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EDITORIAL

Hepatocyte cryopreservation: Is it time to change the strategy?

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Abstract

Liver cell transplantation presents clinical benefit in patients with inborn errors of metabolism as an alternative, or at least as a bridge, to orthotopic liver transplantation. The success of such a therapeutic approach remains limited by the quality of the transplanted cells. Cryopreservation remains the best option for long-term storage of hepatocytes, providing a permanent and sufficient cell supply. However, isolated adult hepatocytes are poorly resistant to such a process, with a significant alteration both at the morphological and functional levels. Hence, the aim of the current review is to discuss the state of the art regarding widely-used hepatocyte cryopreservation protocols, as well as the assays performed to analyse the post-thawing cell quality both *in vitro* and *in vivo*. The majority of studies agree upon the poor quality and efficiency of cryopreserved/thawed hepatocytes as compared to freshly isolated hepatocytes. Intracellular ice formation or exposure to hyperosmotic solutions remains the main phenomenon of cryopreservation process, and its effects on cell quality and cell death induction will be discussed. The increased knowledge and understanding of the cryopreservation process will lead to research strategies to improve the viability and the quality of the cell suspensions after thawing. Such strategies, such as vitrification, will be discussed with respect to their potential to significantly improve the quality of cell suspensions dedicated to liver cell-based therapies.

Key words: Hepatocyte; Cryopreservation; Quality; Mitochondria; Intracellular ice formation

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INTRODUCTION

Liver cell transplantation (LCT) is able to correct inborn errors of liver metabolism by supplying viable and functional hepatocytes[1-5]. This innovative therapeutic approach is currently accepted as a bridge to transplantation during the waiting time for a graft. The efficacy of

this alternative treatment varies according to the etiology of the disease and remains principally dependent on the quality of the initial liver cell suspension used.

The current great challenge of LCT is the constant availability of the cell suspension. Besides sterility, the cell suspension should present high viability and metabolic activity levels. If freshly isolated cells can be ready to use 5-8 h after the treatment of the organ, data of sterility tests are rarely available before transplantation. Nevertheless, most of the quality control tests performed and documented in the literature reveal a good quality profile of freshly isolated cells, both *in vitro* and *in vivo* after transplantation. However, their availability remains significantly limited by ongoing organ shortages. Furthermore, even if freshly isolated cells could be transplanted the same day, we are limited by the quantity of cells that can be infused in one single session. Extensive research based on two different strategies has been developed to efficiently store the isolated liver cells: cold preservation, which could happen during the first 24 h post-isolation and cryostorage. This later strategy remains the sole practical method for the long-term storage of hepatocytes and leads to (1) the development of a readily available cell bank, even in emergency cases, such as metabolic decompensation; (2) the use of fully analysed cell suspensions, including bacterial and viral safety assays; and (3) an efficient planning of future transplantation. In 1999 an "international panel of experts" recognized that "research should continue to improve the liver cell cryopreservation procedures"[6]. Ten years later, only a few cryopreservation protocol improvements have been documented, and hepatocyte post-thawing quality remains poor.

The aim of this review is to discuss current developments regarding the major cryopreservation/thawing (C/T) protocols used in the field. Pre-C/T management of the cell suspension and post-thawing *in vitro* and *in vivo* analyses will be discussed and reviewed. Understanding the biophysical properties of the cryopreservation protocol might supply key and useful information to build efficient strategies. Intracellular ice formation (IIF) or exposure to hyperosmotic solutions, which remain the major C/T damages initiators will be reviewed in detail, regarding their effects on the decrease or loss of cell function, and on cell damages and cell death. Finally, technological developments, such as vitrification, which avoids the crystalline state, or encapsulation, which confers mechanical protection, are currently considered to be exciting new perspectives for the improvement of the cell suspension quality dedicated to clinical LCT.

PRE-CRYOPRESERVATION/THAWING CRITICAL FACTORS

Donor organ and isolation step

An initial high quality cell suspension after isolation remains essential prior to cryopreservation. Indeed, key factors that compromise the quality of the isolated hepatocytes include high liver fat content, prolonged warm ischemia and/or storage of the organ[6].

Liver cell isolation is mainly performed using the two-step collagenase perfusion protocol. At 37℃, the first solution, which contains a calcium chelating agent, is perfused to weaken the intercellular junctions of liver cells by removing extracellular calcium ions. The second solution contains collagenase and calcium, essential for the collagenase activity, and disaggregates the extracellular compartment to easily release both non-parenchymal and parenchymal cell fractions. The isolated hepatocyte suspension is obtained after mechanical dissociation, filtration and low speed centrifugation[7].

Isolation is thus the first cause of cell trauma, probably due to oxidative stress, as demonstrated in ischemia/reperfusion of the liver, with impaired mitochondrial functions, consequent intracellular adenosine triphosphate (ATP) depletion (personal unpublished data), and production of reactive oxygen species, leading to hepatocyte death. Addition of anti-oxidant molecules to the isolation medium, such as curcumin, ameliorates the post-isolation quality in terms of metabolic activity and plating. However, such compounds did not show any beneficial effect after cryopreservation/thawing[8].

Detachment from the extracellular matrix has also been shown to promote apoptosis, called anoikis (loss of adhesion molecule). This early cell death could not be totally reversed after *in vitro* culture of hepatocytes because cells already engaged in this process will die in the hours following the isolation procedure[9,10]. Anoikis is possibly a consequence of the recently described isolation oxidative stress. In conclusion, cell damage, due to the isolation process itself, is already evidenced prior to C/T. However, if plated, the cells have the opportunity in culture to recover and maintain a good metabolic activity.

Post-isolation management of the cell suspension

Suspension pre-culture: To allow the hepatocytes to recover from the isolation stress and improve their quality post-isolation, authors proposed culturing (no attachment culture conditions) freshly isolated hepatocytes prior to C/T. Cold (4℃) or warm (37℃) non-attached, stirred, culture conditions (bio-artificial liver) were developed to avoid later addition deleterious detachment of plated cells. Using pig hepatocytes, Darr *et al*[11,12] demonstrated that 24 h pre-culture in a spinner bioreactor at 37℃ leads to a detectable increase in albumin production after C/T as compared to non pre-cultured hepatocytes. This beneficial effect decreased after 48 h of pre-culture, showing that the recovery of cell quality post-thawing remains difficult. Indeed, non-attached culture conditions might, over time, lead to additional cell damage. Furthermore, albumin production levels remained markedly lower following cryopreservation as compared to freshly isolated cells, even with 24 h pre-incubation. The utility of pre-culture was confirmed by Gómez-Lechón *et al*[13] who demonstrated that high post-thawing quality hepatocytes of other species (rat, dog and human hepatocytes) were possible using non-attached pre-culture. Criteria such as viability, adaptation of hepatocytes

to culture, drug-metabolizing capability and cytochrome P450 (CYP) activity were assessed. The influence of a non-supplemented pre-culture step on hepatocyte quality was not confirmed by data published by Lloyd *et al*[14] with pig hepatocytes cultured in a bioartificial liver.

Pre-incubation with antioxidants: ATP cellular boosters or antioxidants have been proposed to supplement pre-incubation medium and to potentialize the beneficial effects of pre-culture. Several pre-culture conditions, type of ATP booster and culture at 4℃ or 37℃, were evaluated by Terry *et al*[15,16]. Two hours hyperosmotic glucose 100-300 mmol/L pre-incubation has been shown to improve the viability and attachment efficiency of rat hepatocytes, as well as the viability of human hepatocytes post-thawing. Fructose 100-300 mmol/L pre-incubation also improved the viability and attachment efficiency of rat hepatocytes. On human hepatocytes, fructose improved their attachment efficiency, but not their viability. Pre-incubation with the anti-oxidant alpha-lipoic acid at 0.5-5 mmol/L improved the viability and attachment efficiency of both rat and human hepatocytes. The beneficial effects of this pre-treatment (at lower concentration: 15 mmol/L glucose for 30 min at 37℃) in human hepatocytes were demonstrated by Silva *et al*[17]. They found that the response of CYP enzymes to typical inducers was significantly improved in the pre-incubated rat and human hepatocytes. The pre-incubated hepatocytes showed a significantly higher plating efficiency compared with hepatocytes cryopreserved without pre-incubation. Finally, Gómez-Lechón *et al*[18] recently demonstrated that the optimal preservation of isolated cells (cell viability, attaching capacity, and functionality, particularly GSH and glycogen levels, as well as drug-metabolizing cytochrome P450 enzymes) was found in media supplemented with 2 mmol/L N-acetyl-cystein (anti-oxidant molecule) and 15 mmol/L glucose, confirming the importance of anti-oxidant protection after isolation.

In conclusion, based on the literature and on our experience, we believe that liver cell isolation represents an important oxidative stress, potentially controlled by the addition of anti-oxidants to the isolation media and/or by non-attached time-limited culture in an anti-oxidant supplemented medium. Quality of cells prior to C/T is therefore increased. However, all the damage related to C/T is not avoided by these pre-C/T steps. Furthermore, in clinical settings, pre-culture adaptation is difficult.

STANDARDISED HEPATOCYTE CRYOPRESERVATION/THAWING PROTOCOL: STATE OF THE ART

After isolation and related oxidative stress, the obtained cell population is re-suspended in cryopreservation media and distributed at specific concentrations in special freezing vials. The hepatocytes are then ready for the cooling process and storage in liquid nitrogen. In this chapter, we will review the literature data for liver cell freezing solution as well the documented cooling and thawing process.

Concentration of hepatocytes and type of vials

In most studies, the hepatocyte concentration varied from 10^6 to 10^7 cells/mL[19]. In this range, Lloyd *et al*[14] did not find any significant superiority of cell concentrations investigated, when evaluating porcine hepatocytes after thawing. This was confirmed by analyzing, hepatocyte attachment, lactate dehydrogenase (LDH) leakage, bilirubin conjugation, and CYP3A4 activity. However, De Loecker *et al*[20,21] revealed that a decreased cell density of rat hepatocytes correlated with an increased post-thawing viability, as estimated by viability trypan blue exclusion assay. These data suggest that higher cell densities might increase membrane-membrane contacts and subsequent cell damage. Therefore, unless high cell density will save space and is useful for the development of cell banks, cryopreservation at a low cell density (less than 10^7/cell) is recommended.

Besides cell density, type and volume of vials used for liquid nitrogen storage might also influence post-thawing cell quality; however, few data are available in the literature. Based on the trypan blue exclusion test, cytochrome activity, and tetrazolium inner salt assays, bags of 50 mL seem to give better quality pig hepatocytes, post-thawing, than bags of 100 mL[22].

The lack of recently published data on the concentration of hepatocytes is considered as a minor point for reaching the best post-thawing quality.

We also confirm that, in our hands, in several different volumed vials (from 2 to 100 mL) and varying cell densities, cell density does not influence post-thawing cell quality.

Freezing solution

Cryopreservation medium: University of Wisconsin solution (UW) is the gold standard cryopreservation medium for isolated hepatocytes. It was originally developed as a cold storage solution for transplant organs. The principal cryoprotectants of the UW solution are Lactobionate (100 mmol/L), a large molecular weight anion impermeable to most membranes and supposed to suppress hypothermia-induced cell swelling, and Raffinose (30 mmol/L), which allows additional osmotic support[23]. Dexamethasone, another compound in UW solution, is used to stabilise cell membranes[24].

The superior beneficial effect of UW solution was demonstrated by comparing UW to three other freezing media [all were supplemented with 12% dimethylsulfoxide (DMSO)], Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and commercial solutions, Cell Banker 1 and Cell Banker 2. Parameters including viability, plating efficiency, LDH release, ammonia removal test, and lentiviral gene transfer were shown to be highly maintained in hepatocytes cryopreserved with UW solution[25].

