 5 cm (P < 0.001), tumor type (poorly defined) (P < 0.001), and lipiodol dose (> 7 mL, P < 0.001).

CONCLUSION: PEF has no impact on survival at 20 mo or radiologic response. However, the ALT level after TACE and the lipiodol dose represent significant risk factors for PEF.

Key words: Chemoembolization; Therapeutic; Fever; Carcinoma; Hepatocellular; Prognosis; Progression-free survival

Jun CH, Ki HS, Lee HK, Park KJ, Park SY, Cho SB, Park CH, Joo YE, Kim HS, Choi SK, Rew JS. Clinical significance and risk factors of postembolization fever in patients with hepatocellular carcinoma. *World J Gastroenterol* 2013; 19(2): 284-289 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i2/284.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i2.284

INTRODUCTION

Hepatocellular carcinoma (HCC) is the seventh most common carcinoma worldwide and the third most common cause of cancer-related mortality[1]. In South Korea, the age-standardized incidence rate of HCC is 46.5 per 100 000 individuals[2]. Recent advances in treatment, including liver transplantation, surgical resection, percutaneous ethanol injection therapy, radiofrequency ablation and transcatheter arterial chemoembolization (TACE) have improved the prognosis for patients with HCC[3,4]. In addition, beneficial therapeutic options that may affect long-term cure include surgical resection, liver transplantation, and percutaneous ablation[5]. However, these curative therapies are feasible only for a small subset of patients with HCC[6]. Among the noncurative therapies, only chemoembolization, the most widely used treatment for unresectable HCC, has demonstrated a positive effect on survival[7]. In contrast, TACE can be employed for any type of HCC irrespective of tumor size, location, or number[8].

However, TACE inevitably results in a hypoxic insult to the HCC and the surrounding liver tissue[9], and postembolization syndrome is common[10]. Postembolization syndrome, which consists of temporary fever, ileus, and abdominal pain, is the most common side effect of chemoembolization, affecting 60% to 80% of patients with HCC[5,11]. Postembolization fever after TACE is the most significant adverse effect, and it frequently affects the duration of hospitalization and causes the needless administration of antibiotics, although the fever is self-limited in most cases. However, few data about postembolization fever have been reported. Therefore, we evaluated the risk factors and prognostic significance of postembolization fever in patients with HCC.

MATERIALS AND METHODS

Patients and methods

Four hundred forty-three HCC patients who underwent the first session of TACE between January 2005 and December 2009 were analyzed retrospectively. The diagnosis of HCC was confirmed histologically or based on consistent findings obtained from at least two imaging techniques: ultrasonography, computed tomography (CT), magnetic resonance imaging, and/or selective hepatic arterial angiography[12,13]. Clinical staging was determined based on the American Joint Committee on Cancer tumor, node, metastases (TNM) classification of malignant tumors[14].

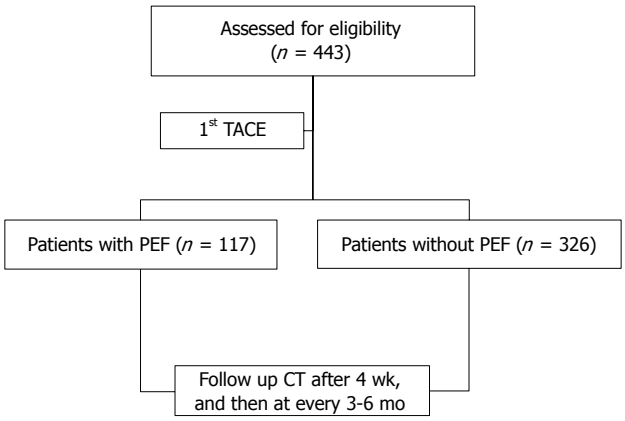

Figure 1 Flowchart of study patient enrollment. PEF: Postembolization fever; TACE: Transcatheter arterial chemoembolization; CT: Computed tomography.

Clinicoradiological variables were compared between two groups (patients with PEF *vs* patients without PEF). The host-related variables included age, sex, viral status, cause of HCC, Child-Pugh score, Eastern Cooperative Oncology Group (ECOG) performance status, white blood cell counts, aspartate aminotransferase (AST), alanine aminotransferase (ALT), α-fetoprotein (AFP), and 20-mo mortality. The tumor-related variables included maximal tumor size, number of tumors, TNM stage, radiological findings (poorly defined or well defined), portal vein thrombosis, and CT response after 1st TACE.

All patients were admitted before their 1st TACE, and blood samples were obtained from all patients before and after treatment. Serum AFP, CRP, blood chemistry and ECOG score at admission were measured. After the 1st TACE, the patients were carefully followed. Dynamic CT was performed after 4 wk and then every 3 to 6 mo (Figure 1).

Our institutional review board did not require approval because the procedures were performed for clinical reasons. Informed consent was obtained from all patients after the nature and purpose of the TACE procedure had been fully explained.

Chemoembolization procedure

An arterial catheter was inserted into the femoral artery using the Seldinger method and placed in the hepatic artery. Tumor-feeding vessels were superselected whenever possible, and a solution containing 10 to 40 mg of doxorubicin hydrochloride (ADM; Dong-A Pharmacy, Seoul, Korea) and 0 to 40 mL of iodized oil (Lipiodol; Guer-

Table 1 Comparison of transcatheter arterial chemoembolization sessions with or without postembolization fever

Variables	Patients with PEF (n = 117)	Patients without PEF (n = 326)	P-value
Sex (M/F)	94/23	276/50	0.309
Age (yr)	62.26 ± 10.79	62.16 ± 9.98	0.971
Cause of HCC (HBV/HCV/alcohol/other)	66/25/14/11	180/44/73/25	0.088
Tumor type (well defined/poorly defined)	57/44	202/90	0.021
Portal vein thrombosis (yes/no)	9/93	39/253	0.292
Poor blood glucose control (yes/no)	29/88	116/210	0.039
Favorable tumor response	61 (52.5%)	155 (48.5%)	0.516
20-mo mortality	60 (51.7%)	172 (53.4%)	0.828
Initial WBC/mm^3	5969 ± 3497	5547 ± 2535	0.194
Initial AST U/L	68.87 ± 45.49	64.11 ± 44.79	0.358
Initial ALT U/L	50.84 ± 41.58	43.89 ± 33.16	0.129
Initial CRP mg/dL	1.23 ± 1.66	0.86 ± 0.93	0.116
Initial AFP IU/mL	2778 ± 8014	2600 ± 8656	0.857
Child-Pugh score	5.66 ± 0.72	5.77 ± 0.97	0.296
TNM stage	1.95 ± 1.05	1.83 ± 1.07	0.305
Interval from 1st TACE to 2nd TACE (d)	157 ± 225	180 ± 270	0.412
Total TACE sessions	1.48 ± 3.45	4.02 ± 3.62	0.230

TACE: Transcatheter arterial chemoembolization; AFP: α-fetoprotein; PEF: Postembolization fever; TNM: Tumor, node, metastases; CRP: C-reactive protein; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HCC: Hepatocellular carcinoma; M: Male; F: Female; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

bet, Aulnay-sous-Bois, France) with absorbable gelatin particles (Gelfoam; Upjohn, Kalamazoo, Michigan) was injected through the catheter (5F) or microcatheter (2.8 or 3F). The doses of doxorubicin and iodized oil were individually determined according to tumor size, tumor extent, and the patient's underlying liver function.

Monitoring and management of postembolization fever

For the purpose of this study, we defined postembolization fever as a body temperature greater than 38.0 ℃ during the 3 d after TACE. Body temperature was measured *qid* by nurses using an axillary thermometer. Bacterial cultures from blood and urine and chest X-rays were performed for patients who had fevers after TACE to detect any potential infectious agents. Empirical broad-spectrum intravenous antibiotics were used to treat potential infections if there was fever but were discontinued when bacterial cultures did not reveal any causative agent and the fever had subsided. Ultrasonography or CT scans were performed if the fever persisted despite the use of antibiotics to detect the possible formation of an abscess. Acetaminophen or nonsteroidal anti-inflammatory drugs were used for symptom control, if necessary.

Response assessment

The efficacy of TACE was evaluated by comparing the CT scans obtained before and after chemoembolization in terms of iodized oil uptake patterns in the tumor that could be considered necrotic[12] and tumor extent. The iodized oil uptake was considered compact if the oily contrast medium was clearly dispersed through all viable target tumors but was noncompact in all other cases[8]. Tumor response to TACE was defined as a compact uptake of iodized oil or at least a 30% decrease in the sum of the largest diameters of viable tumors, despite noncompact iodized oil uptake.

Definitions

PEF was defined as a body temperature greater than 38.0 ℃ that developed within 3 d of TACE without evidence of infection. Poor blood glucose control was defined as a mean blood glucose level > 200 mg/dL. Poorly defined tumor type was defined as diffuse-type HCC, whereas well-defined tumor type was defined as nodular HCC. Tumor progression-free survival was defined as the interval during and after treatment in which a patient remained alive and the disease did not worsen (in this case, the interval from the 1st TACE to the 2nd TACE).

Statistical analysis

Comparisons were performed using the student's t test for continuous variables and Pearson's χ^2 test. Factors that were significant in the univariate analysis were entered into a stepwise multivariate analysis to identify the most significant risk factors. The hazard function data were estimated using the Kaplan-Meier curve and compared using the log-rank test. Multivariate analyses were performed using the Cox proportional hazards model to identify prognostic factors. We performed statistical analyses using SPSS 17.0 (SPSS Inc., Chicago, United States). A P-value less than 0.05 was considered statistically significant.

RESULTS

Fever after TACE and clinical features

The study population consisted of 370 (85.4%) men and 73 (14.6%) women with a mean age of 62.29 ± 10.35 years. A total 1836 sessions of TACE were conducted in 443 patients between January 2005 and December 2009. Each patient received between 1 and 27 (mean, 4.14 ± 3.57) sessions of TACE. The mean follow-up duration was 22.23 ± 19.6 mo (range: 0-81 mo).

One hundred seventeen episodes of postembolization fever (26.41%) occurred in 443 HCC patients after the 1st TACE session. Most of the postembolization fever episodes peaked within the first two days after TACE. The post-TACE fever was usually self-limiting, with durations ranging from 1 to 10 d (mean: 1.72 ± 1.11 d). The infectious complication rate was 0.16% (3/1836 cases). Two cases of bacteremia and one case of liver abscess developed after 1836 TACE sessions. A comparison of the TACE sessions with and without fever is presented in Table 1.

Association of PEF with clinical variables

A univariate analysis indicated that diffuse-type HCC (P < 0.05), large tumor size (≥ 5 cm) (P < 0.05), lipiodol

Table 2 Multivariate and univariate analysis for postembolization fever, 20-mo mortality, and tumor progression-free survival

Variables	HR	95%CI	*P*-value
Multivariate analysis[1]			
ALT value after TACE	1.002	1.00-1.005	< 0.05
Lipiodol dose (< 7 mL)	0.539	0.329-0.881	< 0.05
Univariate analysis[2]			
Poor BS control	2.673	1.77-4.034	< 0.01
Portal vein thrombosis	3.048	1.536-6.06	< 0.01
Poor CT response	2.638	1.785-3.891	< 0.01
Initial AST	1.006	1.001-1.011	< 0.05
Initial CRP	1.348	1.011-1.796	< 0.05
Tumor size	1.31	1.198-1.433	< 0.01
TNM	1.555	1.26-1.92	< 0.01
Lipiodol dose	1.12	1.058-1.185	< 0.01
Multivariate analysis[3]			
Tumor size	1.252	1.108-1.414	< 0.01
Poor BS control	2.442	1.310-4.55	< 0.01
Portal vein thrombosis	3.344	1.021-10.98	< 0.05
Cox regression analysis[4]			
Poor CT response after TACE	0.302	0.192-0.765	< 0.01
Lipiodol dose (≥ 7 mL)	0.494	0.279-0.874	< 0.05

[1]Predictors for postembolization fever; [2]Risk factors for 20-mo mortality; [3]Predictors for 20-mo mortality; [4]Predictors for tumor progression-free survival. HR: Hazard ratio; CRP: C-reactive protein; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TNM: Tumor, node, metastases; TACE: Transcatheter arterial chemoembolization; BS: Base of support.

dose (≥ 7 mL) ($P < 0.01$), poor blood glucose control ($P < 0.05$), ALT value after TACE ($P < 0.01$) and CRP value after TACE ($P < 0.05$) were possible risk factors correlated with postembolization fever in patients with HCC.

A multivariate analysis using logistic regression showed that the ALT value after TACE ($P < 0.05$) and the lipiodol dose (≥ 7 mL) ($P < 0.05$) were independent predictive factors of postembolization fever (Table 2).

PEF was not associated with 20-mo survival ($P = 0.754$), 10-mo survival ($P = 0.524$) and CT response ($P = 0.461$) in the univariate analysis.

20-mo mortality

The univariate analysis revealed that poor blood sugar control ($P < 0.01$), portal vein thrombosis ($P < 0.01$), favorable CT response after TACE ($P < 0.01$), initial AST ($P < 0.05$), initial CRP ($P < 0.05$), tumor size ($P < 0.01$), TNM stage ($P < 0.01$) and lipiodol dose ($P < 0.01$) were possible risk factors correlated with 20-mo mortality (Table 2). A multivariate analysis using logistic regression showed that tumor size ($P < 0.01$), poor blood glucose control ($P < 0.01$) and portal vein thrombosis ($P < 0.05$) were independent risk factors for 20-mo mortality (Table 2).

Tumor progression-free survival

The progression-free survival in the poorly defined tumor (diffuse) type group was significantly shorter than in the well-defined (nodular) tumor type group ($P < 0.01$). Additionally, large size (size ≥ 5 cm, $P < 0.01$), no antiviral treatment ($P < 0.05$), poor CT response ($P < 0.01$), lipiodol dose (dose ≥ 7 mL, $P < 0.01$), antibiotic use

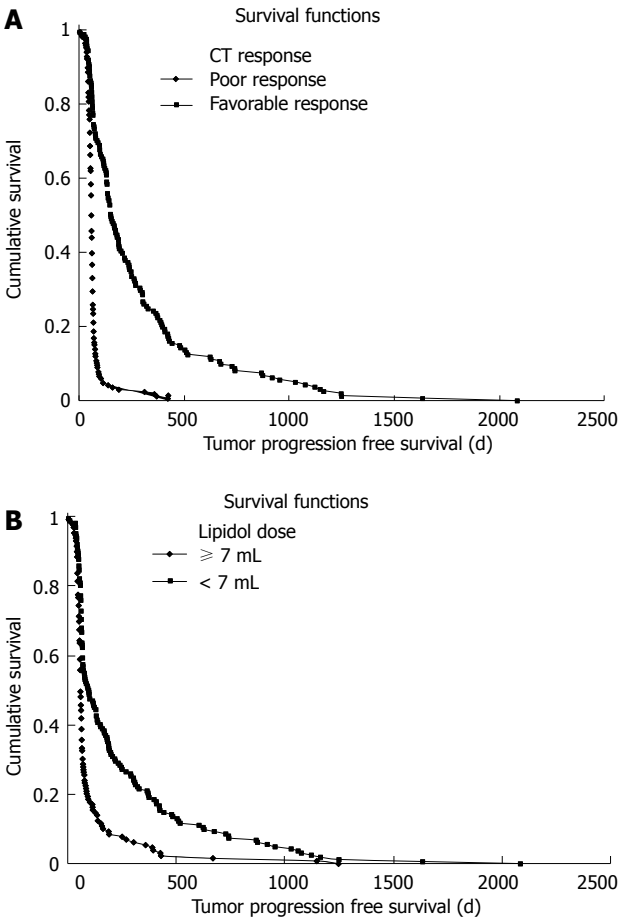

Figure 2 Kaplan-Meier curves of progression-free survival. A: According to computed tomography response (log-rank test, $P < 0.001$); B: According to lipiodol dose (log-rank test, $P < 0.001$).

($P < 0.05$) and CRP (CRP ≥ 1 mg/dL, $P < 0.01$) were statistically significant factors in the univariate analysis (Figure 2A and B). In the multivariate Cox proportional hazard model for progression-free survival, CT response ($P < 0.01$) and lipiodol dose (≥ 7 mL) ($P < 0.05$) were identified as independent factors (Table 2).

DISCUSSION

TACE is one of the major treatment methods for unresectable HCC and has demonstrated survival benefits[4,10-15]. Chemoembolization acts by obstructing the hepatic artery with embolization agents, usually gelatin, and introducing antitumor agents (e.g., cisplatin, doxorubicin, and mitomycin C) emulsified in iodized oil, thereby inducing extensive necrosis in large vascularized HCC tumors[7]. TACE complications can be categorized as hepatic injuries, including deterioration of hepatic function, hepatic infarction, or intrahepatic biloma, and liver abscess; extrahepatic complications, including gastrointestinal bleeding, gallbladder or spleen infarction, and pulmonary embolism; and systemic complications, including postembolization syndrome and septicemia[16]. The most frequent complication of chemoembolization is postembolization fever, which can typically be satisfactorily

alleviated with symptom treatment[11,16]. Nevertheless, PEF frequently troubles patients, family members and physicians, and few data have been published concerning postembolization fever; therefore, we investigated the risk factors and clinical significance of PEF that developed after TACE in patients with HCC.

Infectious complications are very rare because of the standard antiseptic procedures associated with TACE. Another study reported that only 0.26% of HCC patients developed liver abscesses after TACE[17]. Thus, antibiotic prophylaxis is usually not necessary in patients with HCC who are undergoing TACE[10]. Although fevers were common (27%) in this study, they were generally not caused by an infectious process because very few patients had bacterial infections (0.16%). These fevers can often be adequately controlled with antipyretics, and in most cases, antibiotics are not necessary.

The pathogenesis of PEF remains unclear and complicated. The main aspects are as follows: (1) lipiodol-induced embolisms may result in ischemia, hypoxia, and necrosis in some normal hepatic cells; (2) chemotherapeutic drugs themselves have toxicities[18]; (3) the procedure itself can lead to a considerable release of inflammatory factors[19]; and (4) such stimuli as injury and drugs can contribute to stress responses in the human body. In the present study, the occurrence of PEF was closely associated with several clinical and laboratory variables, including poor blood glucose control, large tumor size (> 5 cm), poorly defined tumor type, post-TACE CRP level, lipiodol dose higher than 7 mL, and post-TACE ALT level in a univariate analysis. However, the multiple regression analysis showed that a lipiodol dose over 7 mL and the post-TACE ALT level were independent risk factors, which is similar to the result of another recent study[20]. No difference was found between favorable CT responses and unfavorable CT responses regarding the presence of PEF, which is in concordance with another study showing that post-TACE fever was not associated with an enhanced tumor response in patients with HCC[20-22].

PEF is thought to reflect extensive tumor necrosis, thereby representing the efficacy of chemoembolization[22-24]. However, we observed no other robust association between PEF and survival in this study. Moreover, PEF did not independently affect progression-free survival, which may be an indirect indicator of treatment efficacy. These findings suggest that the previously described correlation between PEF and the extent of tissue necrosis cannot always be justified because the extent of tissue necrosis after chemoembolization is proportional to tumor mass, a factor that is independently associated with PEF.

Raoul *et al*[25] suggested that factors associated with poor TACE outcomes included Child-Pugh score, reduced liver function, AFP level, tumor size, tumor number, tumor type, portal vein thrombosis, multiple TACE sessions and lobar embolization. In our study, large tumor size, poor blood glucose control and portal vein thrombosis were independent risk factors for 20-mo mortality. However, unlike in other studies, the Child-Pugh score was not a significant prognostic factor in our study. It was likely that most of the patients enrolled in our study had Child A (the Child score for patients with PEF was 5.66 *vs* 5.77 for those without PEF), and as a result, liver function did not affect the prognosis. According to Shim *et al*[22], hepatitis B virus infection, modified UICC stage (Stage 1), and response to chemoembolization are independent predictive factors for the tumor progression-free interval. In good agreement with other studies, we found that the TACE response and lipiodol dose (< 7 mL) were closely associated with tumor progression-free survival. Because the lipiodol dose was dependent on tumor size, progression-free survival was closely associated with tumor size and response to TACE.

This study had several limitations, including its retrospective design. Although we performed laboratory and culture studies of blood, urine, and ascites to detect hidden infections, it was impossible to rule out all infective complications in patients with PEF. In addition, the tumor response to chemoembolization may have been overestimated in patients with poorly defined HCC, particularly those with the diffuse infiltrative type of HCC, because of the difficulty of evaluating the degree of viable tumor. Finally, it may be difficult to determine whether the development of PEF after a single session of TACE was exclusively associated with overall survival because the mortality of TACE-treated HCC patients is subject to many other factors.

In conclusion, ALT levels after TACE and lipiodol dose were independent risk factors for postembolization fever in HCC patients. However, postembolization fever after TACE had no impact on survival at 20 mo or on the radiologic response.

COMMENTS

Background

Postembolization fever (PEF) is thought to reflect extensive tumor necrosis and thereby represent the efficacy of transcatheter arterial chemoembolization (TACE) in hepatocellular carcinoma (HCC). The aim of this study was to assess the tumor response and survival of patients with PEF after TACE and to determine the risk factors for PEF.

Research frontiers

TACE has improved the prognosis of patients with HCC. However, TACE inevitably results in a hypoxic insult to the HCC and the surrounding liver tissue, and postembolization fever is common. However, the clinical meaning of and the risks factor for PEF are not known.

Innovations and breakthroughs

PEF has been associated with overall survival in HCC patients. However, there is limited published data about PEF and the risk factors for PEF. Unlike other studies, their demonstrated that PEF was not associated with overall survival or radiological tumor response. Furthermore, this study demonstrated that alanine aminotransferase (ALT) levels after TACE and lipiodol doses were risk factors for PEF.

Applications

The results of this study imply that PEF is associated with lipiodol dose and ALT levels after TACE but does not affect prognosis; therefore, patients undergoing TACE who have risk factors for PEF should receive active or prophylactic antipyretics for PEF control.

Terminology

Postembolization fever was defined as a body temperature greater than 38.0 ℃

that developed within 3 d of TACE without evidence of infection. Poorly defined tumor type was defined as diffuse-type HCC, whereas well-defined tumor type was defined as nodular HCC. Tumor progression-free survival was defined as the interval of time during and after treatment in which a patient remained alive and the disease did not worsen (in the case of this study, the interval from the 1st TACE to the 2nd TACE).

Peer review

Postembolization fever is a clinically relevant problem in the treatment of patients with HCC. The authors addressed potential causes and risk factors and found that the ALT level after TACE and the lipiodol dose served as independent risk factors for PEF after TACE and that PEF had no impact on 20-mo survival in HCC patients. These results were extracted and calculated from a large group of 443 patients who were treated and observed within a 5-year period.

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BRIEF ARTICLE

IL28B polymorphism and cytomegalovirus predict response to treatment in Egyptian HCV type 4 patients

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Abstract

AIM: To test whether the status of positive cytomegalovirus (CMV) DNA detection adds to the predictive value of IL28B and to further categorize C/T allele carriers.

METHODS: This study included 166 chronic hepatitis C (CHC) patients who received combined interferon and ribavirin therapy for 48 wk, 84 spontaneous hepatitis C virus (HCV) resolvers who were positive for IgG anti-HCV antibody and negative for HCV RNA, and 100 healthy subjects who were negative for both HCV antibodies and RNA as controls. Genomic DNA from peripheral blood was used for IL28B rs.12979860 single nucleotide polymorphism (SNP) and CMV DNA detection. A 139 bp fragment containing IL28B SNP was amplified in all subjects by polymerase chain reaction using a specifically designed primer. Then the IL28B rs.12979860 SNP was detected by restriction fragment length polymorphism (RFLP) genotyping. The presence of CMV DNA was tested by amplification of the *gB1* gene using nested polymerase chain reaction. The role of CMV and IL28B rs.12979860 SNP genotypes in determining the response rate to combined interferon therapy and clinical status of patients were statistically analyzed.

RESULTS: Current data showed that 67% of patients carrying the IL28B 12979860 C/C allele had a sustained viral response (SVR) while the genotypes C/T and TT were associated with lower SVR rates, 50% and 48%, respectively. SVR rates for the C/C allele were lower than other HCV genotypes and/or other populations. Genotype CC was associated with the response to interferon (P = 0.025). Genotype C/C was reduced from 48% in controls to 14% in CHC patients suggesting its protective role against progression to chronicity. The majority of spontaneously cleared subjects (86%) were C/C, confirming its protective role. The C/T allele was present in 71% of CHC patients compared with 38% of controls, so the use of IL28B SNP genotyping only in these patients may be of little value as a predictor of response. CMV reactivation occurred in 40% of CHC patients. Co-infection with CMV seriously diminished the response to interferon (IFN) therapy, with SVR rates in C/C genotypes 87.5% in CMV-negative patients and 12.5% in CMV-positive patients (P < 0.0001). SVR rates among C/T carriers were reduced to < 50% in patients with positive CMV DNA while the non-response rate doubled. These data indicate that a supplemental assay for CMV viremia adds to the prognostic value of IL28B genotyping.

CONCLUSION: The results suggest that both genetic (i.e., spontaneous) and therapeutic (IFN-based therapy) arms are complementary in the battle against HCV. CMV DNA testing may be of value to better predict the response to IFN, particularly in IL28B C/T carriers.

Key words: Hepatitis C; Interleukin 28B; Genetic polymorphisms; Human cytomegalovirus; Spontaneous clearance

El Awady MK, Bader El Din NG, Tabll A, El Hosary Y, Abdel Aziz AO, El Khayat H, Salama M, Abdelhafez TH. IL28B polymorphism and cytomegalovirus predict response to treatment in Egyptian HCV type 4 patients. *World J Gastroenterol* 2013; 19(2): 290-298 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i2/290.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i2.290

INTRODUCTION

The current standard-of-care for chronic hepatitis C virus (HCV) infection includes weekly injections of pegylated interferon-α (peg-IFN) combined with daily oral ribavirin[1,2] for 48 wk[3]. During the first 3 mo of therapy, HCV viral loads usually fall to undetectable levels in response to interferon-α. However, in non-responder (NR) patients, HCV viral loads persist at or near pretreatment levels. Among those patients with an initial response to treatment, known as early virological responders, up to 50% will relapse after treatment is discontinued and are known as relapsed responders, whereas the remainder will have a sustained virological response (SVR) as determined by the absence of detectable viremia 6 mo after treatment has stopped[4]. In Egypt, several reports stated that approximately 50% of patients infected with genotype 4, the most common among Egyptian HCV patients, achieve a SVR to this regimen. Genome-wide association studies have recently revealed that single nucleotide polymorphisms (SNPs) within or adjacent to IL28B (19q13) that codes for interferon-λ3, predict spontaneous resolution of HCV[5,6] and a likely SVR to IFN-based treatment in chronic hepatitis C (CHC) patients infected with genotypes other than type 4[4,5,7-9]. Therefore, IL28B SNPs may have strong predictive value for the outcome of IFN-based therapy in the difficult to treat HCV genotype 4 patients, with the hope that IL28B SNPs will improve future decision-making in the management of CHC in Egypt. Nevertheless, it was estimated that IL28B variations account for about 15% of the inter-individual variability of SVR, thus supporting the necessity for additional predictor(s) of the response to treatment. Recent predictors include vitamin D deficiency, IFN-c-inducible protein-10 serum levels, steatosis/insulin resistance[10-17], or IFN-stimulated genes (ISGs) such as OAS1[18] and MxA[19]. Therefore, biological models for the prediction of SVR should include a variety of parameters besides IL28B genotyping, as this would offer better accuracy compared with IL28B typing alone[13]. Recently two predictors for SVR in genotype 4 patients were reported; first, reactivation of CMV infection[20] and second, SNP at exon 7 splice acceptor site of 2' 5'OAS gene[18]. The role of several genetic factors that influence spontaneous or treatment-induced HCV clearance, such as ISGs and genes encoding the natural killer cell receptor KIR2DL3 and its ligand, and human leukocyte antigen C group 1[21-23] should be re-analyzed parallel with the IL28 genotype. A major difficulty in decision-making for prediction of the response at the start of treatment is the high frequency of heterozygous carriers rs 12979860 C/T among chronic HCV patients, ranging from 45% to 70% of the CHC populations and provide vague predictive results. This means that the predictive value of rs 12979860 SNP will not be useful in almost half the patient population. The search for additional predictive factors for response is mandatory. Co-infection with other pathogens is, in some instances, an interfering factor against host genotype-based prediction. Several studies revealed comparable associations between IL28B variations and treatment-induced HCV clearance in patients co-infected with human immunodeficiency virus (HIV)[5,24-26]. For example, one study in HIV co-infected patients reported 2-3-fold higher SVR rates for HCV genotypes 1 and 4 according to rs12979860 C/C *vs* C/T, T/T, but no difference for HCV genotype 3 according to rs12979860 C/C *vs* C/T, T/T[26]. Recently, active CMV infection was shown by our laboratory[20] to dramatically interfere with SVR rates in genotype 4 CHC patients, probably by inhibiting the JAK-STAT cascade. We, therefore, hypothesize that the role of CMV in the IL28B association with SVR should be a strong candidate for further categorization of C/T genotype carriers.

MATERIALS AND METHODS

Subjects

The present study comprised 350 subjects, including 166 CHC patients, 84 spontaneous HCV resolvers and 100 healthy control subjects.

Chronic HCV patients: All 166 patients had mild liver disease F0-F3 according to the Metavir classification[27]. All subjects had undetectable HBV surface antigen (HBsAg), Anti-schistosoma antibodies (Abs), and autoimmune Abs. None of the patients had a history of long-term drug use, aflatoxins consumption or alcohol intake. All patients were infected with HCV genotype 4. Patients included 105 males and 61 females, with an age range of 19-57 years. None had uncontrolled type II diabetes mellitus, thyroid dysfunction or obesity. All patients received weekly injection of peg-IFN-α plus daily oral ribavirin treatment for 48 wk. Eighty six patients achieved a SVR, namely undetectable HCV RNA 24 wk after the end of

the treatment response that was achieved after 48 wk, i.e., a total of 72 wk. The remaining 80 patients were NR who failed to achieve a SVR, i.e., either did not respond to therapy after 12 wk or achieved undetectable viremia but failed to maintain the state of no viremia till 48 wk (breakthrough) or post 48 wk (viral relapse).

Spontaneous HCV resolvers: Eighty four subjects who tested positive for IgG anti-HCV Abs (3rd generation) and negative for HCV RNA were enrolled in the study. Patients included 53 males and 31 females, with an age range of 18-55 years.

Controls: One hundred healthy subjects who tested negative for both IgG anti HCV Abs and HCV RNA served as controls for the IL28B SNP and CMV reactivation studies. Also, they were negative for HBsAg, anti-schistosoma Abs, autoimmune Abs and CMV DNA. Subjects included 50 males and 50 females, with an age range of 18-64 years. None of subjects had a history of liver insult, either viral, metabolic or drug exposure.

Methods

Extraction of peripheral blood DNA: Peripheral blood was withdrawn from all subjects into EDTA solution and DNA was extracted using genomic DNA extraction kit (Qiagen, Milan Italy). Plasma samples were separated before DNA extraction and utilized for testing HBsAg, anti schistosomiases Abs and autoimmune Abs (ANA, AMA, LKM1, ASMA). Purified genomic DNA was used for IL28B SNP analysis and for testing the presence of CMV DNA.