The effectiveness of UW solution as a cryoprotectant

agent suggests that metabolic, as well as ultrastructural, factors might be important in the effective cryopreservation of isolated hepatocytes[26]. However, UW solution also has limitations, principally its cost and, as demonstrated in the "Parameters for the evaluation of hepatocytes quality after cryopreservation/thawing" paragraph, its incomplete cell protection.

HypoThermosol (HTS), a recently developed freezing solution, is a dextran-based intracellular-type solution. It is used as the carrier solution of the freezing medium. Freshly isolated rat hepatocytes cryopreserved in HTS supplemented with 10% DMSO have been shown to present high viability levels, good long-term maintenance of hepatospecific functions, and good quality response to cytokine challenge at the post-thawing level compared to other supplemented culture media[27]. Further studies confirmed the utility of HTS, allowing a decrease in the DMSO levels within the cryopreservation solution. However, no data comparing UW to HTS are available in the literature[28,29].

Cryoprotectants: Cryoprotectants are essential components of freezing solutions. Two classes of cryoprotectants are described, those that permeate the cell membrane (DMSO, Glycerol) and those that do not such as polymers (Dextran), oligosaccharides (Trehalose), and sugars (Glucose, sucrose or fructose).

DMSO is an important polar permeating aprotic solvent which is less toxic than other members of this class. The use of DMSO in medicine dates from around 1963, when an University of Oregon Medical School team discovered it could penetrate the skin and other membranes without damaging them and could carry other compounds into a biological system. It is able to enter cells and reduce injury by moderating the increase in solute concentration during freezing. In most studies, DMSO is the ideal cryoprotectant, notably giving the best plating efficiency[27,30-37]. Classically, a final concentration of 10% DMSO is described in many protocols, with some exceptions, although higher levels are potentially toxic due to high osmolarity[38]. The rate of addition of the cryoprotectant also appears important to the outcome of cryopreservation. Freezing must be commenced as soon as possible after addition of the cryoprotectant to reduce the possibility of toxicity at ambient temperatures. Hence, DMSO should be added at 4℃, as toxicity of DMSO was demonstrated at 25℃ or 37℃. Some authors proposed adding permeating cryoprotectants slowly to the cell suspension to avoid damages related to osmotic shock and cellular dehydration[33,39]. However, this seems to be a minor point[38].

The use of oligosaccharides with higher molecular weights resulted in greatest improvement in viability. Their combination with DMSO has been shown to allow efficient hepatocyte cryopreservation. Both rat and human hepatocytes exhibit significantly higher viability (as estimated by trypan blue exclusion assay) than hepatocytes previously cryopreserved without oligosaccharides. Moreover, attachment and survival rates in plastic dishes of rat hepatocytes were greater after freezing in the presence of di-, tri-, and tetrasaccharides. Such plating amelioration was not confirmed with human hepatocytes[40]. Metabolic activity was also evaluated after cryopreservation with oligosaccharides. When trehalose was combined with DMSO for the cryopreservation of human hepatocytes, a significant increase in total protein level and secretion of albumin was observed after thawing, as well as decreased levels of aspartate aminotransferase[41].

Those works were inspired from data demonstrating the influence of trehalose on cell quality on bull sperm[42,43]. Similarly, several authors recommended adding sucrose to the cryopreservation medium, with or without trehalose, to ameliorate the quality of the cells after thawing. This allows the concentration of DMSO to be decreased while ameliorating the quality of cells. However, it was evaluated on hematopoietic stem cells and fetal liver hematopoietic stem/progenitor cells[44,45], not on hepatocytes.

The beneficial role of a non-metabolizable glucose derivative as a cryoprotectant that mimicked the natural cryoprotective adaptations observed in freeze-tolerant frogs was also investigated. Primary rat hepatocytes were loaded with 3-O-methyl glucose (3OMG) through endogenous glucose transporters without evident toxicity and cryopreserved according to a controlled rate freezer program down to -80℃ before storage in liquid nitrogen. In this study, hepatocytes cryopreserved with a relatively small amount of intracellular 3OMG (< 0.2 mol/L) showed high post-thaw viability and maintained long-term hepatospecific functions, including synthesis, metabolism, and detoxification. Metabolite uptake and secretion rates were also largely preserved in the cryopreserved hepatocytes, showing that 3OMG must be considered as an interesting cryoprotectant[46].

An interesting report proposed that wheat protein extracts permitted long-term storage and recovery of large quantities of healthy cells that maintain high hepatospecific functions, *via* an osmotic modulation effect[47]. In post-thawing culture, the morphology of hepatocytes cryopreserved with wheat protein extracts was similar to that of fresh cells. Furthermore, hepatospecific functions, such as albumin secretion and biotransformation of ammonium to urea, were well maintained during four-days post-plating. Inductions of CYP1A1 and CYP2B in hepatocytes cryopreserved with wheat extracts were similar to those in fresh hepatocytes. Additional data confirmed the utility of wheat extracts as efficient, non-toxic, economic natural cryoprotectants, superior to DMSO, which has limitations due to potential cellular toxicity[47-49].

Finally, human application does not tolerate the use of animal origin products because of possible zoonosis contamination and/or immune response to animal proteins[50]. Fetal calf serum (FCS) or human albumin, are classical ingredients of the cryopreservation solution, in a proportion of 10% to 90 %. No significant differences in classical viability or drug metabolizing enzyme activities were noted while varying the percentage of serum for (human, pig, and rat) hepatocyte cryopreservation in most

Table 1 Summary of freeze-rate comparison studies

Species	Cryoprotectant	Freeze rate	Storage temperature	Ref.
Human	DMSO	-1℃/min	-80℃	[55]
Rat	DMSO	-1℃/min to -38℃ (with cooling shock) then liquid nitrogen	Liquid nitrogen	[61]
Dog, monkey, human	DMSO	-1.9℃/min from 4℃ to -30℃, then -30℃ from -30℃ to -150℃	Liquid nitrogen	[57]
Rat	DMSO	-38℃/min -2℃/min Slow variable Optimized variable rate	Liquid nitrogen	[59]
Human	DMSO	-1.9℃/min from 4℃ to -30℃, then -30℃ from -30℃ to -150℃	Liquid nitrogen	[51]
Rat	DMSO	Cooling in 10 min down to 0℃, 8 min at 0℃, in 4 min down to -8℃, in 0.1 min down to -28℃, in 2 min down to -33℃, in 2 min up to -28℃, in 16 min down to -60℃, in 4 min down to -100℃ (variable rate)	Liquid nitrogen	[60]
Human	DMSO	Variable rate	Liquid nitrogen	[6]
Dog, monkey, human	DMSO	-1.9℃/min from 4℃ to -30℃, then -30℃ from -30℃ to -150℃	Liquid nitrogen	[58]
Rat	DMSO	Variable rate	Liquid nitrogen	[39]
Pig	DMSO	Optimized[59] Modified variable	Liquid nitrogen	[22]

DMSO: Dimethylsulfoxide.

of the published studies[26,30,33,36,51-53]. We think that a minimal concentration of serum is required for optimal cryopreservation, even if some authors have also successfully cryopreserved porcine hepatocytes without serum. They showed that, after thawing, in appropriate conditions and without serum, the addition of conditioned medium derived from hepatic non-parenchymal cells improved attachment and function of hepatocytes (urea production and CYP activity)[54].

In conclusion, UW solution remains the best and most studied freezing medium and must be supplemented with a permeating cryoprotectant; DMSO remains the gold standard. The addition of a non-permeating cryoprotectant to this solution must also be considered.

Cooling process

Slow freezing protocols are considered to be the best strategy for cryopreserving mature isolated hepatocytes. All the protocols described in this paragraph were developed using DMSO as the cryoprotectant. First protocols included the use of an isopropranolol cooler device, placed in a -80℃ freezer, giving a constant temperature decrease of -1℃/min down to -70℃ or -80℃, before storing in liquid nitrogen[55]. Other slow freezing protocols are described in the literature, varying from -1℃/min to -5℃/min up to -40℃ or -80℃, before storing at -196℃[56]. A decrease in temperature at -1.9℃/min from 4 to -30℃ and then -30℃/min from -30℃ to -150℃ was also adopted by many authors[51,57,58]. More specific protocols were developed by Diener *et al* and by Hengstler *et al*[39,59,60]. Several cooling process protocols, where the temperatures of the vial and of the cryopreserving solution were controlled, were tested on rat hepatocytes. Firstly, shock freezing in liquid nitrogen dramatically decreased cell viability, despite the presence of 10% DMSO. Secondly, a slow freezing protocol with -2℃/min led to much better recovered viability than a cooling rate of -38℃/min. While using the slow freezing protocol, the authors determined that the cell suspension becomes supercooled around -20℃. Indeed, when crystallisation starts, the latent heat of fusion is released and the cell sample is warmed. This heat release may be deleterious; therefore, a freezing program with shock cooling was developed. Analysis of post-thawing viability did not show significant differences of hepatocyte viability (86% viability *vs* 79% according to the slow linear protocol). The same cooling shock can be obtained by clamping the vials, with forceps cooled in liquid nitrogen[61]. However, studies from Lloyd *et al*[14] (measuring LDH release, cell return, attachment, and biochemical assays) and from our team[62] did not show any difference between computer-controlled freeze rate (without frozen shock), the Nalgene propan-2-ol device or simply using -20℃ and -80℃ freezers.

Storage of hepatocytes at -20℃ or -80℃ remains deleterious for cells functions as several proteases might be active at those temperatures. At -130℃, no chemical reaction can occur as there is no more thermal energy. Furthermore, at this temperature, no water, which is at the vitreous or crystalline state, is present at the liquid state. Therefore, -140/-150℃ is the minimum acceptable temperature for long-term storage of cryopreserved hepatocytes[6,34,57,63]. At -140℃ (the vapour phase of liquid nitrogen) or -196℃ (the liquid phase of liquid nitrogen), cells can be stored for long periods[6,34,51,57]. A summary of freeze rate comparison studies is presented in Table 1. The passage of water from one state to another, IIF, is the critical point that might modulate the cell quality. The limitations of these cooling processes will be discussed later in the IIF paragraph.

Thawing procedure

The critical point of this procedure is to avoid the deleterious phenomenon, IIF. Rapid thawing at 37℃ to minimize cellular damage due to reformation of intracellular ice will significantly enhance cell viability. As for cooling, a slow dilution of the cryoprotectant at 4℃ is recommended, to avoid osmotic shock and the toxicity

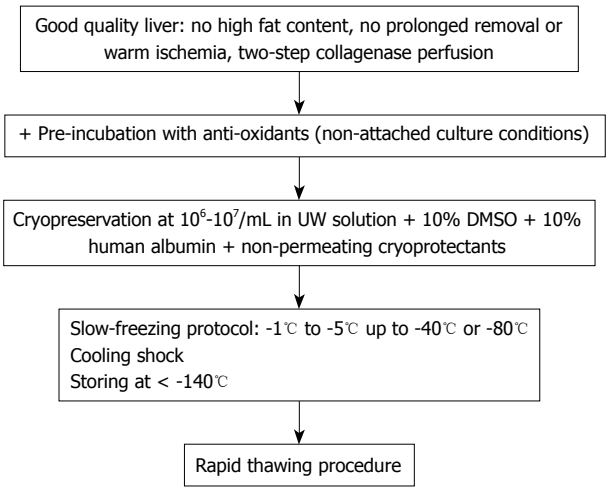

Figure 1 Standardized cryopreservation protocol. UW: University of Wisconsin solution; DMSO: Dimethylsulfoxide.

of the cryoprotectant[38].

The standardized cryopreservation/thawing protocol is summarized in Figure 1.