Detection of IL-28B rs12979860 C/T polymorphism: Genotyping for the IL-28B rs12979860 C/T polymorphism was performed according to Fabris *et al*[24] with minor modification by polymerase chain reaction based restriction fragment length polymorphism (PCR-RFLP). Using purified genomic DNA, a 139 base-pair (bp) product was obtained with the forward primer 5'-CCAGGGCCCCTAACCTCTGCA-3' and the reverse primer 5'-GGGAGCGCGGAGTGCAATTCA-3', newly designed with the aid of NCBI Primer-Blast Tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Amplification was carried out in a total volume of 10 μL containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, Tween-20 0.01%, 0.2 mmol/L deoxyribonucleotides, 2-4 pmol of each primer, 2.0 mmol/L MgCl2, 0.5 units hot-start Taq DNA polymerase (Righ Taq, Euroclone, Milan, Italy) and ~10 ng of genomic DNA. The thermal protocol for amplification included 35 cycles of denaturation (at 95 ℃ for 60 s), annealing (at 64 ℃ for 60 s), and polymerization (at 72 ℃ for 60 s). Ten μL of the amplicons were digested with 1 unit of the BstU-I restriction endonuclease (New England Biolabs, Hitchin, UK) in a total volume of 20 μL at 37 ℃ for 4-6 h or overnight. The fragments were resolved by electrophoresis in 4% agarose gel after staining with ethidium bromide. A band of 139 bp indicated the TT genotype, 109 bp indicated the CC genotype; 139 + 109 bp indicated the CT genotype.

PCR amplification of the CMV *gB* gene: Primers for the first-round PCR were derived from the *gB* region of CMV, and PCR protocols were followed as described previously[28,29] The amplification mixture contained 10 pmol of each primer (CMV1 and CMV2), 0.2 mmol/L from each dNTP (Promega, Madison, WI, United States), 0.1 U Taq polymerase (Promega, Madison, WI, United States), 1 μL Taq buffer, 5 μL template, and distilled water to a final volume of 20 μL. A negative control tube (in which water replaced the DNA sample) was incorporated into each run. A sample from the first PCR product was used as a template in a second-round PCR (nested). Briefly, 1 μL of first round product was added to the reaction mixture, which is the same as the first-round reaction mix except for use of CMV3 and CMV4 as nested primers. The thermal cycling protocol was as follows: 1 min at 94 ℃, 1 min at 55 ℃ and 1 min at 72 ℃ for 30 cycles. Then, the nested PCR products were analyzed with 3% agarose gel electrophoresis and stained with ethidium bromide. A result was considered positive when a clear 100 bp product was visible on the gel.

Primers used for the first and the second-round PCR were CMV1: 5'-GAGGACAACGAAATCCTGTTGGGCA-3'; CMV2: 5'-GTCGACGGTGGAGATACTGCTGAGG-3'; CMV3: 5'-ACCACCGCACTGAGGAATGTCAG-3'; and CMV4: 5'-TCAATCATGCGTTTGAAGAGGTA-3'.

Assessment of SVR status (real-time and RT-PCR of HCV RNA: As reported in our previous study[20,29]. Disappearance of viremia was confirmed by a variety of tests including RT-PCR using nested primers derived from the highly conserved HCV 5'UTR and real-time PCR. These tests were done 3, 6, 12 and 18 mo after the start of IFN therapy. HCV RNA was extracted using the BIOZOL-total RNA extraction reagent kit (Hangzhou Bioer Technology, Hangzhou, China). Nested RT-PCR performed in a 25 μL reaction mixture containing 20 units of avian myeloblastosis virus reverse transcriptase (Clontech, Mountain View, CA, United States), 200-400 ng of total cellular RNA as template, 40 units of RNAsin (Clontech), 0.2 μmol/L from each dNTP (QBIOGENE, Carlsbad, CA, United States), and 50 pmol of the reverse primer P2. The reaction was incubated at 42 ℃ for 60 min and denatured at 98 ℃ for 10 min. Amplification of the highly conserved 5'-UTR sequences was done using two rounds of PCR with two pairs of nested primers. First-round amplification was done in 50 μL of reaction containing 50 pmol from each of the P1 forward primer and P2 reverse primer, 0.2 mmol/L from each dNTP, 10 μL from RT reaction mixture as template and two units of Taq DNA polymerase (Finnzyme, Woburn, MA, Unit-

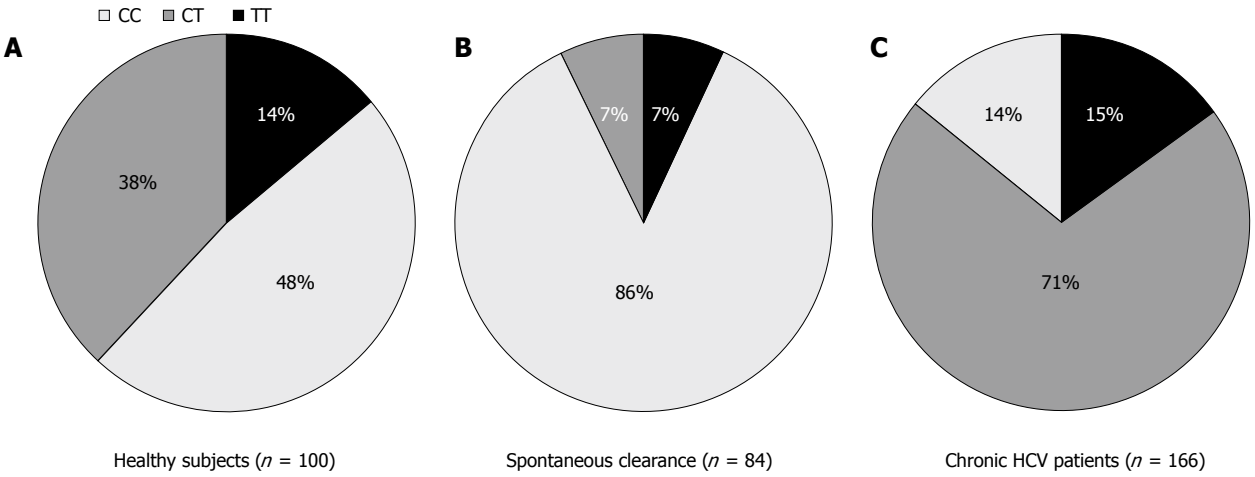

Figure 1 Distribution of LI28B rs12979860 single nucleotide polymorphism alleles among a healthy Egyptian population, spontaneous clearance and chronic hepatitis C patients. A: A hundred healthy subjects representing ethnic backgrounds of the Egyptian population were typed for IL28B single nucleotide polymorphism (SNP) rs 12979860. The distribution of different allele frequencies is depicted as a pie chart; B: Eighty four patients with spontaneous clearance from hepatitis C virus (HCV) infection were typed for IL28B SNP rs 12979860. The distribution of different allele frequencies is depicted as a pie chart; C: A hundred and sixty six chronic hepatitis C (CHC) patients with low F scores (F0-F2) were typed for IL28B SNP rs 12979860. The distribution of different allele frequencies is depicted as a pie chart.

ed States) in a 1 × buffer supplied with the enzyme. The thermal cycling protocol was as follows: 1 min at 94 ℃, 1 min at 55 ℃ and 1 min at 72 ℃ for 30 cycles. The second round of amplification was done similar to the first round, except for use of the nested reverse primer p4 and forward primer p3 at 50 pmol each. A fragment of 171 bp length was identified in positive samples. Primer sequences were as follows: P1: 5'-AACTACTGTCTTCACGCAGAA-3'; P2: 5'-GGTGCACGGTCTACGAGACCTC-3'; P3: 5'-GTGCAGCCTCCAGGACCC-3'; and P4: 5'-ACTCGGCTAGCAGTCTCGCG-3'.

HCV quantitation was performed using the Fluorescence Quantitative Detection kit (Hangzhou Bioer Technology, Hangzhou, China). HCV RNA was reverse transcribed and a specific fragment was amplified with specific primers in a one-step RT-PCR reaction. The products were detected using specific Taqman-MGB Probes. The real-time RT-PCR reaction was done in 50 μL of reaction containing 25 μL RT-PCR mix, 2.5 μL Mn^{2+}, 1.5 μL HCV probe mix, and 1 μL internal control. Then 20 μL of the corresponding HCV RNA template were added. For the standard, 20 μL of the standards control 1-4 were added. All PCR tubes were placed in the real-time PCR Detection Instrument. The thermal cycling protocol was as follows: one cycle (30 s at 90 ℃, 20 min at 61 ℃ and 1 min at 95 ℃) followed by 30 cycles and 50 cycles (15 s at 95 ℃, 1 min at 60 ℃).

Statistical analysis

The significance of the response to IFN therapy was investigated by multivariate logistic regression analysis. CMV significantly affected the HCV response rate in C/T heterozygous carriers. The frequency of rs 12979860 SNP was compared in the control group *vs* the CHC patient group. All statistical analyses were performed using SPSS 9.0 statistical software (SPSS Inc., Chicago, IL, United States). The odds ratio (OR) and 95% confidence interval (CI) were calculated to assess the relative risk confidence. A two tailed *P*-value 0.05 was considered statistically significant.

RESULTS

IL 28 C/C frequency in spontaneous resolvers of HCV infection

To test the hypothesis whether patients who spontaneously cleared HCV infection during or shortly following the acute phase bear the protective C/C allele more frequently than healthy subjects, we typed the IL28 B SNP (rs 12979860) in 84 spontaneous resolvers and 100 healthy subjects. The results of this study were previously described by our laboratory and are shown in Figure 1A and B, which clearly indicate the protective role of the C/C allele in patients who spontaneously cleared the virus, where C/C was present in 86% of spontaneous resolvers *vs* 48% of healthy subjects.

IL28B C/C frequency in chronic HCV patients

To examine the allele frequencies of the C/C genotype according to progression to chronic HCV infection, we compared the IL28B typing data derived from 166 chronic HCV patients with 84 subjects with spontaneous clearance of HCV. The results showed that the frequency of the protective C/C allele was markedly decreased from 86% in spontaneous resolvers to 48% in healthy controls to 14% in CHC patients regardless of their response to IFN therapy (Figure 1A-C). This decline was associated with a comparable increase in the heterozygous C/T from 7% in spontaneous resolvers to 38% in healthy subjects and to 71% in CHC patients.

Table 1 Statistical analyses of the different IL28B genotypes and alleles in non-responder and sustained viral response patients *n* (%)

	Non-responders (NR = 79)	Responders (SVR = 87)	Odds ratio	95%CI	*P* value
CC (*n* = 24)	8 (33.3)	16 (66.7)	4.575	1.376-7.846	0.025
CT (*n* = 117)	58 (49.6)	59 (50.4)	0.858	0.538-1.449	0.589
TT (*n* = 25)	13 (52)	12 (48)	0.978	0.758-1.759	0.464
C (*n* = 165)	74 (44.8)	91 (55.2)	1.636	0.773-3.465	0.198
T (*n* = 167)	84 (50.3)	83 (49.7)	0.798	0.598-1.349	0.604

Frequencies of genotypes and alleles at IL28B SNP were compared between SVR and NR patients. Genotype CC were associated with SVR (*P* = 0.025). SVR: Sustained viral response; NR: Non-responder.

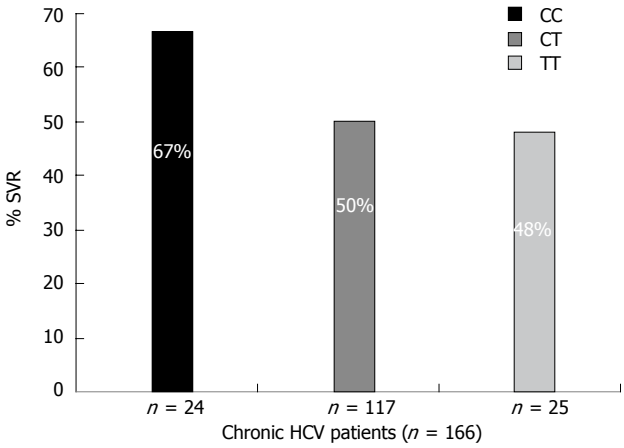

Figure 2 Rate of sustained viral response in IL28B variants. Among 166 chronic hepatitis C (CHC) patients treated with combined interferon (IFN) and ribavirin therapy, 80 patients did not achieve a sustained viral response (SVR) while 86 patients achieved a SVR. Among the 14% (24) of CHC patients bearing the C/C allele, 67% (16 patients) achieved a SVR, while the other two genotypes C/T and TT were associated with lower SVR rates; 50% and 48%, respectively. Genotype CC was associated with response to IFN (*P* = 0.025). HCV: Hepatitis C virus.

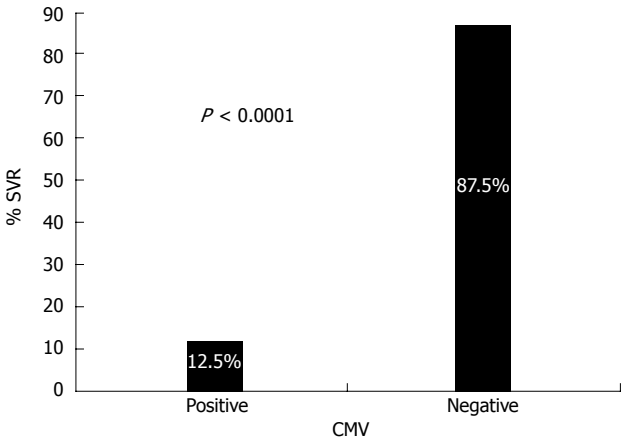

Figure 3 Association of cytomegalovirus reactivation with sustained viral response rates in C/C patients. The data shown clearly demonstrate that cytomegalovirus (CMV) reactivation has dramatically reduced the sustained viral response (SVR) rates in C/C genotypes as represented by a 12.5% SVR rate in CMV-positive patients compared with 87.5% in CMV-negative patients (*P* < 0.0001).

Rate of SVR in patients with the rs12979860 C/C allele

Among 166 CHC patients treated with combined IFN and ribavirin therapy, 79 patients did not achieve a SVR while 87 patients achieved a SVR. The effect of different IL28B genotypes and alleles on the HCV response to IFN treatment are shown in Table 1. The genotype CC was associated with response to IFN ($P = 0.025$). Of the 24 CHC patients bearing the C/C allele, 16 (67%) achieved a SVR, while the other two genotypes C/T and TT were associated with lower SVR rates: 50% and 48%, respectively (Figure 2).

Association of CMV reactivation with SVR rates in C/C patients

The PCR results of the CMV *gB* gene showed that 66/166 (40%) of chronic HCV patients have detectable CMV DNA. Since we have previously shown that coinfection with CMV seriously diminished the response to IFN therapy, it was tempting to examine whether this co-infection is associated with lower SVR rates in C/C holders. The data shown in Figure 3 clearly demonstrate that positive CMV DNA dramatically reduced the SVR rates in C/C genotypes as represented by a 12.5% SVR rate in CMV-positive patients compared with 87.5% in CMV-negative patients ($P < 0.0001$).

Sorting of C/T genotype according to association with CMV infection

Since the C/T allele represents the majority of CHC patients (117/166) (71%), the use of IL28B SNP genotyping may be of little value as a predictor of response prior to starting the treatment regimen. Therefore, there is a great need to include another factor that was previously known to affect SVR rates. Table 2 shows the results of CMV testing in CT genotype patients. Specifically, CMV DNA was detected in 71% of NR patients and in 29% of SVR patients. In Figure 4, the SVR rates among C/T carriers was reduced to < 50% of its value in cases where patients had positive CMV DNA On the other hand, the NR rate doubled in cases of positive CMV DNA. These data indicate that a supplemental assay for CMV viremia adds to the prognostic value of IL28B genotyping.

DISCUSSION

Several predictive factors have been reported recently for SVR rates for combination therapy in HCV infection. Viral factors, other than viral loads and genotypes were reported, including substitutions at core amino acids 70 and 91[30,31], IFN sensitivity determining region variations[32] and IFN and ribavirin response determining region variations[33]. SNPs within a number of host genes were found to be associated with different treatment outcomes to therapy. These include IFN-α pathway genes[34] and IFN-stimulated genes such as OAS[18]

Table 2 Influence of cytomegalovirus infection on response rate to interferon combined therapy in chronic hepatitis C virus infected patients with IL28B CT genotype *n* (%)

HCV response to treatment (*n* = 117)	IL 28B genotype (CT) +ve CMV patients (*n* = 45)	IL 28B genotype (CT) -ve CMV patients (*n* = 72)
NR (*n* = 59)	32 (71)	27 (38)
SVR (*n* = 58)	13 (29)	45 (62)

Reactivation of cytomegalovirus (CMV) was assessed by amplification of viral genome in all patients. Association of IL28B genotype CT and CMV emia with response to interferon based therapy is illustrated in this table. SVR: Sustained viral response; NR: Non-responder.

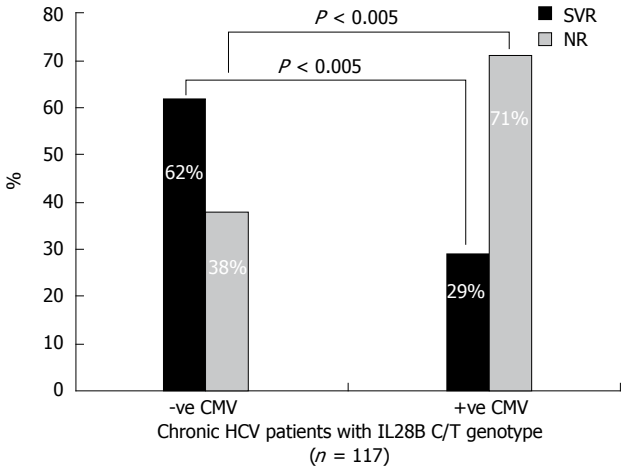

Figure 4 Sorting of C/T genotype according to association with cytomegalovirus infection. The sustained viral response (SVR) rates among C/T carriers, is reduced to < 50% of its value in cases where patients had cytomegalovirus (CMV) reactivation. On the other hand, the non-response rate is increased 2-fold in cases of CMV reactivation. HCV: Hepatitis C virus.

and MxA[19]. The widely studied IL28B SNP allele was reported to correlate with a favorable therapeutic response and was also associated with spontaneous clearance of HCV[6]. Thomas *et al*[6] reported that the allele related to HCV clearance, namely rs 12979860 C/C, was the major allele in the majority of Asian and European countries. Conversely, patients of African ancestry had lower C/C allele frequency and SVR rates. The current data indicate that the Egyptian population infected mainly with genotype 4 has an intermediate response between that of the populations of African and European ancestries, i.e., the C/C allele frequency in normal subjects was 48% *vs* > 50% in Europeans and ~35% in Africans while SVR rates in the currently studied CHC patients were 67% *vs* ~80% in Europeans and ~50% in Africans. One of the most interesting aspects of the current study is the dramatic decline in C/C frequency in CHC patients (from 48% in controls to 14% in genotype 4 infections), thus allowing speculation that protective IL28B variations provide a more substantial advantage in acute HCV genotype 4. In a similar study on a German cohort, the rs12979860 C/C genotype was found in 49% of uninfected subjects and declined in chronic infection to 42.7% of HCV genotype 2 and 3 patients and 33.9% of HCV genotype 1 patients[35]. Taking together the data from the German cohort and the data of the current study, we may speculate that HCV genotype 4 has the highest advantage of viral clearance during acute infection followed by genotype 1 and lastly genotypes 2 and 3.

Strong evidence from the current data on the significant protective effect of the IL28 B variant in acute HCV 4 is the high percentage of the C/C genotype among spontaneously cleared individuals, i.e., positive HCV Abs and negative HCV RNA compared with controls and CHC patients (86% *vs* 48 and 14%, respectively). The lack of a protective role of the C/C genotype in 14% of CHC patients suggests that, in CHC patients with the C/C genotype, other factors are involved in development of chronic infection. It is tempting to hypothesize that IL28B C/C in those 24 CHC patients was not sufficient to provide substantial tendency towards viral clearance and consequently explains the relatively lower figure for the C/C association with SVR rate in the present cohort compared with Asian and European studies. In a recent study from our laboratory co-infection with active CMV viremia induced severe failure to achieve acceptable rates of SVR[20]. Infection with CMV has evolved multiple mechanisms for disrupting the IFN-stimulated JAK/STAT signal transduction. It appears to inhibit IFN-α responsiveness by decreasing JAK1 protein, which is an essential component of IFN-α signaling[36]. It was reported that CMV blocks IFN-stimulated gene factor 3 (ISGF3)-dependent (MHC class I, 2',5'-OAS, and MxA) and ISGF3-independent gene expression in infected cells. Moreover, the essential component of ISGF3, p48, is significantly decreased by CMV. Therefore decreased JAK1 and p48 protein would inhibit IFN-α stimulated signal transduction, transcription factor activation, and gene expression, thus it is likely to globally block IFN-stimulated responses in CMV-infected patients[37]. The dual analysis of both roles of IL28B SNP and CMV co-infection clearly showed that C/C carriers not infected with CMV have a 7-fold higher rate of SVR than those C/C patients co-infected with CMV. One of the major problems in decision-making on an individual patient basis is the overwhelming preponderance of the IL28B C/T genotype among the current CHC cohort as well as other cohorts[38,39]. The present results indicate that the IL28B SNP is not the only factor that influences the IFN-induced antiviral activities of host cells against viral infection. The present results showed a 2-fold increase in SVR rate in CMV-negative over CMV-positive C/T carriers and clearly highlight the significance of additional testing for CMV viremia besides IL28 genotyping to set a model for accurate prediction of response to combined therapy in C/T carriers.

In conclusion the current results allow us to speculate that both genetic (i.e., spontaneous) and therapeutic (IFN-based therapy) arms are complementary in the battle against HCV where poor spontaneous clearance is

associated with higher IFN-mediated response as in genotypes 2 and 3. On the other hand, the difficult to treat genotypes 1 and 4 have clearly better chances for spontaneous resolution. Furthermore, CMV DNA testing may be of value at the moment to make better prognostication on response to IFN particularly in heterozygous carriers. Needless to say the search for supplemental predictive host factors will never stop at least in the near future.

COMMENTS

Background

The current recommended treatment for chronic hepatitis C virus (HCV) infection is the combination of pegylated α-interferon (IFN) and ribavirin given for 48 wk. Both viral and host factors may contribute to the phenomenon that some patients with chronic hepatitis C respond well to IFN therapy but others do not. The host genetic factors mediate the vigor of the immune response of the host through the control of inter-individual differences in the production of intracellular antiviral proteins or some cytokines. Different studies revealed that single nucleotide polymorphisms (SNPs) within or adjacent to IL28B (19q13) that codes for interferon-λ3, predict spontaneous resolution of HCV and likely SVR to IFN based treatment in chronic HCV patients infected with genotypes other than type 4. Since more than 93% of patients in Egypt are infected with HCV genotype 4, the authors investigated whether IL28B SNPs may be of predictive value for the outcome of IFN-based therapy in the difficult to treat HCV genotype 4 patients. Also the authors tested whether the status of positive cytomegalovirus (CMV) DNA detection added to the predictive value of IL28B and to further categorization of C/T allele carriers.

Research frontiers

HCV is a global health problem that infects more than 170 million people around the world and more than 15% of the Egyptian population. The response rate to combined IFN therapy does not exceed 50% of patients infected with HCV genotype 4, the most prevalent subtype in Egypt. Timing and rules for this therapy without major complications are not very well defined, so that infected patients not only suffer from severe adverse effects but there is also a contribution to evolution of more resistant strains of the virus. A research focus is how to determine and understand which HCV patients are likely to develop persistent infection, progressive liver disease or do not respond significantly to IFN therapy. These criteria are of utmost importance in disease control programs and treatment strategies. Therefore, there is an urgent need for biological models containing a variety of prognostic parameters to predict the response to IFN treatment in HCV-infected patients and to help make better decisions on treatment strategy.

Innovations and breakthroughs

The use of IL28B SNP analysis in predicting the response to combined IFN therapy is informative only in cases of CC or TT genotypes. Since the heterozygous genotype CT represents more than 70% of chronic HCV patients, there is a need for supplemental markers for making a decision on whether the HCV patient is a potential responder to combined IFN therapy or not. The authors' data clearly improve the decision-making by detecting CMV reactivation in the CT genotype group. CMV reactivation has been shown to dramatically interfere with the response to combined IFN treatment. The data presented in this manuscript are indeed important for hepatologists regarding the anticipated response to standard combined therapy for HCV genotype 4 patients.

Applications

The data of this manuscript help hepatologists make better decisions on response rate to combined interferon therapy for HCV genotype 4 patients.

Peer review

In this manuscript, the authors aimed to investigate whether the status of positive cytomegalovirus (CMV) DNA detection increases the predictive value of IL28B and to further categorize C/T allele carriers. The authors have found that CMV DNA testing might be valuable at the moment to make better decisions on response to IFN particularly in IL28B C/T carriers. Overall, this topic is very interesting.

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CASE REPORT

Lubiprostone induced ischemic colitis

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Abstract

Ischemic colitis accounts for 6%-18% of the causes of acute lower gastrointestinal bleeding. It is often multifactorial and more commonly encountered in the elderly. Several medications have been implicated in the development of colonic ischemia. We report a case of a 54-year old woman who presented with a two-hour history of nausea, vomiting, abdominal pain, and bloody stool. The patient had recently used lubiprostone with close temporal relationship between the increase in the dose and her symptoms of rectal bleeding. The radiologic, colonoscopic and histopathologic findings were all consistent with ischemic colitis. Her condition improved without any serious complications after the cessation of lubiprostone. This is the first reported case of ischemic colitis with a clear relationship with lubiprostone (Naranjo score of 10). Clinical vigilance for ischemic colitis is recommended for patients receiving lubiprostone who are presenting with abdominal pain and rectal bleeding.

Key words: Lubiprostone; Ischemic colitis; Gastrointestinal bleeding; Rectal bleeding; Colonoscopy

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INTRODUCTION

Ischemic colitis in patients with nonocclusive vascular disease is thought to result from a transient low flow state through an inadequate vascular system. Advanced age, aortic surgery, diabetes mellitus, hypertension, and peripheral vascular disease have been also suggested to be predisposing factors for ischemic colitis[1]. It occurs more often in the splenic flexure and rectosigmoid junction which are known as the watershed areas. These areas are the distributions of the superior mesenteric artery and the inferior mesenteric artery for the splenic flexure and between the inferior mesenteric artery and the internal iliac artery for the rectosigmoid junction[1].

The pathophysiology of developing ischemic colitis includes transient hypoperfusion that involve segments of the colon. Affected patients are often elderly or have a cardiovascular condition (congestive heart failure or an arrhythmia) which puts them at risk to develop a transient reduction in bowel perfusion. Less common causes of ischemic colitis include hypercoagulable states, vasculitis, embolus, colonic obstruction (strictures, colon cancer, diverticulitis)[1,2]. Other causes include medications and substances such as amphetamines, alosetron, catechol-

amines (epinephrine, norepinephrine), cocaine, digitalis, nonsteriodal anti-inflammatory drugs, pseudoephedrine and triptans, *etc*[3].

Lubiprostone was initially approved in 2006 by the US Food and Drug Administration for the treatment of idiopathic chronic constipation at a dose of 24 mcg twice daily[4-6]. In 2008, it was approved for women with constipation predominant irritable bowel syndrome at dose of 8 mcg twice daily[4,5,7]. Lubiprostone is a prostaglandin E1 derivative which acts by activating chloride channel type-2 (CLC-2) in the apical membrane of the intestinal epithelial cells. Consequently, it produces chloride rich fluid into the intestinal lumen with a passive sodium and water shift which increases the liquidity of the stool and promotes the gastrointestinal tract motility which in turn accelerates the bowel transit[8]. Lubiprostone acts locally on the bowel epithelium and is minimally absorbed. It is well known to cause nausea, diarrhea, abdominal distention, and abdominal pain. Here we describe the first case report of ischemic colitis associated with lubiprostone usage.

CASE REPORT

A 54-year old female presented with a two-hour history of nausea, non-bloody, non-bilious vomiting associated with crampy generalized abdominal pain located to the epigastrium and left upper quadrant. She did not have any bowel movements 3 d prior to her presentation. In the emergency department, she had one bowel movement with hard stool followed by 6 watery, bloody stools. She did not have a fever or urinary symptoms. She did not have a history of recent antibiotic use, travel outside the United States, recent diet changes, or history of symptoms suggestive of inflammatory bowel disease. She suffers from diabetes mellitus, hypertension, hypercholesterolemia, hypothyroidism and chronic constipation. Her current medications included metformin (taking for 8 years), lisinopril (taking for 3 years), atorvastatin (taking for 4 years) and replacement thyroxin (taking for 4 years). She did not smoke or use illicit drugs.

On admission, her vital signs were essentially normal with a blood pressure of 134/70 mmHg without orthostatic changes, a heart rate of 61 bpm, temperature of 97.7 F°, respiratory rate of 16/min, oxygen saturation of 97% on room air, height of 162 cm, weight of 60.8 kg, and body mass index of 23.2. She did not have any episodes of hypotension either in the emergency department or during her hospitalization. Physical examination revealed a soft abdomen with mild epigastric and left upper quadrant tenderness. There was no distension, guarding, rebound tenderness, or organomegaly. The bowel sounds were normal. Rectal exam was unremarkable except for positive occult blood. The remainder of physical exam revealed no findings.

The laboratory studies were unremarkable including complete blood count, comprehensive metabolic panel, amylase, lipase, urinalysis, and urine toxicology. The stool studies were negative for ova, parasites, clostridium difficile, and pathogenic bacteria. The hemoglobin A1c was 6.5%. A plain abdominal X-ray was unremarkable. A computed tomography (CT) scan of the abdomen and pelvis revealed thickening of the colonic wall involving the transverse and the proximal portions of the descending colon near the splenic flexure (Figure 1A). A colonoscopic examination showed mucosal changes consistent with ischemic colitis (mucosal edema and hyperemia with areas of hemorrhage, erosions, and frank ulcerations) affecting the descending colon, between 30 to 40 cm from the anal verge (Figure 1B-D). The histopathology examination showed ischemic loss of crypts with acute hemorrhage in lamnia propria associated with acute fibrinopurulent exudate at colonic epithelial surface, consistent with ischemic colitis (Figure 1E, F).

A diagnosis of ischemic colitis was made based on the above findings. To clarify the etiology, a detailed history was taken including the use of over the counter and herbal medications. She stated that she had been taking lubiprostone for a period of 2 mo prior to her admission after she failed to respond to conventional laxatives for chronic constipation. The patient was initially started on 24 mcg of lubiprostone, but several hours after taking the drug, she developed nausea, vomiting and abdominal pain with bloody diarrhea which lasted for two days. She did not undergo any investigations, but her dose of lubiprostone was changed to 8 mcg daily as needed after she called her primary care physician. Twenty-four hours prior to her current admission she had taken a total of 24 mcg of lubiprostone. Her symptoms developed two hours after the last dose. This temporal relationship between the recurrence of her symptoms and the rechallenging of the drug confirms the diagnosis of ischemic colitis in the light of absence of other causes of ischemic colitis. This case scored a 10 on the Naranjo Nomogram for adverse drug reactions which places lubiprostone as a definitive cause of ischemic colitis in this case (definitive $\geqslant$ 9). The patient was treated with discontinuation of lubiprostone, intravenous fluids, bowel rest and intravenous antibiotics. Her hospitalization course was uneventful and she was discharged home without any complications 4 d later.

DISCUSSION

Ischemic colitis accounts for 6%-18% of the causes of acute lower gastrointestinal bleeding[2]. The causes of ischemic colitis vary from systemic hypotension, aortoiliac surgery, atherosclerosis, thromboembolic events, vasculitis, and medications[9].