PARAMETERS FOR EVALUATING HEPATOCYTE QUALITY AFTER CRYOPRESERVATION/THAWING

The aim of this paragraph is to review the principal assays that may help to standardize the post-thawing evaluation step process.

The viability assays (Trypan blue exclusion test, LDH release, mitochondrial functions and necrotic/apoptotic markers) are important quality markers. Most of the "metabolic" assays investigate only some specific hepatocyte functions, notably drug metabolizing enzymes activities, essential for drug industry application; but also the hepatocytes' capacity to plate to collagen coated dishes. Other assays should be developed to allow a rapid evaluation of LCT-related critical parameters.

Viability assays

Necrosis was first described to occur following C/T[31], whereby intracellular organelles, most notably the mitochondria, and the entire cell swell and rupture (cell lysis). This phenomenon begins with an impairment of the cell's ability to maintain homeostasis, leading to an influx of water and extra-cellular ions. Due to the breakdown of the plasma membrane, the cytoplasmic contents, including lysosomal enzymes, are released into the extracellular fluid. This can be tested by LDH release from the cytoplasm to the extracellular medium and reflects cell membrane integrity. However, we found that LDH release was unaffected after C/T of both human and mice hepatocytes and does not adequately assess viability after C/T[62].

Apoptosis, a programmed cell death which has been well characterized, both at the morphological and biochemical levels, was also described following C/T. Annexin V staining, *in situ* TUNEL assay combined with confocal laser scanning microscopy, or deoxyribonucleic acid (DNA) fragmentation are the most popular apoptosis assays[28,31,64]. These assays should be carefully interpreted if performed *in vitro* or *in vivo*.

The mitochondrion is a key player in the initiation of apoptosis and recent studies highlighted its role in C/T-induced cellular damage[65]. Disruption of mitochondrial membrane potential ($\Delta\Psi$) was reported following C/T, which is followed, within hours after thawing, by cytochrome c extra-mitochondrial release, caspase-3 activation, and DNA fragmentation. Addition of caspase inhibitors (IDN-1965 or ZVAD-fmk) to the medium during cryopreservation and static culture rescued cells from apoptosis and was associated with increased phase 1 and phase 2 metabolism[64,66].

As mitochondria are the major source of reactive oxygen species (ROS), induction of apoptosis by oxidative stress was also proposed to be involved in the impairment of hepatocytes after C/T[67,68]. In fact, the combination of antioxidant medium containing a caspase inhibitor allowed significant improvements in viability and function in treated rat hepatocytes[68]. Similarly, other authors proposed the addition of S-adenosylmethionine to the cryopreservation medium, to avoid glutathione and viability decrease during cold preservation or cryopreservation in liquid nitrogen[69]. Finally, C/T decreased mitochondria-related cellular respiration and oxygen consumption rate. This effect was evidenced on oligomycin (ATP synthase inhibitor)-sensitive respiration, suggesting that it could result from alteration of a mitochondrial process linked to ATP synthesis, rather than an intrinsic modification of mitochondrial membrane proton permeability (leak). In permeabilized hepatocytes, a marked impairment of mitochondrial oxidative phosphorylation following C/T was observed in *in situ* mitochondria with substrates for complex 1, under basal mitochondrial respiratory rate. Interestingly, the inhibition of basal mitochondrial respiration was not revealed with complex 2 substrates[62]. The respiratory-chain complex 1 is one of the largest known membrane protein complexes, and is also the major source of mitochondrial ROS[70,71]. Thus, specific alterations of complex 1 subunit(s), which comprise the hydrophilic domain containing the redox centres of the enzyme, and/or deregulation of ROS production leading to oxidative stress, could constitute one of the start-points of the C/T-induced damage. This could explain the oxygen consumption and $\Delta\Psi$ decrease, leading to ATP depletion and later cytochrome c release. The intracellular ATP concentration, which is an indirect mitochondrial marker, is probably the easiest and most rapidly measurable parameter for detecting early cellular damages related to cryo-storage.

Detailed information regarding cryopreserved hepatocyte death *in situ* after transplantation remains poorly investigated in LCT *in vivo* models.

Plating

Attachment of isolated hepatocytes *in vitro* (collagen coated dishes) is widely used for the evaluation of their quality. The low plating efficiency is often documented

in cryopreserved cells. This remains a major problem because engraftment of the transplanted hepatocytes in the recipient liver parenchyma is also dependent on the proteins involved in extracellular matrix adhesion mechanisms[17,23,25,63,72]. Structural membrane damage observed after cryopreservation might contribute to such alterations. Recently, it was demonstrated that the process of cryopreservation leads to down-regulation of cell adhesion at the gene and the protein level (β1-integrin and E-cadherin, amongst others)[73]. This is relevant and probably begins to explain the observed low plating efficiency. Another team was able to demonstrate that when hepatocytes are cryopreserved with wheat extracts instead of DMSO, there was a clear protective effect against loss of β1-integrin, E-cadherin, and β-catenin[74]. We must also recognize the high plating variability from one liver to another.

Hepato-specific functions

Conjugation and secretion of biliary acids seems to be maintained following C/T of human hepatocytes. The uptake of taurocholate in cryopreserved hepatocytes of was found to range from 10% to 200% of that observed in freshly isolated cells immediately after thawing at 37℃[75].

The characterization of freshly isolated and C/T monkey hepatocytes demonstrated the maintenance of various hepato-specific functions, but at a low level. The ability to synthesize proteins, glucose, and glucose-6 phosphatase activity was decreased after deep-freeze storage[30]. Concerning protein synthesis, data from the literature show that this important hepatic function is often impaired in hepatocytes after C/T. De Loecker *et al*[61] demonstrated that cryopreserved human hepatocytes albumin production was reduced to half that of freshly isolated hepatocytes. Glycogen synthesis in cryopreserved porcine hepatocytes was reduced by about 30% after 24 h of culture and about 47% after 48 h of culture compared to freshly isolated hepatocytes. Reduced basal levels of glycogen and of glycogen synthesis could be explained by an increased energy demand in cryopreserved hepatocytes to repair damage caused by cryopreservation. Glycogenolysis was reduced to about 50% in cryopreserved hepatocytes and gluconeogenesis to about 40% of the glucose production in freshly isolated hepatocytes at day 1 and 2 post-thawing. Incubation with glucagon (90 min) increased the glucose production from glycogenolysis and gluconeogenesis in both freshly isolated and cryopreserved hepatocytes[76].

Urea production also seems to be reduced following C/T, according the majority of the papers[30].

There is no apparent significant change in drug metabolizing enzyme activities between freshly isolated and cryopreserved hepatocytes for the major drug-metabolizing pathways. The cryopreservation of human hepatocytes isolated from 17 donors was shown not to alter their capability to metabolize substrates for the major CYP isoforms (CYP1A2, CYP2A6, CYP2C9, CYP2Cl9, CYP2D6, and CYP3A4), as well as the phase Ⅱ enzymes UDP glucuronyltransferase, and 7-HC sulfation for sulfotransferase[77]. Steinberg *et al*[78] showed that phase I drug-metabolizing enzyme activities analyzed in cryopreserved human, rat, and mouse hepatocytes were very similar to those of freshly isolated hepatocytes; while phase Ⅱ enzyme activities were affected by cryopreservation.

Other studies show better stability of drug metabolizing activities in monkey than in rodent hepatocytes. After thawing, Phase I and Phase Ⅱ activities (CYP, ethoxycoumarin-O-deethylase, aldrin epoxidase, epoxide hydrolase, glutathione transferase, glutathione reductase, and glutathione peroxidase) were well preserved[79]. The decrease in the activity of phase Ⅱ enzymes, documented in several studies, might be related to the loss of the corresponding cofactors; however, the hypothesis of physical cell alteration is not excluded[78]. The cytosolic enzymes, notably glutathione S-transferase, are more exposed to intracellular ice formation and related C/T damages, even if some mechanical protection can be given by microsomal membranes[55].

Finally, if thawed hepatocytes cultures are sensitive to CYP inducers (rifampicin, rifabutin, phenobarbital, omeprazole, and β-naphthoflavone) the induced activity remains lower as compared to freshly isolated cells, with an increased delay induction time[6,17,60,80-83]. This is summarized in Table 2.

In conclusion, the standardized "literature based" C/T protocols have limitations, as demonstrated by the collected data in the last four paragraphs. If some progress were made in the assessment of specific hepatic functions, then the true effects of pre-C/T incubation with anti-oxidants or the addition of non-permeating cryoprotectants to the freezing solution on the poor quality of hepatocyte post-C/T could be properly tested. Finally, cell death, probably not a reversible mechanism following C/T, is initiated due to mitochondrial impairment. ATP concentration evaluation, as a mitochondrial operation marker, is a crucial test to evaluate the quality of C/T cells.

INTRA-CELLULAR ICE FORMATION: THE START POINT OF THE OBSERVED CELL DAMAGE?

Post-thawing cell quality remains poor. How can we explain the observed damages? The passage from a liquid stage of the intracellular and extracellular water, to a crystalline state probably holds the key to understanding C/T damages. In the cryopreservation of cells or tissues, each system has its specific optimal cooling rate, showing a decreased survival at both too low (slow cooling damage) and too high cooling rates (fast cooling damage). During freezing, the transition phase of water leads to a decrease of the extracellular water content. Water can pass through the plasma membrane, which will in turn lead to water efflux and cell dehydration. Slow cooling damage has been attributed to such phenomena as the increase in the external and internal solute (salt) concentration, the small size of the

Table 2 ***In vitro*** **evaluation of post-thawing quality of hepatocytes**

Hepatocyte *in vitro* model	Cryopreservation protocol	Parameters evaluated: impairment following C/T	Parameters evaluated: no impairment following C/T	Ref.
Rat and human	Pre-incubation -20℃, -70℃, liquid nitrogen	Plating	CYP induction	[17]
Porcine	Slow freezing protocol up to -80℃	Trypan blue exclusion test Plating Ammonia clearance		[23]
Rat	Slow freezing protocol up to -80℃	Trypan blue exclusion test Plating Ammonia clearance		[25]
Human	20% DMSO, 40% FCS Slow freezing protocol	Trypan blue exclusion test Plating LDH release MTT	ATP Urea synthesis	[63]
Porcine	Freezing boxes or slow freezing protocol	CYP Glycogen synthesis Glycogenolysis Gluconeogenesis	Plating	[72]
Rat and mouse	Slow freezing protocol	Plating Uptake of neutral red Protein synthesis		[41]
Porcine	Immediate cryopreservation Serum free	Protein synthesis Gluconeogenesis CYP activity Urea synthesis	Trypan blue exclusion test	[30]
Rat (monolayer culture post-thawing)	Not available	Protein synthesis		[61]
Human	Storage in liquid nitrogen		Conjugation and secretion of biliary acids	[75]
Human, rat, rabbit, dog and monkey	Slow freezing protocol		CYP activity Phase 2 enzymes	[77]
Human, rat and mouse	Slow freezing protocol	Phase 2 enzymes	Phase 1 enzymes	[78]
Monkey	Slow freezing protocol	LDH release Plating	Phase 1 enzymes Phase 2 enzymes	[79]
Human	Storing at -80℃	Cytosolic enzymes: glutathione S-transferase	CYP activity	[55]
Rat	Slow freezing protocol	CYP induction		[60]
Human	Slow freezing protocol	CYP induction		[80]
Rat	Slow freezing protocol		CYP induction	[81]
Human	Not available	CYP induction		[82]
Human	Not available	CYP induction		[83]

C/T: Cryopreservation/thawing; FCS: Fetal calf serum; LDH: Lactate dehydrogenase; MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide; CYP: Cytochrome P450.

channels of unfrozen solution, or the mechanical stress of cell shrinkage and destabilisation of membranes and proteins at low water potential. At high cooling rates, the intracellular dehydration (by water efflux) cannot keep pace with the extracellular dehydration (by phase transition of water). As a consequence, higher cooling rates result in higher levels of intra-cellular supercooling, higher trans-membrane differences in osmotic pressure and solute concentrations, and higher rates of water efflux through the membrane. This fast cooling damage seems to be due particularly to IIF[84-89].

We recently demonstrated that cryopreservation at -20℃ for 20 min followed by rapid thawing induced a dramatic drop of ATP levels in cryopreserved/thawed hepatocytes, correlated with a decreased oxygen consumption rate and altered mitochondrial complex 1 activity (personal unpublished data).

These results suggest that during the cryopreservation damage, complex 1 impairment occurred early in the cryopreservation process by mechanical alteration of mitochondria due to IIF or exposure to hyperosmotic solutions. However, IIF might also occur during the thawing process.

CRYOPRESERVED/THAWD HEPATOCYTES FOR LIVER CELL TRANSPLANTATION

Animal

According to the data available in the literature, the ability of cryopreserved/thawed hepatocytes to engraft and to repopulate the recipient liver is not definitively demonstrated. In the eighties, Fuller *et al*[90] described that fewer cryopreserved (slow freezing protocol in DMSO) autologous hepatocytes cells were detected one month

post-transplantation in the recipient liver as compared to freshly isolated cells. David *et al*[91] in Nagase analbuminemic transplanted rats, found few clusters of C/T cells three months post-transplantation as compared to freshly isolated hepatocytes, with no significant production of albumin. Dunn *et al*[92] showed in a Dalmatian dog model that sequential intrasplenic LCT can provided a significant but transient, 22 d, correction of urinary uric acid excretion that was similar with either freshly isolated or C/T (slow freezing protocol in DMSO, post-thawing viability around 60%) hepatocytes. The protocol of Papalois *et al*[93] has demonstrated that cryopreserved pig hepatocytes, at -20℃ without cryoprotective medium in Hank's solution, have adequate viability (around 60%) after one month of storage to support hepatic function in animals with severe acute liver failure by hepatoproliferative factors produced by the hepatocytes engrafted in the spleen.

Besides metabolic supply, intrasplenic transplantation of C/T hepatocytes (at -80℃ in UW and DMSO) in rats pre-treated with D-galactosamine improved survival to 60% after seven days (as compared to 100% obtained using freshly isolated hepatocytes)[23].

However, in a transgene-induced liver disease model, an environment that is permissive for clonal expansion of donor cell populations, C/T hepatocytes (stored up to 32 mo in liquid nitrogen) have been shown to possess clonal replicative potential identical to that of freshly isolated hepatocytes. C/T hepatocytes constituting 0.1% of the total adult hepatocyte number in the recipient could repopulate a mean of 32% of recipient liver parenchyma[94]. Furthermore, transplantation of woodchuck hepatocytes into the liver of urokinase-type plasminogen activator/recombination activation gene-2 mice demonstrated that cryopreserved (slow freezing protocol in DMSO, viability up to 70%-80%) cells, retained the ability to divide and to repopulate a xenogenic liver three months post-transplantation. Notably, *in vivo* susceptibility to infection with woodchuck hepatitis B virus and the proliferative capacity of frozen/thawed woodchuck hepatocytes in recipient mice were identical to those observed by transplanting freshly isolated hepatocytes[95].

The efficiency of C/T (slow freezing protocol in DMSO and HTS, post-thawing viability around 60%) for human hepatocytes was also evaluated in an animal NOD/SCID mice model. Cho *et al*[96] demonstrated that transplanted cryopreserved human liver cells engrafted in the peritoneal cavity as well as the liver, retained hepatic function (glycogen storage and Glucose-6 phosphatase activity) and proliferated in response to liver injury by carbon tetrachloride. This effect was greater two hours and three days post-transplantation as compared to 7, 14 and 40 d post-transplantation, suggesting some loss of transplanted cells at later times.

Based on the above data, we may conclude that C/T hepatocytes, in a favourable environment, are transiently able to maintain hepatocyte function *in vivo*, engraft the liver and proliferate at low levels, as compared to freshly isolated cells.

Human

Metabolic diseases: Immediate and medium term metabolic efficacies, decrease of the ammonia levels and urea synthesis were observed in our hands in two urea cycle disorder patients using C/T hepatocytes[97,98]. This was correlated, in one case, with effective demonstration of engraftment up to one year after cell infusion, using Fluorescence *In Situ* Hybridization (FISH) for the Y chromosome. This four year-old arginosuccinate-lyase deficiency girl was transplanted with C/T cells and underwent a first liver biopsy after infusion of C/T male hepatocytes, which showed a XX/XXYY chimerism, with 4.7% Y-positive cells. This cell lineage was further described on several post-transplant biopsies, reaching more than 10% of the recipient cells, while she received additional fresh and C/T hepatocyte infusions. At King's College Hospital, one patient with an inherited factor Ⅶ deficiency was entirely transplanted with C/T hepatocytes, which led to a transient reduction to 20 percent of the requirements for factor Ⅶ therapy[99]. To our knowledge, all other published case reports used an infusion protocol at least partially comprising freshly isolated hepatocytes, preventing any conclusion regarding the respective efficiency of C/T *vs* freshly isolated cells.

In conclusion, as in animal models and based on few reported data, C/T hepatocytes seem able to transiently support deficient hepatocyte function, justifying their use to stabilise metabolically unstable patients while waiting for a liver graft.

PERSPECTIVES ON HEPATOCYTE CRYOPRESERVATION

In this final section, we will analyze and discuss several ways to ameliorate the C/T protocols. We think that these new techniques applicable to C/T protocols are the best hopes for changing the future of cryopreservation/thawing.

New hepatocyte culture configurations

Encapsulation-*in vitro*, *in vivo*: Encapsulation, by conferring a mechanical protection, was investigated with success for hepatocyte cryopreservation protocols.

Firstly, and before considering LCT, several *in vitro* studies showed that encapsulation of freshly isolated hepatocytes in specially designed multi-component capsules (alginate, cellulose sulphate, and poly (methylene-co-guanidine) hydrochloride) retained their specific functions (transaminase activity, urea synthesis and protein secretion) over the first days of culture. Furthermore, most detoxifying enzymes were also expressed (in cryopreserved alginate-entrapped hepatocytes) at levels close to those in unfrozen encapsulated hepatocytes[100]. Long-term, up to 120 d of cryopreservation, preservation of drug metabolism and transport activities was demonstrated using microencapsulated rat hepatocytes[101]. Moreover, cold-induced apoptosis in hepatocytes can be significantly reduced following their entrapment within alginate gel beads, as demonstrated by measurement of

caspase-3-like activity[102]. Finally, cryomicroscopy studies showed that the alginate microencapsulation technique protected the hepatocytes from physical damage caused by the growth of extracellular ice crystals[103].

How can we adapt the encapsulation of cells to the LCT protocol? In 1993, in the Gunn rat model, the authors proposed intraperitoneal transplantation of cryopreserved alginate-encapsulated hepatocytes, allowing significantly reduced hyperbilirubinemia, as well as freshly isolated encapsulated hepatocytes, up to 28 d following transplantation[104]. In a severe liver failure model, two-stage 95% hepatectomy, with xenogenic hepatocytes and without immunosuppression, the authors demonstrated the utility of intrasplenic encapsulated hepatocytes[105].

Intraperitoneal transplantation of cryopreserved or fresh encapsulated rat hepatocytes significantly increased the survival rate to 66% and 80% in the ALF model (acetaminophen administration and 30% hepatectomy). Intraperitoneal transplantation of cryopreserved or fresh encapsulated immortalized hepatocytes improved survival, in this model, to 50% and 55%, respectively. Histopathology revealed that encapsulated hepatocytes were viable, but for a limited period (up to two weeks post-transplantation)[106]. Recently, Baldini *et al*[107] showed the retention of biological activity and significant viability of porcine encapsulated hepatocytes transplanted intraperitoneally in rats without immunosuppression, confirming the utility of encapsulation to avoid rejection. Moreover, Aoki *et al*[108] demonstrated that poly-L-lysine entrapped cryopreserved human hepatocytes survived and expressed albumin in rat spleen after transplantation. Finally, Mei *et al*[109] confirmed these data by showing an increased rate of survival in a mouse model of fulminant hepatic failure after xenogenic transplantation of pig hepatocytes.

In conclusion, cryopreservation of encapsulated hepatocytes is a promising tool for the establishment of banks for the supply and storage of hepatocytes, by mechanically conferring protection. However, the main problem of this technique remains the adaptation to LCT, the problem of injection site and adaptation to the treatment indication (size of capsule pore). Furthermore, this can be only be proposed for ALF or metabolic unstable patients, as the efficacy of the transplantation remains time-limited. Repeated injections must therefore be considered. The time-limited effect is notably due to the hepatocyte de-differentiation, observed with freshly isolated or C/T cells, in this kind of configuration. New projects must evaluate the utility of co-encapsulation of hepatocytes with mesenchymal bone marrow cells or pancreatic islets, as a new type of feeder cells to avoid de-differentiation.

Vitrification

Vitrification (from the Latin, vitreus, glassy) is essentially the solidification of a supercooled liquid by adjusting the composition (high concentration of cryoprotectant) and cooling rate (fast freezing protocol) such that the crystal phase is avoided. The process involves a progressive and marked increase in viscosity during cooling and prevention of ice nucleation and growth. The system is stabilized in the glassy state as translational molecular motion is essentially halted. Vitrification eliminates the biologically damaging effects associated with freezing. No appreciable degradation occurs over time in living matter trapped within a vitreous matrix. Vitrification is potentially applicable to all biologic systems. As the major problem with the current protocols remains IIF, alternatives such as vitrifying hepatocytes are an interesting strategy for attaining the best post-thawing cell quality. Vitrification of precision cut-slices, tissue engineered pancreatic substitute, jugular veins/vessels constructs, and embryonic kidneys has already been performed, allowing the absence of ice into the vitrified samples and an excellent post-thawing quality and/or viability[110-115].

Classically, tissues (vessels constructs and embryonic kidneys) are vitrified at cooling rates of > 40℃/min in a specific solution, comprising DMSO, formamide and 1,2-propanediol in EuroCollins solution (VS55) or a polyethylene formulation consisting of propanediol, DMSO and polyethyleneglycol 400[110,113]. Best viability results were obtained with the VS55 solution. To obtain cooling rates of > 40℃, tissues contained in vials, are cooled to -100℃ in an isopentane bath (conductive cooling, freezing point -160℃) placed in a -135℃ freezer, removed from the 2-methylbutane bath and vitrified to -120℃ in the -135℃ freezer (convective cooling).

Re-warming is performed under controlled conditions, and the chemicals removed in a stepwise manner. However de-vitrification might occur during warming from the vitrified state. To prevent de-vitrification, the vitrified material must be warmed uniformly as fast as possible [slowly re-warmed to -100℃ using convection followed by rapid re-warming achieved by placing the vial in a DMSO/H_2O mixture at room temperature (225℃/min)] so that ice does not have the opportunity to form in significant quantities.

Vitrification of encapsulated hepatocytes in M or G-collagen was recently proposed as an alternative freezing protocol[116]. Wu *et al*[117] proposed a rapid stepwise introduction of microencapsulated hepatocytes to vitrification solution (40 % v/v ethylene glycol, 0.6 mol/L sucrose in the medium) and their direct immersion in liquid nitrogen. Using this technique, they obtained 100% retention of hepatocyte functions, correlated with excellent viability, and no detectable damage to the microcapsules. If vitrification was also proposed as successful cryopreservation protocol for isolated cells, as has been done for human amnion derived mesenchymal stem cells[118]; however, vitrification of non-encapsulated hepatocytes has not yet been studied. Therefore, further investigations are needed to confirm the potential of vitrification for LCT protocols.