The medications that have been implicated in the development of ischemic colitis have different mechanisms including decreasing blood flow *via* systemic hypotension with angiotensin-converting enzyme inhibitors, vasospasm with pseudoephedrine, thromboembolic events with oral contraceptives, vasculitis secondary to gold salts, and increasing intracolonic pressures with alos-

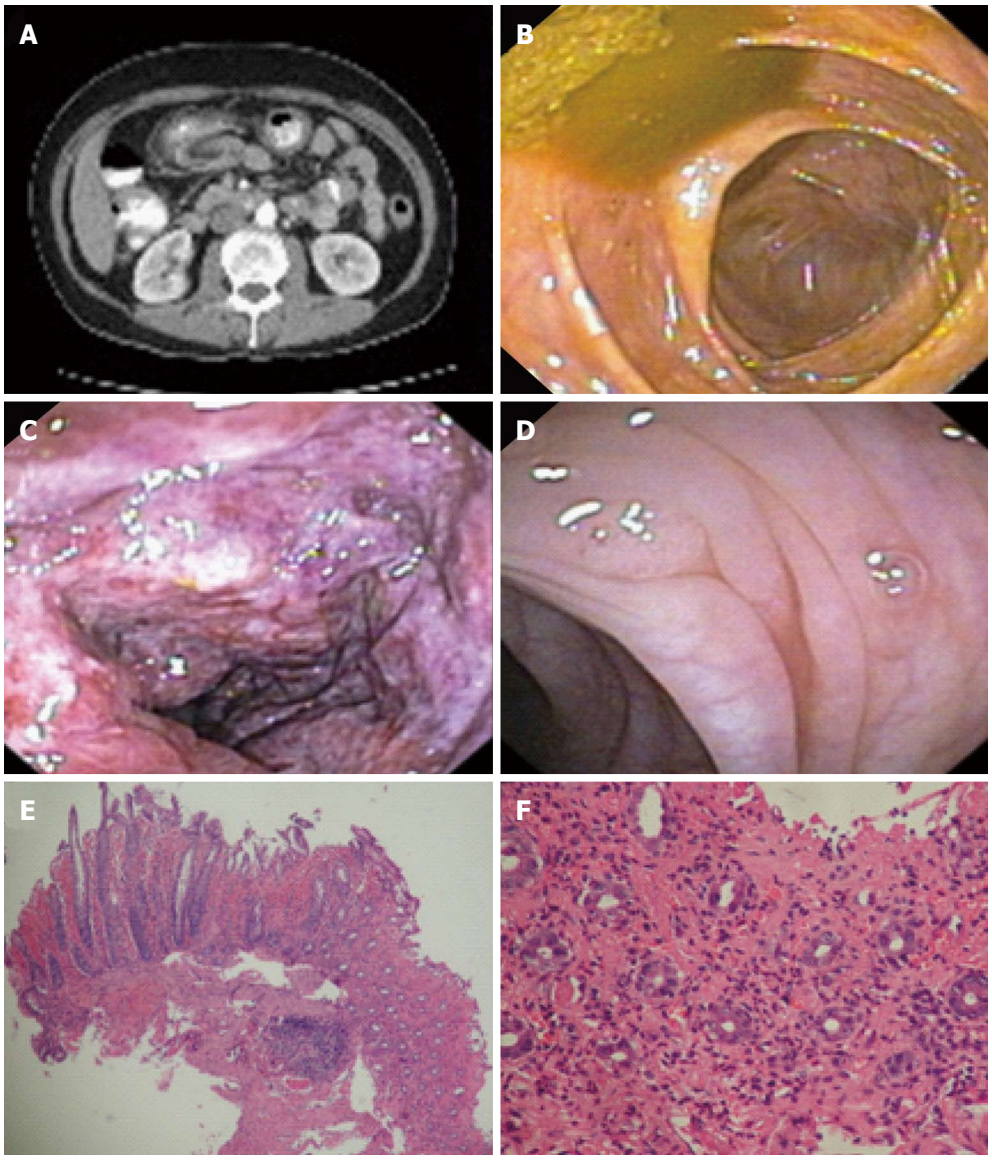

Figure 1 The laboratory studies results. A: Computed tomography scan shows thickening of the colonic wall involving the splenic flexure of the colon; B: Normal mucosa in the right colon (appendiceal orifice); C: Ischemic colitis features in the splenic flexure of the colon; D: Normal mucosa in sigmoid with a small polyp; E, F: Histopathology shows ischemic loss of crypts and acute hemorrhage in lamnia propria (acute hemorrhagic necrosis replacing glands) associated with acute fibrinopurulent exudate at colonic epithelial surface, consistent with ischemic colitis.

etron[3]. The mechanism of some drugs reported to cause ischemic colitis has not yet determined. Table 1 shows a list of medications and their mechanisms for causing ischemic colitis[3].

Many case reports have linked ischemic colitis and other unusual associations such as herbal remedies (ma huang/ephedra), weight loss medications such as phentermine, following screening colonoscopies, certain chemotherapy agents such as bevacizumab and irinotecan, hepatitis C therapy with peginterferon and ribavirin, scuba diving, flying, snake bites, acute carbonic monoxide poisoning, using electrical muscle stimulation devices on the abdominal wall, and following long distance running[3].

Lubiprostone has been shown to improve symptoms in patients with chronic idiopathic constipation. In a 48 wk prospective, multicenter, open-labeled trial performed by Lembo *et al*[10] patients with chronic idiopathic constipation were taking lubiprostone 24 mcg twice daily as needed and the most common side effects were nausea (19.8%), diarrhea (9.7%), abdominal distension (6.9%), headache (6.9%), and abdominal pain (5.2%).

Lubiprostone has also been approved for the treatment of constipation predominant irritable bowel syndrome (IBS-C). In a randomized, placebo controlled trial, the overall rate of response based on a patient ranked assessment tool was significantly higher in the lubiprostone group using 8 mcg twice daily compared to placebo. The side effects were similar in both groups[11].

In a phase 2, placebo controlled trial by Johanson *et al*[12] lubiprostone was superior to placebo in IBS-C regarding the severity of constipation, stool consistency,

Table 1 Medications associated with ischemic colitis

Agent	Mechanism
Amphetamines	Vasoconstriction
Alosetron	
Catecholamines (epinephrine, norepinephrine)	
Cocaine	
Cyclosporine	
Digitalis	
Dopamine	
Ergot derivatives	
Nonsteriodal anti-inflammatory drugs	
Pseudoephedrine	
Triptans (Naratriptan, Rizatriptan, Sumatriptan)	
Vasopressin and vasopressin analogues	
Glycerin enema	Local vasospasm effect
Phosphosoda solution	
Angiotensin-converting enzyme inhibitors	Systemic hypotension
Antipsychotic (chlorpromazine)	
Beta blockers	
Barbiturates	
Diuretics	
Interleukin-2	
Tricyclic antidepressants	
Amphetamines	Vasculitis
Gold compounds	
Estrogens	Thrombotic lesion induction
Progestational agents	
Alosetron	Increased intracolonic pressure
Danazol	
Glycerin enema	
Carboplatin	Undetermined
Flutamide	
Glutaraldehyde	
Hyperosmotic saline laxatives	
Interferon-alpha	
Mycophenolate mofetil	
Paclitaxol	
Simvastatin	
Tegaserod	

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frequency, straining, bloating, and abdominal pain in 1, 2, and 3 mo follow ups. Both diarrhea and nausea occurred more often in the lubiprostone group, especially with 24 mcg twice daily compared to 8 mcg twice daily.

Lubiprostone is a bicyclic fatty acid, derived from prostaglandin E1. It is a member of a class of drugs called prostones which, unlike prostaglandins, are not believed to cause direct smooth muscle contraction. It is metabolized in the upper part of the gastrointestinal tract by the microsomal carbonyl reductase system. Lubiprostone acts locally in gastrointestinal epithelium and its active metabolite, M3, absorbs systemically. The half-life of M3 is 0.9-1.4 h. M3 is used for the pharmacokinetic parameters of lubiprostone because the drug itself has limited systemic absorption and low bioavailability[4,8].

Lubiprostone mainly acts on CLC-2 activators in the intestinal epithelium which is one of the nine types of chloride channels in the body which also includes the cystic fibrosis transmembrane conductance regulator (CFTR) (Figure 2)[13].

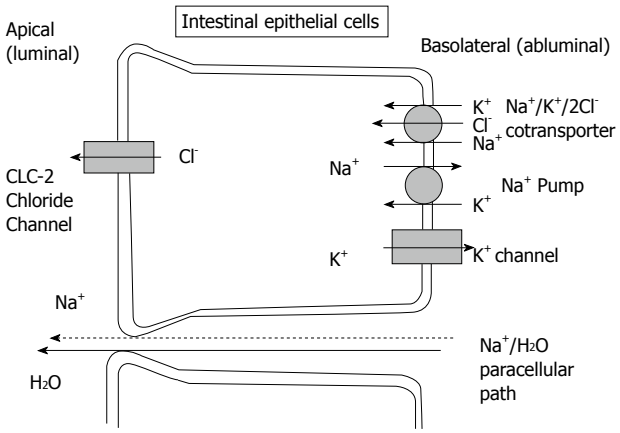

Figure 2 Mechanism of action of lubiprostone across epithelial intestinal cells[8].

Activation of CLC-2 in the apical membrane of intestinal epithelial cells causes an active secretion of chloride ions from cells into the intestinal lumen followed by a passive secretion of sodium ions and water which results in increased isotonic fluid in the lumen which promotes small bowel and colonic transit by stimulating stretch receptors and smooth muscles in the intestine (Figure 2)[8].

There is now controversy whether lubiprostone activates CLC-2 in be basolateral or apical aspect of intestinal epithelium, whether it activates mainly CFTR or CLC-2, or whether it directly stimulates smooth muscles *via* prostaglandin receptors[8]. Bao *et al*[14] showed in an experimental study that lubiprostone activates CLC-2 at low concentrations but activates CFTR at higher concentrations. It has also been shown that lubiprostone weakly activates prostaglandin receptors in different levels of the GI tract which could be responsible for some side effects such as nausea[15].

In our patient, despite having other risk factors for ischemic colitis such as chronic constipation which has been shown to increase the risk of ischemic colitis by 2.7 folds in some studies[16], the close temporal relationship between lubiprostone and occurrence of the symptoms of ischemic colitis makes the drug the most likely culprit of this event. When applying the Naranjo Nomogram in our patient, a score of 10 was obtained indicating a definite likelihood of lubiprostone as the cause of ischemic colitis in this case (Table 2).

To the best of our knowledge, this is the first report of lubiprostone induced ischemic colitis. We report that a direct causal relation by exclusion of other causes, the disappearance of symptoms after cessation of lubiprostone, recurrence of symptoms with self rechallenge, and a Naranjo Nomogram score of 10 all go in favor of the drug-induced ischemic colitis. The actual mechanism of lubiprostone causing ischemic colitis is not known, however, fast fluid shift into the intestinal lumen causing local hypoperfusion, rapid increase of the intracolonic pressure due to rapid fluid secretion into the bowel, or direct vasoconstriction caused by lubiprostone in higher doses

Table 2 Naranjo adverse drug reaction nomogram in our patient

	Yes	No	Our patient
Are there previous conclusive reports on this reaction?	1	0	0
Did the adverse event appear after the suspected drug was administered?	2	-1	2
Did the adverse reaction improve when the drug was discontinued or a specific antagonist was administered?	1	0	1
Did the adverse reaction reappear when the drug was readministered?	2	-1	2
Are there alternative causes (other than the drug) that could have, on their own, caused the reaction?	-1	2	2
Did the reaction appear when a placebo was given?	-1	1	1
Was the drug detected in the blood (or other fluids) in concentration known to be toxic?	1	0	0
Was the reaction more severe when the dose was increased or less severe when dose was decreased?	1	0	0
Did the patient have a similar reaction to the same or similar drugs in any previous exposure?	1	0	1
Was the adverse event confirmed by any objective evidence?	1	0	1
			10

Definite: score ⩾ 9; Probable: 5-8; Possible: 1-4; Doubtful: ⩽ 0.

by stimulating prostaglandin receptors are the proposed pathophysiological mechanisms. In conclusion, lubiprostone may cause ischemic colitis, especially in some susceptible persons and greater awareness of this side effect is warranted with the increasing use of lubiprostone.

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CASE REPORT

Lesson from an intriguing case of cryoglobulinemia

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Abstract

Cryoglobulinemia is a pathological condition usually associated with hepatitis C virus (HCV) chronic liver disease and less commonly with autoimmune or lymphoproliferative disorders. The possible association of cryoglobulinemia with hepatitis B virus (HBV) infection is not widely accepted. In our patient, serum negativity for HCV markers initially led us to consider two other causes of cryoglobulinemia. Myelodysplastic disorders were excluded on the basis of hematological studies, while serum markers for active HBV infection were positive. Surprisingly, the detection of HCV RNA in the cryocrit, even in the absence of anti-HCV antibodies, suggested a pathogenetic role of HCV in this case of cryoglobulinemia. Negative "first level" tests for HCV in the serum do not completely exclude HCV involvement in the pathogenesis of cryoglobulinemia. Analysis of the cryoprecipitate is always essential for diagnosis.

Key words: Cryocrit; Hepatitis B virus; Hepatitis C virus; Myelodysplastic disorders; Cryoglobulinemia

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INTRODUCTION

Cryoglobulinemia is a pathological condition usually associated with hepatitis C virus (HCV) chronic liver disease[1,2]. Cases of cryoglobulinemia associated with autoimmune or lymphoproliferative diseases are less common[2], while the association with hepatitis B virus (HBV) infection is not widely accepted, the HBV virus being mostly associated with other immunocomplex-related disorders[3-5].

Cryoglobulins are proteins that can precipitate at low temperatures (< 4 ℃). The final product of this precipitate, termed the cryocrit, can be characterized on the basis of its composition: polyclonal immunoglobulins, monoclonal immunoglobulins or the presence of rheumatoid activity (RA). Three types of cryoglobulinemia have been identified, namely: type 1, including monoclonal cryoglobulins (IgM, IgG and IgA) without any RA; type 2, that includes a monoclonal component (usually IgM with RA) and a polyclonal component (usually IgM/IgG); type 3, that includes several polyclonal components and a component with RA (usually IgG/IgM)[6].

Normally, cryoglobulinemia type 1 is associated with lymphoproliferative/myelodysplastic diseases such as multiple myeloma, Waldenstrom macroglobulinemia, chronic lymphatic leukemia, non-Hodgkin lymphoma, *etc.* Cryoglobulinemia type 2 is associated not only with lymphoproliferative diseases and plasma cellular dyscrasias but also with infectious and autoimmune diseases (rheumatoid arthritis, Sjogren's syndrome, *etc.*). Finally, cryoglobulinemia type 3 is frequently associated with autoimmune or infectious diseases. The mechanism responsible

for cryoglobulin formation during lymphoproliferative or autoimmune diseases is known, but the etiopathogenesis of the forms defined as "essential cryoglobulinemias", which occur as isolated events, has still to be clarified. Among the essential forms, the most common variant is cryoglobulinemia type 2[2]. The amount of cryoglobulins, expressed as a percentage of the serum volume, can vary significantly among patients and may change over time. It is therefore useful to evaluate the cryocrit, also during follow-up, in order to assess prognosis and treatment. Cryoglobulinemia characterization by protein electrophoresis and immunofixation is equally important to define the type[2]. In 1966, Meltzer *et al*[7] described a clinical triad characterized by palpable purpura, arthralgia and asthenia associated with nephropathy and neuropathy. It is widely accepted that presentation of the complete triad is rare in clinical practice. In fact, most patients are asymptomatic and purpura, often fleeting, is the only clinical manifestation. The frequent association of HCV infection with the great majority of cryoglobulins, initially defined as essential, suggested the involvement of this virus in the pathogenesis of mixed cryoglobulinemia. Actually, mixed cryoglobulinemia (type 2 or 3) is found in 50% of patients with chronic HCV infection[1,4]. Therefore, in patients with mixed cryoglobulinemia, the possibility of HCV infection should always be considered, and serum tests should be performed for the detection of anti-HCV antibodies and HCV RNA.