CONCLUSION

Using current protocols, C/T of hepatocytes induces cell alteration. *In vitro* functions of C/T hepatocytes remain poorer than those of freshly isolated hepatocytes, while

the efficacy *in vivo* seems to be time-limited, both in animal models and in humans. Hepatocyte mitochondria are very sensitive to C/T, with marked complex 1 activity impairment following thawing. This leads to low intracellular ATP concentration, an excellent and easily obtaining post C/T viability marker. Related cytochrome c release induces cell death within hours by apoptosis.

The IIF or exposure to hyperosmotic solutions are probably the start point of the observed damage. New adapted cryopreservation protocols have therefore to be urgently developed. Interesting perspectives such as vitrification, to avoid the crystalline state, with or without encapsulation, conferring a mechanical protection, must be validated in the future, while considering the problem of their clinical translation.

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OBSERVATION

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Limitations in assessment of mucosal healing in inflammatory bowel disease

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Abstract

An emerging parameter to define the effectiveness of new therapeutic agents in clinical trials, and by extension, for use in day-to-day clinical practice has been labeled mucosal healing. It has been hypothesized that complete healing of the intestinal mucosa in inflammatory bowel diseases should result in reduced disease complications, reduced hospitalization and reduced surgical treatment. By implication, the natural history of inflammatory bowel disease might then be altered. Measurement of mucosal healing, however, is largely observational, requiring repeated invasive endoscopic examinations, sometimes with mucosal biopsies. Other indirect imaging methods may play a role in this assessment along with other surrogate markers, including intestinal permeability. These measurements may have significant limitations that prohibit precise correlation with symptom-based disease activity indices in clinical trials. This likely reflects the dynamic nature of this evolving and individualized inflammatory process that tends to be focused, but not limited, to the mucosa of the intestinal tract.

Key words: Intestinal mucosa; Digestive system endoscopy; Clinical trials

INTRODUCTION

Ulcerative colitis and Crohn's disease are inflammatory bowel disorders; both with no known cause. Curative treatment is still needed. As such, management has focused largely on ameliorating symptoms, and reducing hospitalization and the need for surgical treatment. In clinical trials, reductions of symptom-related numerical endpoints have been used [e.g. the Crohn's Disease Activity Index (CDAI)] as evidence of treatment effectiveness and their possible role in translation to clinical practice has been discussed previously[1-5]. Another treatment goal for these diseases is improving quality of life, based upon any means that this parameter might be clinically defined or measured. Now, an emerging measurement to define the effectiveness of new therapeutic agents in clinical trials, and by extension, for use in day-to-day clinical practice has been popularly labeled "mucosal healing".

In practical terms, the assessment of mucosal healing is based largely on observational evaluation, which requires the use of repeated endoscopic studies before and after a defined treatment period, sometimes in conjunction with histological examination of mucosal biopsies, or other more indirect imaging methods, other surrogate markers or miscellaneous methods, such as measurements of intestinal permeability. Logically, however, but not yet conclusively shown, complete healing of the intestinal mucosa should result over the long term in

reduced disease complications, hospitalization and surgical treatment. This proposed hypothesis further suggests that, if mucosal healing can be induced by treatment, then hopefully, the natural course and history of the disease in an individual patient might be modified, and by implication, improved. For example, in a Norwegian study[6], Crohn's disease or ulcerative colitis first diagnosed between 1990 and 1994 (before the use of biological agents) were examined endoscopically for up to 5 years. Mucosal healing after 1 year of treatment was reported in almost 50% of 495 treated patients that could be followed. Mucosal healing also appeared to predict reduced subsequent disease activity and a decreased need for active treatment in ulcerative colitis, but not Crohn's disease. Of note, the study also has demonstrated that other environmental factors may play an important role in mucosal healing (e.g. smoking, level of education).

ENDOSCOPIC INDICES

Earlier historical studies from Europe remain very important. These have shown considerable variability in endoscopic changes detected by experienced observers caring for patients with inflammatory bowel disease[7]. Moreover, the correlation between the patient's clinical status and endoscopic (and histopathological) changes in the colorectal mucosa was limited[8]. Later, using more modern measurements of disease activity (e.g. CDAI), there was a poor correlation between colonoscopic (or histological) findings and indices of disease activity, which implies that these were not reliable measures of disease severity or extent[9]. Similar results have been published by French investigators in a prospective evaluation of ileocolonic and colonic Crohn's disease[10]. In a later study[11], however, specific lesions were identified for evaluation that included: erythema, superficial and deep ulceration, stenoses and pseudopolypoid changes. Then, an index was calculated (Crohn's Disease Endoscopic Index of Severity; CDEIS), based on the percentage of involvement of different ileocolonic segments, for use in clinical trials of new therapeutic agents. A good correlation with lesion severity was reported with positive inter-observer agreement, but these investigators were very experienced and well trained for their study[11]. In routine day-to-day clinical practice, however, the reproducibility of this measurement seemed to be less helpful. As a result, other simplified endoscopic activity measures were proposed and applied in some clinical trials for Crohn's disease[12] and ulcerative colitis[13,14]. A detailed and excellent review of treatment indices, including endoscopic endpoints used in inflammatory bowel disease, specifically ulcerative colitis, has appeared elsewhere[15].

Definition of mucosal ulcers or erosions (or their apparent complete absence as a marker of mucosal healing) has been viewed by some clinicians with skepticism, given the highly fluid and dynamic nature of the inflammatory process in inflammatory bowel disease. Also, other factors may influence endoscopic evaluation, particularly for inflammatory bowel disease and its treatment (e.g. bowel preparation effects on the inflamed intestinal mucosa may differ from non-inflamed mucosa). In addition, the depth or extent of small-intestinal penetration at the time of visualization during ileocolonoscopy may not be well defined in some studies. For example, capsule endoscopy has demonstrated mucosal erosions or ulcerations distributed throughout the small intestine in Crohn's disease that are not appreciated well by other imaging modalities, including routine ileocolonoscopy[16]. Finally, a recent prospective evaluation in Crohn's disease confirmed that clinical response of the patient seemed to correlate poorly with capsule evaluation of the surface mucosa for assessment of healing[17].

Similarly, for ulcerative colitis, few well validated and well accepted endoscopic criteria for endoscopic mucosal healing have been evaluated for clinical trials. A large degree of overlap is evident within historical definitions of mild, moderate and severe endoscopic changes and, the degree of intra- and inter-observer error has been validated poorly in clinical trials, especially in multicenter studies with multiple observers involved in the evaluation of oral, intravenous or topical treatment regimens. In contrast, some studies have reported good inter-observer agreement for some, but not all endoscopic changes in ulcerative colitis, with experienced[18] as well as well-trained observers[19].

HISTOPATHOLOGICAL EVALUATION

In theory, microscopic definition of the mucosa provides precise evaluation of mucosal healing in response to treatment. However, this microscopic evaluation is not only dependent on endoscopic (or macroscopic) evaluation (for selection of the biopsy site), but is also prone to the impact of pathological inter- and intra-observer error. In Crohn's disease, this may be an especially significant problem owing to the focal or segmental nature of the inflammatory process. Even in ulcerative colitis, a disorder often characterized as a continuous inflammatory process, there may be a non-uniform pattern of mucosal healing. Little information is available on the temporal resolution of the inflammatory process, but it not likely to be uniform.

Moreover, the evaluation of the depth of inflammation may also be crucial to precise monitoring of treatment response. In Crohn's disease, this transmural dimension makes complete histopathological definition virtually impossible because endoscopic biopsies provide only mucosa for pathological evaluation. After treatment, this transmural pattern in Crohn's disease may be especially difficult to evaluate since medications may not affect the inflammatory process in a consistent or uniform fashion. Even with ulcerative colitis, a process thought to demonstrate a more continuous and mucosally based pattern of inflammation, variability in the histopathological severity within the colonic mucosa occurs. More precise studies are still needed that define the

mucosal response to different forms of injury and the healing response to different forms of treatment.

OTHER IMAGING METHODS

Invasive imaging studies, particularly repeated endoscopic studies, are normally not appealing to patients, and potentially, although rare, can still result in a procedure-related complication. Indeed, complications in patients with active inflammatory disease may exceed reported rates in otherwise healthy individuals undergoing screening procedures, and have been studied or reported poorly, particularly from treatment trials of new agents. Other less invasive approaches have often also been used in clinical practice, especially for repeated evaluations to assess the effects of therapy. These include imaging methods, such as computerized tomography (CT) and magnetic resonance imaging (MRI), usually with complete enterography. As with older barium imaging, however, there may be some inherent limitations. For example, these more modern imaging methods still have difficulty differentiating the inflammatory component of an intestinal stricture from its more established fibrotic component. CT may correlate with endoscopic evaluation for detection of ileal disease, but substantially increased radiation exposure results with repeated studies[20,21]. While both CT and MRI have limitations, multi-detector spiral CT enteroclysis may be more sensitive than MR enteroclysis for suspected bowel disease. In contrast, pelvic MRI has emerged as a standard for evaluation of perianal inflammatory disease or sepsis, particularly for fistula assessment and treatment[22]. Further correlation of these imaging modalities with other measures of intestinal healing are still needed.

OTHER NONINVASIVE METHODS

A number of surrogate markers have been promoted, including leukocytosis, thrombocytosis and C-reactive protein levels[23,24], but these are more clearly systemic rather than intestinal markers of the inflammatory process. Some of these markers also have been correlated with other indices. Other luminal markers, such as fecal lactoferrin or calprotectin[25], along with functional permeability measurements are available, and may provide a potentially important option for evaluation of healing, but need further evaluation.

TREATMENT ASSESSMENT

Placebo response and remission

In patients with inflammatory bowel disease, spontaneous clinical improvement or remission without treatment may occur. As a result, randomized placebo-controlled trials are done to determine if the investigative agent is superior to placebo treatment. Both patient and investigator are blinded to obviate bias. Placebo-based trials usually produce a positive effect even with placebo, in part, because of repetitive attention provided by caregivers to the trial subject. The placebo response is known to be powerful and, in a meta-analysis of placebo rates for inflammatory bowel disease clinical trials, rates up to 40% have been noted[26]. A superimposed issue in a clinical trial is the need to provide a proven form of therapy (while also testing the trial treatment). As a result, the placebo may, by necessity, be a standard therapy, not an inert treatment, while the treatment may include the standard therapy plus the trial treatment. For some medications, it may be difficult to hide the treatment because of known systemic effects (e.g. sulphasalazine or steroids). As noted elsewhere[26], placebo remission rates may also be influenced by trial length, number of study visits, use of strict remission definitions and enrollment favoring patients with more active disease.

Historical steroid studies

Early clinical trials with steroids have noted reduced clinical symptoms and improved appearances of the colonic mucosa[27,28]. Later trials with steroids have shifted the emphasis to the persistence of inflammatory changes, even though reduced symptoms were evident[29,30]. Unfortunately, the longer term role, if any, of steroids in mucosal healing and curbing the inflammatory process is understood poorly. In clinical practice, physicians limit the duration and dosage of systemic corticosteroids and taper these rapidly within weeks. This may not permit sufficient time for steroids to cause complete restitution of the mucosal surface. In a pooled treatment analysis of a first-pass metabolized steroid, budesonide, mucosal healing was reported to be limited in Crohn's disease after 1 year[31]. Budesonide, however, differs substantially in its chemical structure, metabolism and other properties from other steroids, therefore, generalization to other steroids may be premature. Some have hypothesized that steroids *per se* might be potentially deleterious to the mucosal healing process[32], but there is no evidence to support this view. It is possible that the observed healing effects of steroids only reflect the clinical tendency to minimize duration and dosage of systemic steroids because of fear of potential side effects.