CASE REPORT

Our patient was in apparent good health until the age of 73 when he underwent percutaneous angioplasty for acute non-Q myocardial infarction, after which he was prescribed anticoagulant and antihypertensive therapy. At the age of 77, he had an outpatient medical visit because of the first manifestation of purpura of the lower limbs, arthralgia and a sense of postural instability, raising the suspicion of a cryoglobulinemic syndrome. At this time, the cryocrit was positive (35%), and the patient underwent several serological and molecular investigations that excluded HCV (negative anti-HCV and HCV RNA) and HIV infection and demonstrated chronic HBV infection (surface antigen of the hepatitis B virus (HBsAg) 11 700 IU/mL, anti-HBsAg negative, hepatitis B core antibody positive, anti-hepatitis Be antibody positive, HBV-DNA 2 410 000 IU/mL, anti-HDV negative). Immediately after, he was admitted to our unit because of the onset of a hypertensive crisis that was not controlled by the administration of calcium antagonists, beta-blockers and loop diuretics. Renal function monitoring demonstrated the following values: creatinine 1.85 mg/dL, creatinine clearance 20 mL/min and 24-h proteinuria 1.3 g/24 h. Echotomographic examination showed ultrasonographic signs of cirrhosis, and hepatic elastometry yielded a stiffness value of 47.2 kPa. Other laboratory tests demonstrated aspartate aminotransferase × 6.7 upper limit of normal (ULN), alanine aminotransferase × 4.9 ULN and gamma-glutamyl transpeptidase × 5.2 ULN, and total bilirubin 1.8 mg/dL. On the basis of these data, the patient was started on antiviral treatment (0.5 mg entecavir every 72 h) and prednisone, 50 mg/d, to be tapered to 10 mg/d. Although the hypertensive crisis could presumably be ascribed to a cryoglobulin-induced nephropathy attributable to HBV, considering that the serum β2-microglobulin levels were markedly increased (7.2 mg/L, < 2 ULN) and gamma-globulin showed a monoclonal peak, we also considered the possibility of cryoglobulinemia associated with a myeloproliferative disorder. The characteristics of the cryoprecipitate were therefore evaluated in order to define the type of cryoglobulinemia. We found rheumatoid factor positivity, lower C3 and C4 levels, 24% cryocrit, while serum immunofixation showed a K monoclonal IgM component, and cryocrit immunofixation a K monoclonal IgM component with polyclonal IgG. On the basis of these elements we made a diagnosis of mixed cryoglobulinemia type 2, that did not exclude a myeloproliferative disorder, so a peripheral blood smear and bone marrow biopsy were performed, and these excluded a myeloproliferative disease. During the period of hospitalization we observed an improvement in renal function (creatinine decreased to 1.4 mg/dL, 24-h creatinine clearance increased to 35 mL/min, and 24-h proteinuria decreased to 0.9 g) and a striking reduction of the cryocrit (6.1%). Surprisingly, cryoprecipitate characterization demonstrated the presence of HCV RNA (807 copies/mL, by m2000 Real Time System, Abbott, IL, United States, Figure 1) and for this reason, we rechecked the serum anti-HCV antibodies and HCV RNA, and the absence of both was confirmed (HCV RNA undetectable using 50 IU/mL as cut-off).

DISCUSSION

In the present case, the detection of HCV components in the cryocrit cast some doubt on the pathogenesis of the cryoglobulinemia that we initially attributed to HBV infection.

It has been reported that in chronic HCV patients with mixed cryoglobulinemia, HCV RNA may be undetectable in the plasma (due to the entrapment of HCV RNA in the cryoprecipitate) and the diagnosis of HCV infection is based on the presence of anti-HCV antibodies[8]. However, in our patient anti-HCV antibodies were undetectable in the serum. This could be explained by interference between the two viruses that may even lead to inhibition of HCV replication in patients with chronic hepatitis C with a superimposed HBV infection[9,10].

This experience suggests that negative tests for HCV detection in the serum (anti-HCV antibodies and HCV RNA) do not completely exclude the involvement of HCV in the pathogenesis of cryoglobulinemia, and show that analysis of the cryoprecipitate is always essential, since detection of HCV RNA in the cryoprecipitate is the most sensitive method. Once HCV infection has been excluded, investigations must take into account other causes

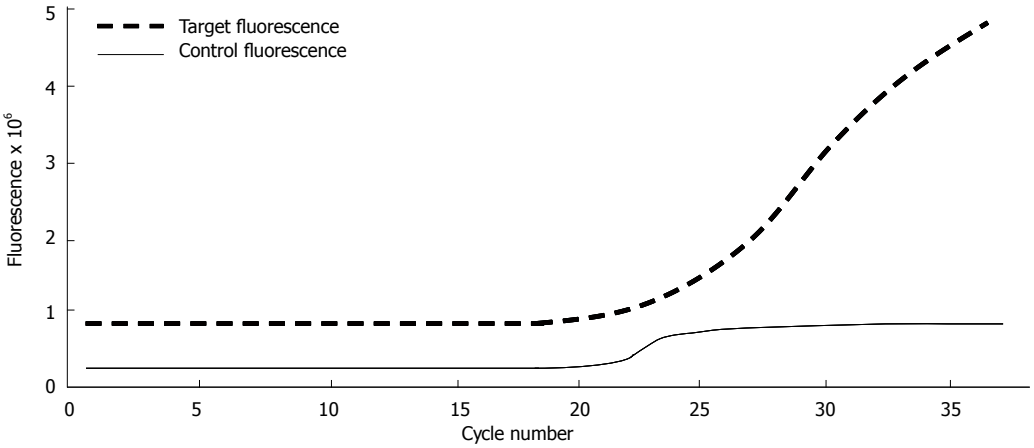

Figure 1 Real time polymerase chain reaction amplification plot for hepatitis C virus RNA. The presence of viral RNA started to become evident after 19.4 cycles and the amount of RNA was 807 copies/mL.

of cryoglobulinemia, such as autoimmune and myelo- or lymphoproliferative disorders and HBV infection.

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CASE REPORT

Recurrent cervical esophageal stenosis after colon conduit failure: Use of myocutaneous flap

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Abstract

A 53-year-old male developed cervical esophageal stenosis after esophageal bypass surgery using a right colon conduit. The esophageal bypass surgery was performed to treat multiple esophageal strictures resulting from corrosive ingestion three years prior to presentation. Although the patient underwent several endoscopic stricture dilatations after surgery, he continued to suffer from recurrent esophageal stenosis. We planned cervical patch esophagoplasty with a pedicled skin flap of sternocleidomastoid (SCM) muscle. Postoperative recovery was successful, and the patient could eat a solid meal without difficulty and has been well for 18 mo. SCM flap esophagoplasty is an easier and safer method of managing complicated and recurrent cervical esophageal strictures than other operations.

Key words: Cervical esophageal stenosis; Corrosive stricture; Esophagus; Esophageal conduit; Esophagoplasty; Myocutaneous flap; Surgery

Sa YJ, Kim YD, Kim CK, Park JK, Moon SW. Recurrent cervical esophageal stenosis after colon conduit failure: Use of myocutaneous flap. *World J Gastroenterol* 2013; 19(2): 307-310 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i2/307.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i2.307

INTRODUCTION

A variety of total esophageal reconstruction procedures have been described; however, there is no universally-accepted method[1]. Cervical reconstruction for esophageal stenosis of any etiology is complicated and can be more challenging in cases of recurrent cervical stenosis with previous colon conduit failure. Recently, we successfully treated upper cervical stenosis caused by corrosive strictures using colon conduits. However, these operations have been followed by recurrent stenosis. Many different approaches have been utilized in the treatment of cervical stenosis. These include local fasciocutaneous flaps, pedicled myocutaneous flaps, pedicled visceral flaps, free flaps, and free fasciocutaneous flaps[2]. We present a successful patch-plasty with myocutaneous flap of sternocleidomastoid (SCM) muscle for treatment of recurrent cervical esophageal stenosis.

CASE REPORT

A 53-year-old male developed recurrent cervical esophageal stenosis 1.5 years after undergoing esophageal bypass surgery using a right colon conduit. He had received corrosive esophageal strictures three years earlier after a suicide attempt by potassium hydroxide ingestion. His presentation was complicated, with multi-level stenotic segments down the entire esophagus. He underwent

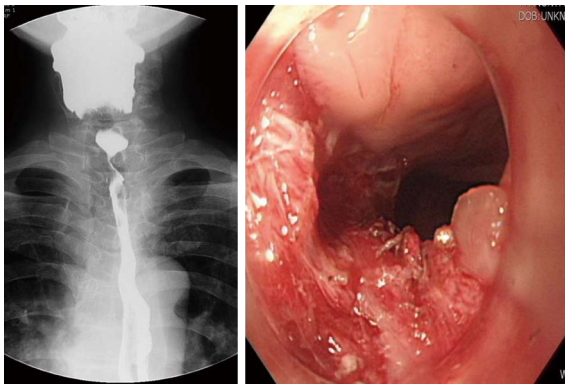

Figure 1 Esophagogram and endoscopic findings before patch esophagoplasty. The native esophagus was visualized with the esophagogram, but the colon conduit from bypass surgery was not visible. After insertion of an Endoscopic Varix Ligation tube, a stenotic lesion of the esophago-colonic anastomosis site was opened. Focal luminal stenosis by fibrosis was evident. Endoscopic dilatation was repeated using electrocauterization.

repeated balloon dilatation, but one cervical stricture progressed to near-complete obstruction just below the entrance of the esophagus as seen on endoscopy and barium esophagogram. The severity of stenosis below the proximal esophagus could not be evaluated. He had severe weight loss of 15 kg and required total parenteral nutrition, afterwards gaining back about 2.5 kg (43 kg and 173 cm, body mass index 14.37). Instead of feeding gastrostomy insertion, esophageal bypass surgery using a segment of the right colon was performed one year later. At surgery, the ileum was anastomosed with the cervical esophagus in a side-to-end manner, and its blood supply was preserved. Postoperative esophagogram showed that barium passed through the native esophagus and newly constructed colon conduit. The patient began to eat 7 d after surgery and returned home 14 d after surgery with a weight gain of 3 kg. On follow-up, he gained weight by additional 3 kg, but suffered from painful swallowing of solid food three mo after discharge. Follow-up endoscopy revealed cicatricial stenosis in the native esophagus proximal to the interposed colon, though the site of gastro-colon anastomosis was patent. Barium esophagogram showed mild stenosis at the level of the thoracic inlet and severe stenosis just above the anastomosis of the esophagus. The patient underwent repeated 7 times esophageal dilatation using Savary-Guillard or balloon dilators, and an episode of electrocauterization ablation to improve swallowing before the 2nd operation. Despite endoscopic dilatation using electrocauterization, he again developed pain on swallowing. Finally, he was admitted for surgical correction. We reviewed the esophagogram and endoscopic findings (Figure 1). The stenotic segment was 3 cm long and located in the proximal esophagus near the site of the previous esophago-colonic anastomosis. Fortunately, the lesion was easily opened with a dilator or balloon. This finding suggested that the stenotic segment was not caused by mechanical stenosis resulting from circumferential fibrosis, but was caused by twisted axis of esophageal anastomosis site possibly due to cicatricial obstruction resulting from colon conduit failure. We planned to perform a patch enlargement operation with a myocutaneous flap of SCM muscle. Under endotracheal anesthesia, the patient was placed supine with neck extension and slight rotation for better exposure. A skin incision in the neck was made along the previous incision while preserving an elliptical skin island over the SCM muscle (Figure 2). Improved exposure of the stenotic cervical esophagus was obtained after SCM division with a skin island near the clavicle. There was a fibrotic band near the proximal esophagus instead of near the colon. The proximal esophagus and hypopharynx were exposed, and a small esophagotomy at the level of the cricoid cartilage was initially made and extended upward to the hypopharynx with a longitudinal incision of about 5 cm (Figure 2). The attached skin island of the SCM myocutaneous flap was rotated medially over the esophagopharyngeal opening and sutured in interrupted fashion with 3-0 black silk. The SCM muscle attached to the skin flap was then tacked into place around the esophagus and pharynx. A drain was placed, and the neck wound was closed after a skin tension-releasing procedure (Figure 2). Seven days later, an esophagogram showed good passage without tracheal aspiration (Figure 3). The patient could eat a solid meal without pain or resistance and returned home in ten days. He then returned to work and has been well with a gradual weight gain about 10 kg for 18 mo after the esophagoplasty.

DISCUSSION

Graft necrosis is the most dangerous complication of colon graft interposition with a reported mortality approaching 90% or more[3]. Most instances of colonic graft ischemia are secondary to arterial insufficiency[3]. Ischemic complications are expressed as frank colon necrosis, anastomotic leakage, and colonic stricture[3]. Colonic segment necrosis and anastomotic leakage usually occur in the first postoperative week[4], but in our case, localized strictures of the cervical region developed 1.5 years after the first bypass operation. The slow course is likely explained by slowly progressive ischemia from graft torsion, resulting in a non-necrotic colonic cicatricial stricture rather than colonic necrosis. This may be the cause of esophageal obstruction in our case. This cicatricial change in the esophageal anastomosis site might twist the esophageal axis, and cause painful swallowing. Also this lesion was easily dilated with esophageal ballooning.

Although dilatation is the primary method of treating esophageal strictures[5], alimentary tract reconstruction is necessary for correction of severe and extensive strictures. Invasive surgeries like stomach pull-up, colon bypass, and free jejunal flap are not always possible when the prior operation was similarly invasive[6].

In our case, the right colon had already been used during the previous bypass operation, and the graft stricture of the neo-esophagus resulted from ischemia. Thus, patch dilative strictureplasty with viable tissue was

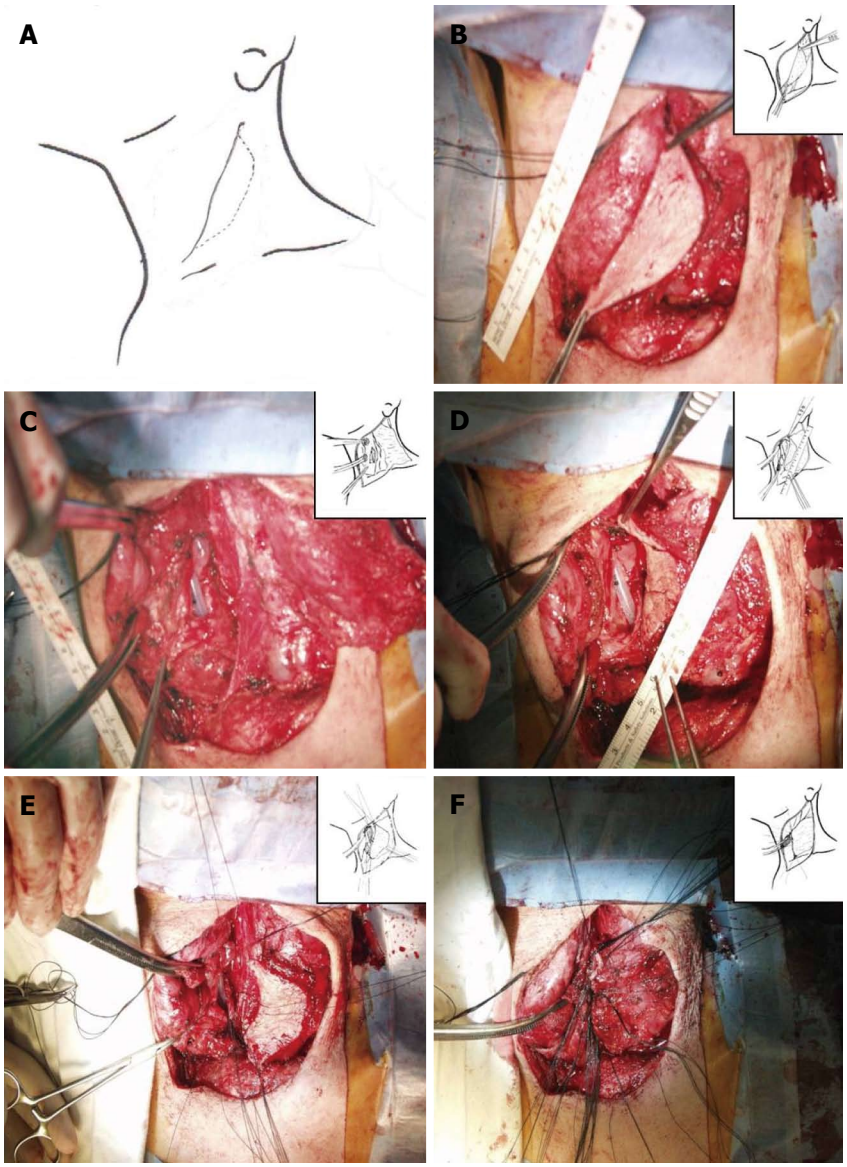

Figure 2 Photograph of the musculocutaneous sternocleidomastoid flap and esophagotomy during the operation. A: A skin incision was designed for an elliptical skin island based on the sternocleidomastoid muscle; B: A skin island (6 cm × 4 cm) on the sternocleidomastoid (SCM) muscle was prepared; C, D: Esophagotomy at the level of the cricoid cartilage was extended upward to pharynx and downward; E: The SCM muscle was severed near the clavicle and rotated. The skin flap was fixated to esophagus with interrupted silk sutures; F: The esophagoplasty site was covered with SCM muscle *via* multiple fixation sutures.