Studies with other agents

Other agents used to treat inflammatory bowel disease, recently summarized in detail elsewhere for ulcerative colitis[33], also have been reported to cause endoscopic mucosal healing. These include 5-aminosalicylates, including a modernized formulation MMX mesalamine[34,35], immunosuppressant agents in Crohn's disease, such as azathioprine and methotrexate[36-40], antibiotics[41,42], and even prolonged courses of anti-mycobacterial treatment in Crohn's disease[43]. Similarly, biological agents are now being evaluated and mucosal healing has been reported as an important endpoint of treatment in the clinical trials[44-46]. Most of these studies, along with initial reports of other biological agents, have been conducted over only limited time frames, relative to the

natural duration of the disease, so positive and negative effects over the long term are not evident. In a recent report from a cohort in a treatment trial that has compared infliximab and azathioprine to conventional therapy with steroids, complete mucosal healing, defined as a simple endoscopic score[12] of 0 after 2 years of treatment predicted a sustained remission 3 and 4 years after therapy in > 70% of patients, compared to almost 30% of those with endoscopic lesions[47]. Of note, the authors also have concluded that achieving mucosal healing (defined by endoscopy) was the sole determining predicting factor and not the treatment *per se*.

FUTURE DIRECTIONS

A number of issues need to be addressed carefully in the near future. Therapeutic trials of differing pharmacological and biological agents in inflammatory bowel disease have shown that mucosal healing may occur with most of the traditional drugs, as well as the emerging biological agents, to a greater or lesser degree, but correlation with the patient's symptoms or other measures of disease activity appear to be limited. The current technology to assess mucosal healing in clinical trials and clinical practice remains limited, tends to be observational, and is not ideal because it does not evaluate transmural inflammation precisely, only the luminal surface mucosa. Repeated invasive endoscopic evaluations may not be optimal, particularly since these are largely one-dimensional. Possibly, this will be improved with the future evolution of confocal endoscopy. The inflammatory process is not a static target and the measured impact of one or the other agent may reflect, in part, this fluidity of the inflammatory process *per se*. As a result, assessing the longer-term effects of old and emerging agents is needed urgently, but may also prove to be particularly challenging. Genome-wide expression differences have been defined using endoscopic pinch biopsies in both ulcerative colitis and Crohn's disease[48]. These ultimately may provide a means for selecting individuals with either ulcerative colitis or Crohn's disease that might be managed optimally with a specific therapy, because multiple genes appear to be involved[49]. New studies have appeared employing microarray technology in animal and human colitis, which have increased our understanding of the basic inflammatory process, along with possible mediators that might be regulated[50-53]. Indeed, very recent genome-wide association studies in ulcerative colitis have identified new susceptibility loci that suggest that changes in the integrity of the mucosal barrier are important in pathogenesis[54]. By recognizing the limitations of current methodology used in clinical trials to assess mucosal healing, the modern day clinician will still have to rely on his or her clinical evaluation and best judgment whenever a new treatment paradigm is contemplated, or a change or cessation in therapy is indicated. Fortunately, however, emerging gene-based technology is likely to lead to better end points for more precise assessment of available treatments.

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ORIGINAL ARTICLE

Loss of CD103$^+$ intestinal dendritic cells during colonic inflammation

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Abstract

AIM: To investigate possible differences in dendritic cells (DC) within intestinal tissue of mice before and after induction of colitis.

METHODS: Mucosal DC derived from intestinal tissue, as well as from mesenteric lymph nodes and spleen, were analyzed by fluorescence activated cell sorting (FACS) analysis. Supernatants of these cells were analyzed for secretion of different pro- and anti-inflammatory cytokines. Immunohistochemistry and immunofluorescence were performed on cryosections of mucosal tissue derived from animals with colitis as well as from healthy mice.

RESULTS: It was shown that DC derived from healthy intestinal lamina propria (LP) represented an immature phenotype as characterized by low-level expression of costimulatory cytokines. In contrast to DC from spleen and mesenteric lymph nodes (MLN) that secreted proinflammatory cytokines, LP-DC produced high levels of the anti-inflammatory cytokine IL-10. After induction of murine colitis in a CD4$^+$CD62L$^+$ transfer model or in chronic Dextran sulfate sodium-colitis, a marked increase of activated CD80$^+$ DC could be observed within the inflamed colonic tissue. Interestingly, in contrast to splenic DC, a significant population of DC within MLN and colonic LP expressed the mucosal integrin CD103 which was lost during colitis.

CONCLUSION: The constitutive secretion of anti-inflammatory cytokines by immature DC within the intestinal LP might regulate the homeostatic balance between mucosal immunity and tolerance. CD103$^+$ DC could mediate this important function.

Key words: Dendritic cell; Colitis; Cytokines; Integrin; CD103

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INTRODUCTION

The intestinal mucosa is continuously challenged by innocuous antigens and potentially harmful pathogens. Therefore, the local immune system has to mount an efficient response towards pathogenic bacteria but must keep the immunological balance during exposure to commensal antigens. Dendritic cells (DC) are most likely

involved within this dual functionality. However, so far only limited data are available regarding intestinal DC (reviewed in[1,2]). Mucosal DC are not only found within Peyer's patches (PP) and mesenteric lymph nodes (MLN) but are also located within smaller isolated lymphoid follicles and within the lamina propria, distributed throughout the wall of the small and large intestine[3-5]. Unusual phenotypic subsets of DC have been described within MLN and PP[6-9] that preferentially stimulate antigen-specific $CD4^+$ T cells to produce IL-10 and/or TGF-β_1[9,10]. This cytokine pattern is similar to that of TR_1 or TH_3 regulatory T cells which have been identified in gut-associated lymphoid tissue of mice fed tolerogenic doses of proteins, and are thought to play an important role in oral tolerance[11].

Considerably more is known about DC in PP and MLN than about DC within the lamina propria of the gut. However, these cells are ideally situated to pick up any material that is transported between or through epithelial cells and have been shown to sample luminal antigens directly by sending dendrites outside the epithelium[12]. It is thought that DC as mobile cells migrate to MLN after antigen-uptake and interact with naïve T cells mainly within lymphatic organs, rather than in the mucosa itself. This rapid and constitutive trafficking of DC from lamina propria to MLN is increased by the presence of inflammatory stimuli[13]. As shown recently, interaction of mucosal DC with T cells generates gut-tropic $CD8^+$ effector T cells that express CCR9 and $\alpha_4\beta_7$[14]. Additionally, DC expressing the mucosal integrin CD103 promote the development of regulatory Foxp3+ T cells through a TGF-β and retinoic acid-dependent mechanism[15]. Together, these findings indicate that lamina propria DC (LP-DC) might be more important for the surveillance of the intestinal milieu and the shaping of intestinal immune responses than previously thought.

Animal model systems of colitis have been used extensively in an effort to determine possible mechanisms that contribute to the initiation and perpetuation of colitis in humans[16]. One model in particular which has been well characterized involves the transfer of $CD4^+CD45RB^{hi}$ T cells. Here, transfer of naïve ($CD4^+CD45RB^{hi}$ or $CD4^+CD62L^+$) T cells into immunodeficient SCID mice induces chronic intestinal inflammation[17,18]. Key factors that drive the pathogenic TH_1-biased mucosal T cell responses within this model are still unknown[19]. However, as described for various other experimental colitis models[20-25], antigen presentation of bacterial antigens plays a role, as only mild colitis develops when lymphocytes are transferred into animals with a restricted enteric flora and no intestinal inflammation is observed after transfer into germ-free mice[26]. Additionally, intestinal bacterial antigens and their presentation were shown to be crucial for the generation and expansion of regulatory T cells in a healthy individual[18], and disruption of the interaction of mucosal DC with activated T lymphocytes by administration of a receptor-blocking antibody against OX40L was shown to ameliorate colitis[27]. Overall, the data indicate that antigen presentation, presumably *via* mucosal DC, plays a role in the pathogenesis of chronic intestinal inflammation. However, the properties of these cells during intestinal inflammation are only beginning to be explored.

The aim of our study was to investigate differences between intestinal DC populations under healthy conditions and after induction of colitis.

MATERIALS AND METHODS

Mice

Balb/c mice and *scid* mice (C.B.-17 SCID) ($H2^d$) were obtained from Charles River (Germany). Animals were housed under conventional animal facility conditions and were generally used at 6-8 wk of age weighing 20-22 g. The animal studies were approved by the local institutional Review Board.

Monoclonal antibodies

The following experimental monoclonal antibodies (mAbs) were purchased from BD Pharmingen (Heidelberg, Germany): anti-CD8, anti-MHC-Ⅱ, anti-B220, anti-CD11b, anti-CD3, anti-CD11c, anti-CD80, anti-CD86, anti-CD40, anti-CD103 ($\alpha_E\beta_7$), anti-CD16/CD32. Directly PE- or FITC-conjugated mAbs were used for FACS analysis. For immunofluorescent staining directly FITC-conjugated anti-CD103 mAb was used in addition to Alexa 546-conjugated tyramide (Invitrogen, Germany).

Isolation of primary dendritic cells

DC were isolated from spleen, MLN and intestinal lamina propria by enzymatic digestion of tissue using collagenase I (Worthington, UK), hyaluronidase and DNase I (both from Sigma-Aldrich, Germany) followed by immunomagnetic selection with anti-CD11c coated microbeads (Miltenyi Biotech, Germany). For intestinal DC, mononuclear lamina propria cells were isolated from digested tissue as described previously[28], followed by enrichment of $CD11c^+$ DC using microbeads. Purity was generally > 85%. Routinely, isolated cells were stained for contaminating T cells and B cells, however virtually no cells could be detected by FACS analysis in the DC preparations.

Colitis models

We adapted a previously described transfer model that resembles the $CD4^+CD45RB^{high}$ model and uses the expression of L-selectin (CD62L) to select for naïve splenic T lymphocytes[18]. Briefly, $CD4^+$ T cells were purified from spleen mononuclear cells of healthy mice by negative depletion of other cell types using anti-CD8, anti-MHC-Ⅱ, anti-B220 and anti-CD11b mAbs and anti-rat-IgG immunomagnetic microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). The resulting $CD4^+$ lymphocytes were separated further into $CD62L^+$ and $CD62L^-$ T cells by CD62L-conjugated microbeads (Miltenyi Biotech). Recipient SCID mice were reconstituted with 0.25×10^6 $CD4^+CD62L^+$ lymphocytes in 200 μL of sterile PBS by intraperitoneal injection. Colitis activity was monitored by changes in weight over time and by histological analysis.

For chronic DSS-colitis, dextran sodium sulfate

(DSS; MW 40 000) was purchased from ICN (Eschwege, Germany) and intestinal inflammation was induced by feeding 3% DSS over 7 d followed by a period of 10 d of water without DSS. Mice received 4 cycles of DSS treatment and animals were sacrificed on day 8 after completion of the 4th cycle[29].

Cytokine ELISA

Dendritic cells from different organs were isolated as described above and 2×10^5 cells/well were incubated in 200 μL complete medium (RPMI-1640, 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, all from GIBCP-BRL, Eggenstein, Germany; and 3×10^{-5} mol/L β-mercaptoethanol, Sigma) for 24 h. Cells were partly stimulated with 5 μg/mL CpG-ODN (Metabion, Martinsried, Germany) or with 1 μg/mL *Salmonella typhimurium*-derived lipopolysaccharide (LPS) (Sigma, Deisenhofen, Germany). Cytokine levels were measured in the supernatant by ELISA (all from Endogene, Woburn, MA, USA), according to the manufacturer's instructions.

FACS analysis

Samples were analyzed using two-color staining as described previously[18]. Briefly, isolated DC were preincubated with 20 μg/mL of anti-CD16/CD32 and 10% FCS to block Fc-Receptors and stained with both FITC- and PE-conjugated mAbs. The cells were washed and analyzed by FACS using an EPICS-XL MCL Coulter.

Immunohistochemistry and immunofluorescence

Tissue samples were snap-frozen in liquid nitrogen, embedded in OCT resin and 5 to 10-μm cryostat-sections cut. For immunohistochemistry, primary antibody application was followed by biotinylated polyclonal anti-rat IgG or anti-hamster IgG (both Dianova, Germany) as secondary antibody. Tissue was stained using the ABC (avidin/biotin complex)-immunoperoxidase kit according to the manufacturer's instructions (Vector Laboratories) and developed with AEC. Sections were counterstained with hematoxylin. For immunofluorescence, sections were incubated with APC-conjugated anti-CD11c and with FITC-labeled anti-CD103 mAbs. The anti-CD11c mAb was visualized by applying horseradish peroxidase-labeled streptavidin followed by Alexa 546-conjugated tyramide, according to the manufacturer's recommendations (Invitrogen, Germany). Slides were counterstained with DAPI.

Statistical analysis

Statistical analysis was performed using the two-tailed Mann-Whitney *U* test. Differences were considered statistically significant when $P < 0.05$.

RESULTS

DC within the healthy intestinal lamina propria show an immature phenotype and produce constitutively the anti-inflammatory cytokine IL-10

$CD11c^+$ DC are found within the lamina propria (LP) of the small and large intestine of healthy mice. Whereas a dense network of cells underlining the epithelium can be detected by immunohistochemistry within the mucosa of the small intestine, only a few scattered cells are found within the colonic LP. To compare the phenotype of DC derived from colonic LP and mesenteric lymph nodes (MLN), $CD11c^+$ DC were isolated from intestinal tissue and MLN of healthy mice. As demonstrated in Figure 1A, no significant levels of costimulatory molecules (CD40, CD80 and CD86) could be detected on the cell surface of freshly isolated DC from the intestinal LP or MLN. This suggests that DC within mucosal tissues demonstrate a rather immature phenotype as compared to splenic DC which show low levels of costimulatory molecules (data not shown). Additionally, isolated primary DC were incubated either unstimulated or in the presence of CpG and secretion of cytokines was detected by ELISA. As shown, MLN-DC and splenic DC differed markedly from LP-DC with regard to their cytokine profile. Unstimulated MLN-DC or splenic DC did not secrete significant amounts of pro- or anti-inflammatory cytokines, however, LP-DC dramatically produced 30-fold higher levels of the anti-inflammatory cytokine IL-10 (MLN-DC: 35.4 ± 5.0; splenic-DC: 12.8 ± 0.7, LP-DC: 1035 ± 270 pg/mL, $P = 0.0235$, Figure 1B). In contrast, large amounts of IFN-γ and IL-12 were secreted by MLN-DC and splenic-DC after stimulation with CpG, whereas LP-DC did not produce significant amounts of proinflammatory cytokines (MLN-DC: 1398 ± 407, splenic-DC: 1087 ± 30, LP-DC: 24 ± 11 pg/mL, $P = 0.0009$). The differences seen were independent from the stimulatory agent used as similar results were detected by using LPS to stimulate primary DC.

Diffuse infiltration of intestinal lamina propria with $CD11c^+$ DC during colitis

As shown in Figure 2A, $CD11c^+$ DC were diffusely distributed throughout the non-inflamed colonic LP and were rarely detected in the submucosa. Chronic colitis was either induced by adoptive transfer of splenic $CD4^+CD62L^+$ T lymphocytes from donor mice into immunodeficient SCID recipients or by cyclic administration of DSS in the drinking water of animals[18,29]. As analyzed by immunohistochemistry using an anti-CD11c antibody in both models of colitis, a dramatic increase in numbers of $CD11c^+$ DC could be detected within the inflamed mucosa of the colon of mice (Figure 2B). MLN of mice with colitis showed a slight increase in the number of DC (Figure 2C and D), whereas no changes in infiltrating DC were seen in Peyer's patches and the spleen (data not shown).

Infiltrating LP-DC in inflamed colonic tissue show a mature phenotype with high expression of CD80 cells and secretion of the regulatory cytokine IFN-α is decreased

As shown above, mucosal DC in healthy tissue are immature. To investigate whether infiltrating $CD11c^+$ DC within the inflamed colon show an activated phenotype,

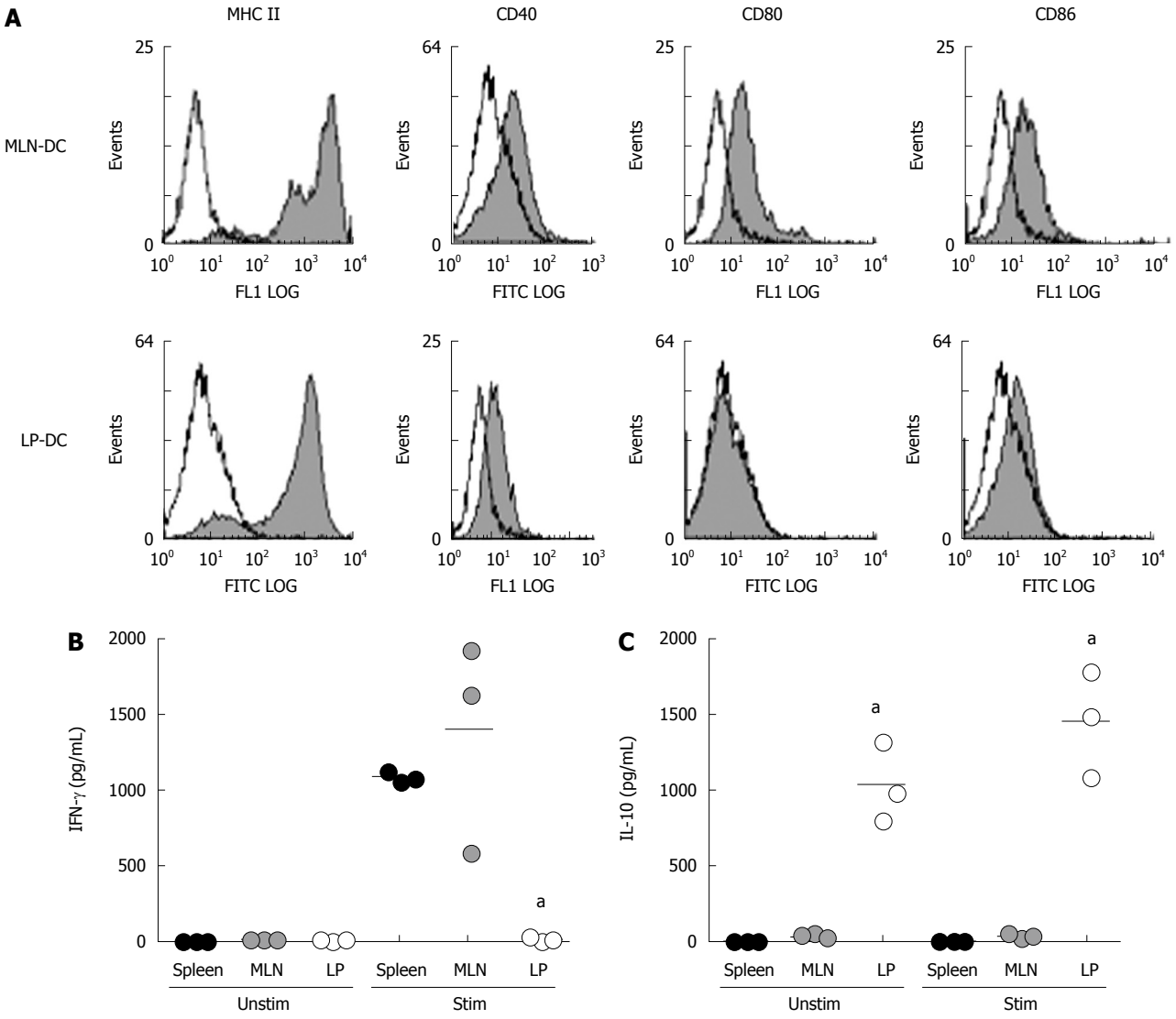

Figure 1 Phenotypic and functional analysis of DC derived from MLN and LP of healthy mice. A: Primary DC were isolated from MLN (MLN-DC) and colonic LP (LP-DC) of healthy mice. CD11c$^+$ DC were stained with FITC-conjugated mAbs for expression of MHC-class II and the costimulatory molecules CD40, CD80 and CD86; B: Isolated DC from spleen, MLN and LP were incubated for 24 h in complete medium partly stimulated with 5 μg/mL CpG (5 wells per situation). Cytokine levels were measured within the supernatant by ELISA. $^aP < 0.05$. Data presented are representative of three independent experiments.

tissue sections were stained for the expression of costimulatory molecules. As demonstrated in Figure 3A, we were able to detect dramatically increased numbers of CD80$^+$ DC within the colonic LP as compared to healthy mucosa. On the other hand, no significant numbers of CD40$^+$ and CD86$^+$ cells were detected. We confirmed the results by FACS analysis after isolation of primary DC from inflamed tissue, demonstrating that LP-DC indeed showed increased cell-surface levels of CD80, whereas no difference in expression of CD40 and CD86 could be observed (Figure 3B).

To investigate whether the cytokine profile of isolated DC from different tissues was changed in animals with colitis we measured the cytokine secretion of primary DC. Secretion of IL-10 by LP-DC was reduced to only 27% of the amount secreted by LP-DC from healthy intestine (colitic LP-DC: 277 ± 27, healthy LP-DC: 1035 ± 270 pg/mL) (data not shown). Additionally, whereas LP-DC from normal colon did not secrete any proinflammatory cytokines as demonstrated above, DC from inflamed intestine were able to produce significant amounts of IFN-γ (397 ± 163 pg/mL) and TNF-α (650 ± 91 pg/mL) (data not shown). Furthermore, IFN-α, a cytokine attributed to plasmocytoid DC with regulatory function, was secreted to a greater extent by LP-DC from healthy mice as compared to splenic or MLN-DC which produced distinctly lower amounts of this cytokines (LP-DC: 143 ± 12, MLN-DC: 71 ± 2, splenic-DC: 67 ± 2 pg/mL, $P = 0.0242$). However, during colitis, production of IFN-α by LP-DC was significantly reduced to 19.6% (Figure 3C, colitic LP-DC: 28 ± 4 pg/mL, $P = 0.0111$).

Numbers of CD103+ intestinal DC are dramatically reduced in colitic mice

The mucosal integrin $\alpha_E\beta_7$ (CD103) is expressed on the cell surface of intraepithelial lymphocytes and mediates

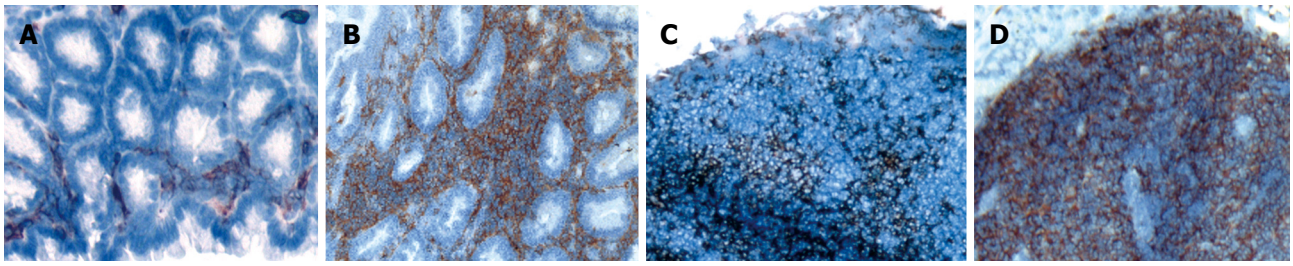

Figure 2 Inflammation induces infiltration of colonic LP and MLN with CD11c$^+$ DC. Tissue was harvested from colonic LP (A/B) and MLN (C/D) of healthy animals (A/C) and mice with colitis (B/D). Immunohistochemistry was performed using an anti-CD11c$^+$ antibody to stain for intestinal DC. Staining with isotype control revealed no background staining (data not shown). Representative sections from 5 mice per group are shown (magnification 100 ×). Staining was performed on tissue derived from colitic animals with transfer colitis as well as chronic DSS colitis and revealed similar results. Sections shown within this figure are derived from animals with transfer colitis.