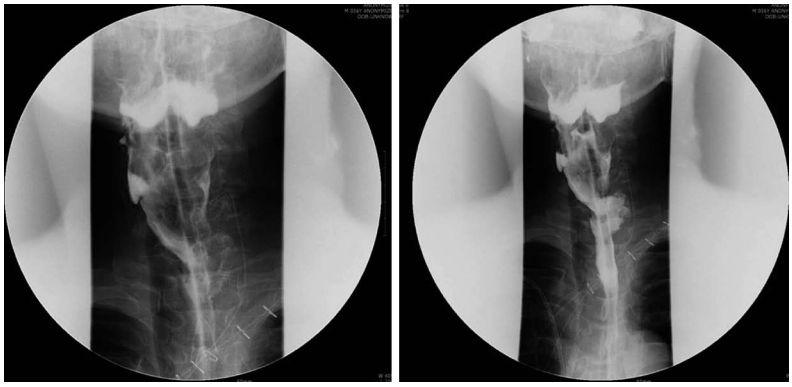

Figure 3 Esophagogram after sternocleidomastoid myocutaneous patch esphagoplasty. The stenotic lesion at the esophago-colonic anastomosis site was widened successfully and the dye passed through the colon conduit without resistance.

thought to be an ideal option for relieving the short, benign, recurrent cervical esophageal stricture.

The choice of tissue to be used as a patch to widen the esophagus varies. Among flaps, a myocutaneous flap has several advantages: its skin provides an adequate patch for the esophageal defect and the well-vascularized muscle forms a seal around the ischemic anastomosis site, obliterates dead space, and conveys antibiotics[7]. The SCM muscle is a logical choice for myocutaneous flap in this case because its anatomic proximity lessens trauma[7]. Because SCM patch esophagoplasty avoids complete transection of the esophagus, coordinated peristalsis is also preserved and vagotomy is avoided[7]. The SCM muscle has a segmental blood supply from branches of three

main vessels: the occipital artery superiorly, the superior thyroid artery in the middle segment, and a branch of the thyrocervical trunk inferiorly. The superior pedicle is more commonly used because it provides greater rotation and allows the SCM to reach the entire length of the ipsilateral cervical esophagus[2]. The SCM myocutaneous flap does, however, have limitations. Safety is a major concern when the SCM flap is used in oncologic patients[8]. In cases of radiation therapy, the SCM myocutaneous flap may be used only if the overlying skin is soft and supple[2]. Furthermore, use of a SCM pedicled skin flap is limited to the cervical esophagus. In conclusion esophagoplasty with SCM myocutaneous flap is thought to be an efficient treatment of recurrent cervical esophageal strictures.

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CASE REPORT

A case of ascending colon variceal bleeding treated with venous coil embolization

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Abstract

A 38-year-old female with a history of alcoholic liver cirrhosis visited our hospital with a massive hematochezia. An esophagogastroduodenoscopy did not demonstrate any bleeding source, and a colonoscopy showed a massive hemorrhage in the ascending colon but without an obvious focus. The source of the bleeding could not be found with a mesenteric artery angiography. We performed an enhanced abdominal computed tomography, which revealed a distal ascending colonic varix, and assumed that the varix was the source of the bleeding. We performed a venous coil embolization and histoacryl injection to obliterate the colon varix. The intervention appeared to be successful because the vital signs and hemoglobin laboratory data remained stable and because the hematochezia was no longer observed. We report here on a rare case of colonic variceal bleeding that was treated with venous coil embolization.

Key words: Colon ascending; Varicose veins; Liver cirrhosis; Therapeutic embolization

Ko BS, Kim WT, Chang SS, Kim EH, Lee SW, Park WS, Kim YS, Nam SW, Lee DS, Kim JC, Kang SB. A case of ascending colon variceal bleeding treated with venous coil embolization. *World J Gastroenterol* 2013; 19(2): 311-315 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i2/311.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i2.311

INTRODUCTION

Colonic variceal bleeding is a very rare cause of lower gastrointestinal bleeding. Fewer than 100 cases of colonic variceal bleeding have been reported in English literature and few case reports exist in South Korea. However, most cases in South Korea were caused by different varix locations, etiologies and treatments. A single case in South Korea with ascending colonic variceal bleeding caused by alcoholic liver cirrhosis was treated with a hemicolectomy[1]. We report the first case of an ascending colon variceal bleed caused by alcoholic liver cirrhosis that was treated with venous coil embolization in South Korea.

CASE REPORT

A 38-year-old female was admitted to our hospital with massive hematochezia and hemodynamic instability. She was diagnosed with alcoholic liver cirrhosis (Child-Pugh classification C) 6 years ago and had multiple hospitalizations with complications of esophageal variceal bleeding and hepatic encephalopathy. Upon physical examination, her initial blood pressure was 80/50 mmHg, her heart rate was 110 bpm, her respiration was 28 bpm and her

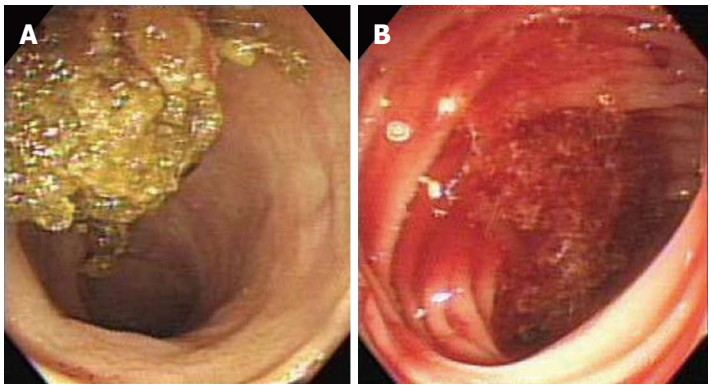

Figure 1 Initial colonoscopy. A: The terminal ileum showing yellowish-colored stool with no evidence of upper gastrointestinal bleeding; B: The ascending colon showing fresh blood without an observable source.

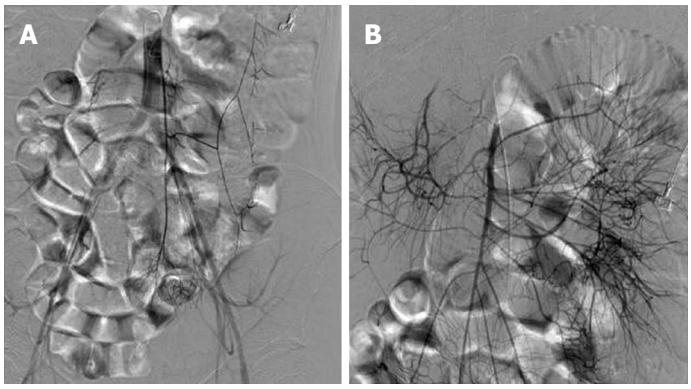

Figure 2 Mesenteric artery angiography. A: The inferior mesenteric artery angiography showing no source of bleeding; B: The superior mesenteric artery angiography showing no source of bleeding.

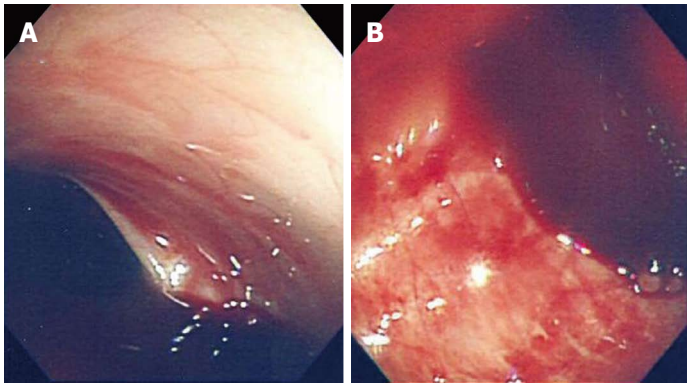

Figure 3 The second colonoscopy. A: Massive hemorrhage within the proximal ascending colon without a definite bleeding focus; B: Fresh blood within the distal ascending colon without a definite bleeding focus.

body temperature was 37 ℃. No evidence of bleeding was detected with nasogastric tube irrigation. Abnormal laboratory data upon admission included the following: hemoglobin 7.0 g/dL, hematocrit 22.3%, platelets 59 000/mm^3, serum creatinine 2.31 mg/dL, international normalized ratio 1.98, and albumin 2.1 g/dL.

An esophagogastroduodenoscopy demonstrated esophageal varices grade Ⅲ but did not show signs of recent bleeding. The colonoscopy showed fresh blood from the ascending colon to the rectum, but there was no evidence of bleeding in the terminal ileum, which demonstrated that the origin of the bleeding could be assumed to be in the ascending colon (Figure 1). Diagnostic angiographies of the superior and inferior mesenteric arteries were performed, but the source of the bleeding could not be found (Figure 2). Even with 2 units of packed RBCs, the hemoglobin level dropped to 5.6 g/dL the next day, and the vital signs remained unstable.

A second colonoscopy was still unable to visualize the bleeding focus and only detected a massive hemorrhage in the ascending colon (Figure 3). We performed an enhanced abdominal computed tomography (CT), which revealed the formation of a varix surrounding and protruding inside the distal ascending colon by the portacaval shunt (Figure 4). The varix was thought to be the source of the gastrointestinal bleeding. We planned an operation, but a right hemicolectomy could not be performed due to poor vital signs and abnormal coagulopathy. Initially, we tried to perform a Balloon-Occluded Retrograde Transvenous Obliteration (BRTO) but failed due to the selection of the portacaval shunt. Because the patient had unstable vital signs that required immediate intervention, we performed a coil embolization and his-

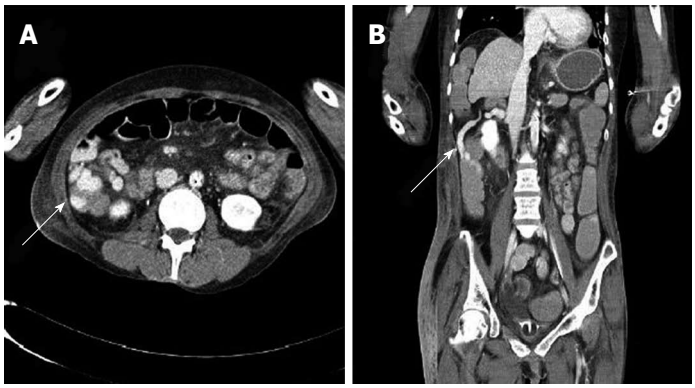

Figure 4 Abdominal computed tomography. A: Portal phase axial view with white arrow showing the varix surrounding and protruding inside the ascending colon formed by portacaval shunt; B: Coronal view with white arrow showing the colon varix.

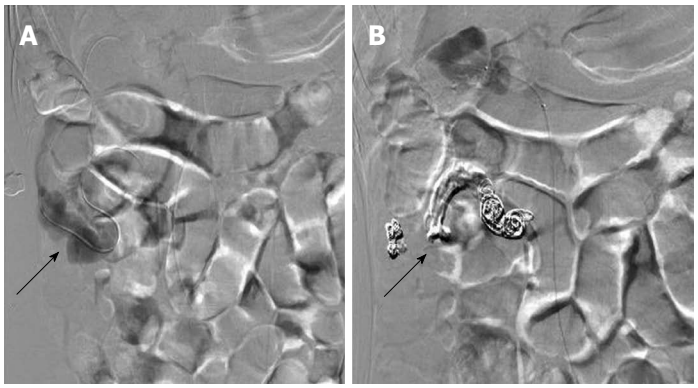

Figure 5 Venous coil embolization. A: Venography showing the varix within the ascending colon (black arrow); B: Coil embolization (black arrow) and histoacryl injection was performed to obliterate the varix.

toacryl injection to obliterate the varix (Figure 5). After the coil embolization and 4 units of packed red blood cells, the patient's hemoglobin level rose to 10.0 g/dL, where it stabilized, and the vital signs (blood pressure and heart rate) recovered. There were no further signs of bleeding, such as hematochezia. However, the patient developed complications of pneumonia and renal failure, which led to multiorgan failure. Even with ventilator care and continuous renal replacement therapy, the patient expired 2 wk after the intervention.

DISCUSSION

Colon varices are a very rare cause of lower gastrointestinal bleeding, with a reported incidence of 0.07%[2]. Colon varices can be associated with several conditions, such as portal hypertension, portal venous obstruction, postsurgical changes and idiopathic factors, but the vast majority of colonic varices are related to portal hypertension caused by cirrhosis or portal vein obstruction. Embryologically derived anastomoses between the portal and systemic systems exist in the esophagus, terminal ileum, colon, retroperitoneum, and anterior abdominal wall[3]. The most common sites of colonic varices are the rectum and cecum[4]. Colon varices with portal hypertension are hypothesized to arise in patients in whom the colonic portosystemic venous collateral network is highly developed[5]. The rate of colonic variceal bleeding that causes lower gastrointestinal bleeding with liver cirrhosis is approximately 1%-8%[6,7]. Thus, if a patient has a history of liver cirrhosis and has massive gastrointestinal bleeding, the possibility of colon variceal bleeding should also be considered. Other than portal hypertension, mesenteric venous or splenic vein obstruction, which may be due to thrombosis, tumor invasion, extrinsic compression, acute or chronic pancreatitis, mesenteric adhesions, or congenital anatomic variations, may cause a colon varix. With no etiology, colon varix is classified as idiopathic or primary[5].

A colonoscopy is the principal method for the diagnosis of colon varices, but with massive bleeding, the diagnostic rate of a colonoscopy is 69% (range: 48%-90%) because the varices may be obscured by blood[8]. Thus, in case of massive hematochezia, magnetic resonance imaging, mesenteric angiography and abdominal CT are alternative diagnostic tools[9,10].

Management of colonic variceal bleeding has not been standardized. In a report describing a case of massive lower gastrointestinal bleeding with some hemodynamic instability caused by colonic varix, an operation was the optimal choice[11]. However, most cases with colonic variceal bleeding are associated with cirrhotic liver, which makes many cases inoperable due to coagulopathy. Thus, interventional therapies are tested depending on the underlying etiology and the distribution of the varices. These therapies include transhepatic intravenous portosystemic shunt (TIPS), BRTO, endoscopic variceal ligation, somatostatin infusion, argon plasma coagulation, histoacryl injection, and coil embolization[12-15]. Four other cases of colon variceal bleeding have been

described in South Korea. Theses cases had different varix locations, etiologies and treatments compared to our case. The first case was a 64-year-old female with liver cirrhosis who had descending colon variceal bleeding that was treated with a left hemicolectomy[16]. The second case was a 24-year-old male who had idiopathic rectal variceal bleeding that was treated with low anterior resection[2]. The third case was a 43-year-old male with ascending colon variceal bleeding due to alcoholic liver cirrhosis. This case was very similar to our case but was treated with a right hemicolectomy[1]. The last case was a 33-year-old woman with a history of systemic lupus erythematosus who developed antiphospholipid syndrome and had ascending colon variceal bleeding that was treated with octreotide, a beta-blocker and warfarin[17]. In our case, the patient was inoperable because of the poor vital signs and coagulopathy caused by the abnormal liver function. We initially tried to perform BRTO to obliterate the portacaval shunt, but the angiographic selection of the portacaval shunt failed. Thus, venous coil embolization along with a histoacryl injection was performed on the ascending colon varix. This technique seemed to be successful because the vital signs (blood pressure and heart rate) along with hemoglobin level remained stable, and no further evidence of gastrointestinal bleeding was observed after the intervention. This result is despite the fact that our patient expired due to complications of multiorgan failure because the patient already had terminal liver cirrhosis and had developed unstable vital signs before arriving in the emergency room. Together, these conditions made the patient susceptible to secondary infection and renal failure. Complications of the varix coil embolization procedure could also be considered after the intervention, such as necrosis, vessel perforation, non-targeting embolization and migration of the coil. However, although rate of complications has not been reported, this rate is thought to be very low because the procedure proceeds through a venous approach, and migration of the coil could be corrected during the procedure, and the patient did not complain of post-interventional symptoms such as abdominal pain, fever, or dyspnea. Thus, acute or delayed complications of the venous coil embolization were eliminated. A similar case in Switzerland included a 43-year-old male patient with ascending colon variceal bleeding due to alcoholic liver cirrhosis. The patient had TIPS initially to reduce the portosystemic pressure gradient, but the bleeding did not stop; the patient then underwent histoacryl injection and venous coil embolization. The patient recovered without further incidents[18]. As noted above, there is no standardized management of colon variceal bleeding, but venous coil embolization could be an effective treatment option for patients with massive hematochezia by the colon varix. Even though our patient could not survive due to multiple organ failure and secondary infection, we believe that the successful venous coil embolization had stopped the bleeding, which prevented a more aggressive progression. Our case is thought to be the first case in South Korea that used venous coil embolization to treat ascending colon variceal bleeding caused by alcoholic liver cirrhosis.

In conclusion, the treatment of colon variceal bleeding has not been well established. However, this case has shown that treatment with venous coil embolization on an ascending colon variceal bleeding caused by alcoholic liver cirrhosis is effective. We report here on the first case of ascending colon variceal bleeding caused by alcoholic liver cirrhosis that was treated with venous coil embolization in South Korea.

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CASE REPORT

Atypical presentation of pseudomembranous colitis localized in adenomatous polyps

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Abstract

The most frequent cause of pseudomembranous colitis is *Clostridium difficile* (*C. difficile*) infection. This type of colitis is characterized by an endoscopic pattern of numerous small, yellowish or whitish plaques diffusely distributed, which typically compromises the rectum extending to proximal colon. Occasionally, the pseudomembranes compromise only the transverse or right colon, but their exclusive localization over polyps has not been reported. In this case report we have described a patient with symptoms compatible with *C. difficile* infection and positive for *C. difficile* toxigenic culture. Colonoscopy examination showed two small polyps with a whitish surface, and histopathological analysis confirmed them to be pseudomembranes over tubular adenomas. The rest of the colonic mucosa was normal and no other cause was demonstrated. We suggest that this particular distribution might be due to a higher affinity for dysplastic cells such as adenomatous polyps of colon by *C. difficile* and/or its toxins.

Key words: *Clostridium difficile*; Pseudomembranous colitis; Adenomatous polyps; Antibiotic-associated colitis; *Clostridium difficile* infections

Hernández-Rocha C, Barra-Carrasco J, Guzmán AM, Paredes-Sabja D, Lezcano G, Zoroquiaín P, Álvarez-Lobos M. Atypical presentation of pseudomembranous colitis localized in adenomatous polyps. *World J Gastroenterol* 2013; 19(2): 316-318 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i2/316.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i2.316

INTRODUCTION

The clinical spectrum of *Clostridium difficile* (*C. difficile*) infections may vary from mild diarrhea to life-threatening pseudomembranous colitis (PMC), which may localize from the proximal colon to the rectum[1]. Localized forms of PMC have been reported to compromise the transverse and right colon[2], but to the best of our knowledge, we have not found cases with exclusive localization of PMC over polyps. Below, we report a case with the pseudomembranes only covering the surface of adenomatous polyps.

CASE REPORT

An 86-year-old female receiving anticoagulant therapy due

to an aortic valve prosthesis with auricular fibrillation secondary to aortic stenosis was admitted with an incarcerated umbilical hernia. A hernioplasty was performed without complication, and she was treated with sodium piperacillin-tazobactam (4.5 g *tid*) intravenously for 7 d and discharged 10 d after surgery. Twenty-five days later, she presented hematochezia with hemodynamic compromise. She was admitted to the coronary unit, and an upper endoscopy showed a hiatal hernia with Cameron's ulcers; she was treated with omeprazole. The next day a colonoscopy showed a clean colonic mucosa, absence of blood, and two sessile 6-mm polyps in the transverse colon and in the right colon. The upper surface of both polyps was covered by an adherent and whitish layer and the surrounding mucosa was normal (Figure 1). The polyps were resected with cold biopsy forceps and sent for histopathological study. Strikingly, the day after colonoscopy, the patient developed fever with no apparent cause in the physical exam, while laboratory results showed a hematocrit of 29%, white blood cell count of 8700 cell/μL without band forms and C reactive protein was 10.2 mg/dL. The chest X-ray was normal and the blood and urine cultures were negative. She was empirically treated with sodium piperacillin-tazobactam, but three days later she presented frequent loose stools and diffuse abdominal pain. An enzyme immune assay (EIA) for *C. difficile* toxin was positive. The antibiotic was stopped and she was treated with oral metronidazole with an excellent response. Presence of *C. difficile* was further confirmed by real time-polymerase chain reaction (PCR) and toxigenic culture. PCR-ribotyping indicated that the clinical isolate that caused *C. difficile* infection was not an epidemic strain (non-ribotype 027 strain). A few days later, the histopathological study of the polyps showed two tubular adenomas with a low-grade epithelial dysplasia and pseudomembranes on their surface with a typical volcanic appearance (Figure 2).

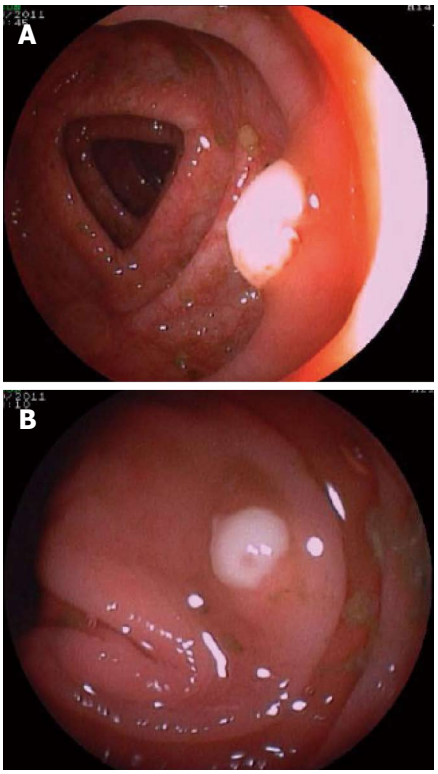

Figure 1 Colonoscopy images showing two polyps, 4 and 6 mm in size, in the right colon with adherent whitish surface. A: Polyp of 6 mm; B: Polyp of 4 mm. The mucosa round the polyps and in the rest of colon is normal.

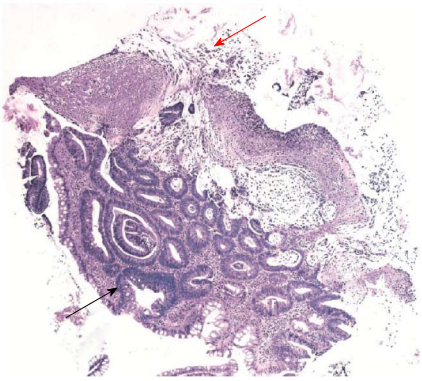

Figure 2 Low power view of a mucosal fragment. Histopathology shows: at the bottom, tubular structures lined by columnar epithelial cells with pseudostratified nuclei, consistent with low grade tubular adenoma (black arrow); at the top, ballooned crypts and intercrypt necrosis, an exudate featuring the classical "volcano" lesion (red arrow) surrounded by a laminated pseudomembrane composed of neutrophils, mucin and fibrin (hematoxylin and eosin, x 100).

DISCUSSION

It is our understanding that this is the first documented case in which the pseudomembranes are exclusively localized over adenomatous polyps with the rest of the colonic mucosa being intact. The term PMC is nonspecific and describes an acute mucosal injury characterized by an endoscopic pattern of numerous, discrete and small (2-5 mm), raised, round and yellowish plaques (pseudomembranes) distributed over an erythematous but unulcerated mucosa[1]. The rectum is frequently involved, but in a few cases the sigmoid and/or right colon is compromised[2]. The histological pattern is characterized by a neutrophil-rich edema fluid in the lamina propria that bursts through tiny breeches to the surface epithelium, like a volcanic eruption, to form a characteristic punctate inflammatory pseudomembrane[1,3]. The PMC is due, in nearly all cases, to *C. difficile*. Other infrequent causes are ischemic or infectious colitis[4]. Piperacillin-tazobactam has been associated at lower rates with *C. difficile* infection than other antibiotics[5]; however, in this case report, the clinical course and the study with EIA, PCR and toxigenic culture confirmed an association with *C. difficile* and the response to treatment with oral metronidazole was concordant.

The specific type of colonic cells where *C. difficile* infections starts is unclear, but it is known that at least enterotoxin A, one of its main virulence factors, has remarkable cytotoxicity for cells derived from human or animal cancer, more than for normal colonic epithelial

cells. One possible explanation, but by no means proven, is that the surface of neoplastic cells possesses a greater density of specific receptors for toxin A and/or toxin B as compared to less sensitive cell lines[6]. Alternatively, it is well known that members of the *Clostridium* genus have a tropism for tumors mainly due to the hypoxic environment as a consequence of poor vascular irrigation[7,8]. Thus, it seems plausible that colonic neoplastic cells might favor *C. difficile* colonization and PMC formation.

In conclusion, we propose that dysplastic changes of the colonic epithelium occurring as adenomatous polyps might represent a site of greater affinity for *C. difficile* toxin and/or tropism of *C. difficile* cells with the consequent formation of pseudomembranes, which were observed by an early colonoscopy in this patient.

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Anesthetic management of the SRS™ endoscopic stapling system for gastro-esophageal reflux disease

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Abstract

The SRS™ Endoscopic Stapling System (Medigus, Tel Aviv, Israel) is a new tool capable of creating a totally endoscopic fundoplication, combined with an endoscope, endoscopic ultrasound and a surgical stapler. SRS™ endoscopic stapling for gastro-esophageal reflux disease is a minimally invasive, outpatient procedure, which requires general anesthesia with positive-pressure ventilation. Keeping the patient on positive end-expiratory pressure (PEEP) may minimize the pressure gradient between the esophagus and the mediastinum, as well as help to prevent air from leaking around the screws and causing pneumomediastinum. In addition, in patients with hiatal hernia, higher PEEP levels may be required to increase intra-thoracic pressure and to force the stomach to slide into the abdomen for ease of endoscopy. We advise smoother emergence from anesthesia, taking precautions for retching, postoperative nausea and vomiting (PONV), while coughing and gagging during extubation and PONV may affect the success of the procedure. Total intravenous anesthesia with propofol and remifentanil seems to be a good choice for these reasons.

Key words: Gastro-esophageal reflux disease; Endoscopy; Anesthesia; SRS™ endoscopic stapling system; Positive end-expiratory pressure

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TO THE EDITOR

Endoscopic ultrasound was developed as a useful diagnostic tool and is being used in the treatment of various gastrointestinal diseases[1,2]. The SRS™ Endoscopic Stapling System (Medigus, Tel Aviv, Israel) is a new tool capable of creating a totally endoscopic fundoplication, combined with an endoscope, endoscopic ultrasound, and a surgical stapler[3]. This modified endoscope fires 3 rows of staples plicating the fundus onto the esophagus. While the anesthesia management of the SRS™ endoscopic fundoplication procedure has not been described, we would like to share our initial experiences with it on 12 patients.

After obtaining written informed consent, patients diagnosed with gastro-esophageal reflux disease, without or with ≤ 3 cm hiatal hernia, were scheduled for this procedure. Following at least 8 h fasting time, patients were premedicated with midazolam, metoclopramide, and a proton pump inhibitor. Total intravenous anesthesia (TIVA) with propofol and remifentanil was used for both induction and maintenance of general anesthesia. After muscle relaxation was achieved with rocuronium, patients were endotracheally intubated. Patients were mechanically ventilated with oxygen/air mixture. During the procedure, the lumen of the stomach was inflated with air for better visualization. To prevent possible air leakage,

positive end-expiratory pressure (PEEP) 4-6 mmHg was applied. If a hiatal hernia was discovered during preliminary gastroscopy, the patient should be tilted 15 degrees head up and the PEEP level must be increased to 7-10 mmHg in order to depress the diaphragm and reduce the gastro-esophageal junction, so that it can be repositioned into the abdomen. Mean interventional time was 75 min; range was between 47-102 min according to the expertise of the operator. Paracetamol 1 g *iv* was administered for postoperative analgesia and ondansetron 4 mg *iv* for postoperative nausea and vomiting (PONV). Recovery was uneventful in all patients.

In conclusion, SRS™ endoscopic fundoplication for gastro-esophageal reflux disease is a minimally invasive, outpatient procedure, which requires general anesthesia with positive-pressure ventilation. Keeping the patient on PEEP may minimize the pressure gradient between the esophagus and the mediastinum and may help to prevent air from leaking around the screws and causing pneumomediastinum. In patients with hiatal hernia, higher PEEP levels may be required to increase intra-thoracic pressure and force the stomach to slide into the abdomen for ease of endoscopy. It must be kept in mind that higher PEEP levels may be associated with arterial hypotension. We advise smoother emergence from anesthesia, taking precautions for retching, PONV, while coughing and gagging during extubation and PONV may affect the success of the procedure. TIVA with propofol and remifentanil seems to be a good choice for these reasons.

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An informative, structured abstract should accompany each manuscript. Abstracts of original contributions should be structured into the following sections: AIM (no more than 20 words; Only the purpose of the study should be included. Please write the Aim in the form of "To investigate/study/…"), METHODS (no less than 140 words for Original Articles; and no less than 80 words for Brief Articles), RESULTS (no less than 150 words for Original Articles and no less than 120 words for Brief Articles; You should present *P* values where appropriate and must provide relevant data to illustrate how they were obtained, e.g. 6.92 ± 3.86 *vs* 3.61 ± 1.67, $P < 0.001$), and CONCLUSION (no more than 26 words).

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Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

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Please write a summary of less than 100 words to outline the most innovative and important arguments and core contents in your paper to attract readers.

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For articles of these sections, original articles and brief articles, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the main text or in Figures and Tables, but not in both.

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Data that are not statistically significant should not be noted. $^{a}P < 0.05$, $^{b}P < 0.01$ should be noted ($P > 0.05$ should not be noted). If there are other series of *P* values, $^{c}P < 0.05$ and $^{d}P < 0.01$ are used. A third series of *P* values can be expressed as $^{e}P < 0.05$ and $^{f}P < 0.01$. Other notes in tables or under illustrations should be expressed as ^{1}F, ^{2}F, ^{3}F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc*., in a certain sequence.

Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

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Format

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English journal article (list all authors and include the PMID where applicable)

1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.00000 35706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*
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