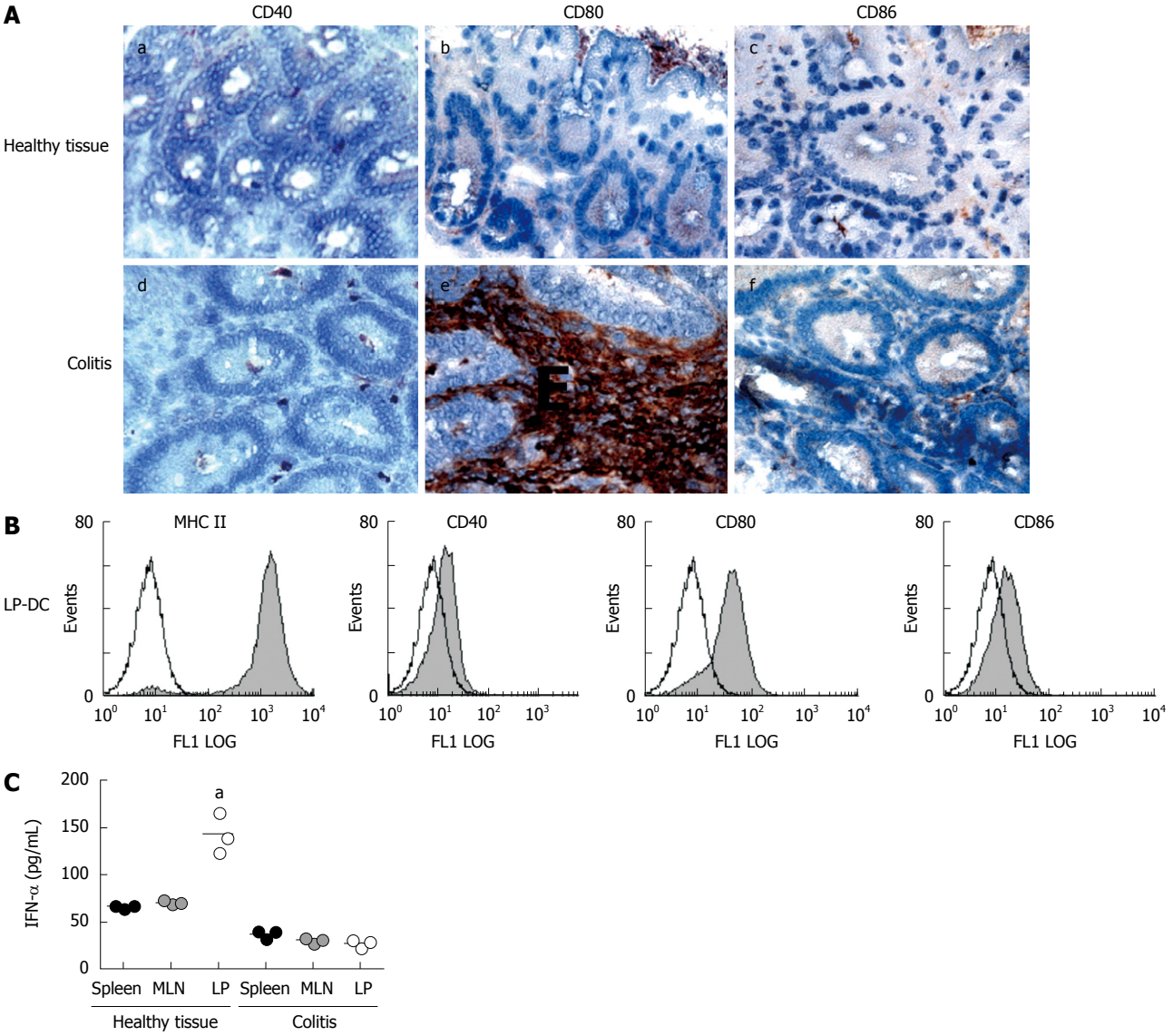

Figure 3 Intestinal DC are activated after induction of colitis. A: Tissue was harvested from LP of healthy animals and from mice with colitis. Immunohistochemistry was performed with antibodies against the following costimulatory molecules: CD40 (a/d), CD80 (b/e), CD86 (c/f). Representative sections are shown (magnification 100 ×). Experiments were performed from 5 animals each group. Sections shown within this figure are derived from animals with transfer colitis, however staining was also performed on tissues derived from colitic animals with chronic DSS colitis and revealed similar results; B: LP-DC were isolated from inflamed intestinal tissue and FACS analysis was performed. CD11c$^+$ DC were stained with FITC-conjugated mAbs for expression of MHC-class II and the costimulatory molecules CD40, CD80 and CD86. Data presented are representative of three independent experiments performed with cells derived from animals with transfer colitis. Similar results were generated in the DSS colitis model; C: Primary DC were isolated from different tissues (spleen, MLN, colonic LP) of healthy animals and mice with colitis. Isolated DC were incubated for 24 h in complete medium and levels of IFN-α were measured within the supernatants by ELISA (5 wells per situation). $^aP < 0.05$. Data presented are from one of three independent experiments performed with cells derived from animals with transfer colitis. Similar results were generated in the DSS colitis model.

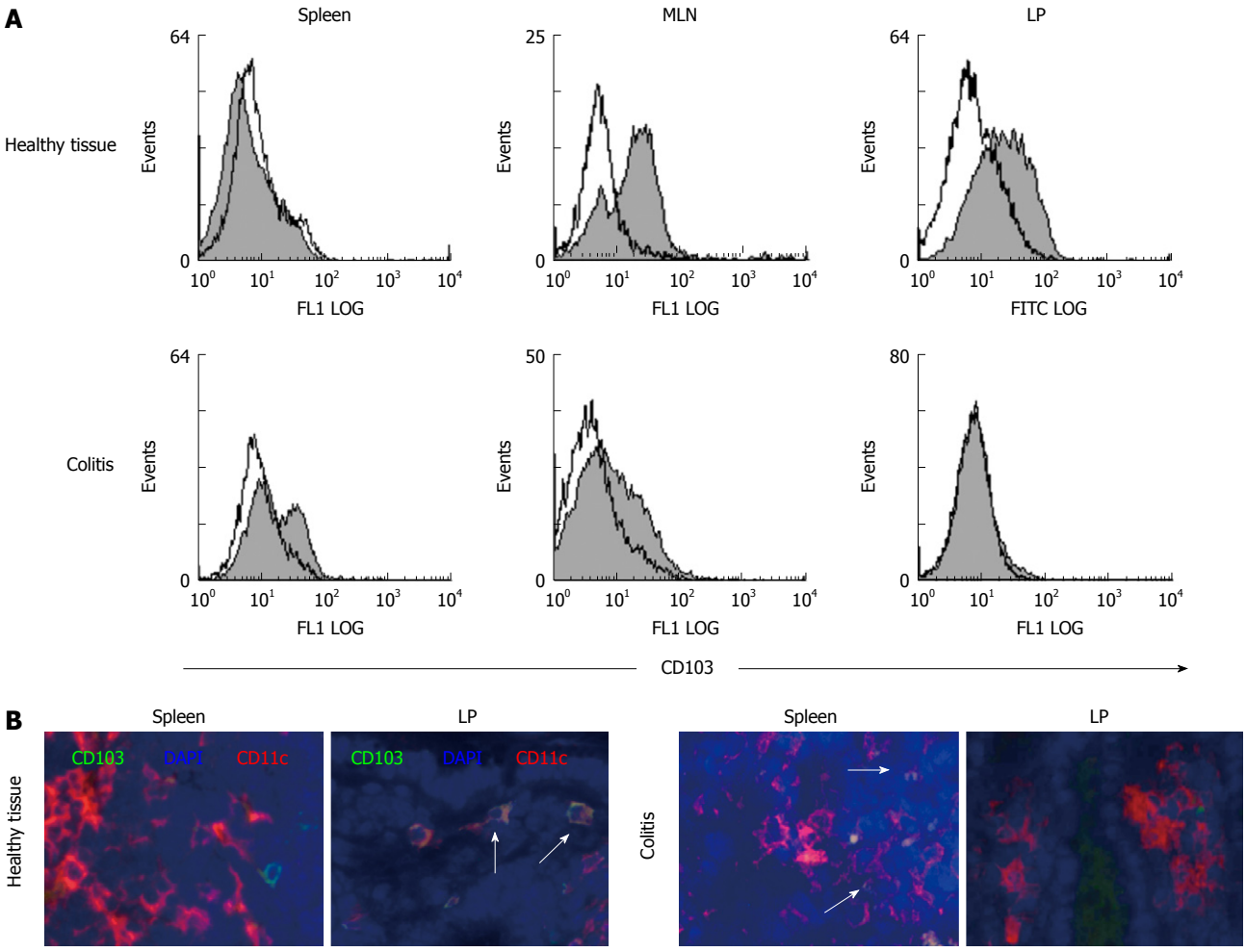

Figure 4 CD103$^+$ DC are found in mucosal tissues of healthy mice and are lost during colitis. A: Primary DC were isolated from different tissues (spleen, MLN, colonic LP) of healthy animals and mice with colitis. FACS analysis was performed after staining of CD11c$^+$ DC with FITC-conjugated mAb against CD103, the integrin $\alpha_E\beta_7$. Data presented are representative of three independent experiments that were carried out with cells derived from animals with transfer colitis. Similar results were generated in the DSS colitis model; B: Tissue was harvested from spleen and LP of healthy animals and mice with colitis. Immunofluorescence was performed using an anti-CD11c$^+$ mAb to stain for intestinal DC and an anti-CD103 mAb to recognize the integrin $\alpha_E\beta_7$. DAPI was used to visualize nuclei. Images show overlays of CD103 (green), CD11c (red) and DAPI (blue). DC coexpressing CD103 appear yellow (indicated by arrows). Representative sections from 5 mice per group are shown (magnification 100 ×). Staining was performed on tissue derived from colitic animals with transfer colitis as well as chronic DSS colitis and revealed similar results. Sections shown within this figure are derived from animals with transfer colitis.

adhesion to epithelial cells *via* binding to E-cadherin[30]. Recently, it was shown that expression of CD103 characterizes an important subset of regulatory T cells[31]. Additionally, CD103$^+$ mucosal DC were suggested to play an important role for the generation of Foxp3+ T lymphocytes within the gut[15]. As this integrin seems to play a role in regulatory immunological functions, especially in mucosal sites, we wanted to investigate whether CD103$^+$ intestinal DC change during intestinal inflammation. As demonstrated in Figure 4, freshly isolated DC from healthy spleen did not contain a significant population of CD103$^+$ DC. However, this was very different in mucosal sites of healthy animals. Here, a large subpopulation of MLN-DC expressed the integrin $\alpha_E\beta_7$ and even more strikingly, almost all LP-DC showed at least low levels of CD103 expression on the cell surface, demonstrating that LP-DC from healthy mucosa did not only differ dramatically in their cytokine secretion potential but also in their phenotype from DC at peripheral sites. However, when we looked for CD103 expression on DC isolated from animals with colitis we observed a dramatic loss of this population in mucosal tissues. Within the chronically inflamed colonic LP, no CD103$^+$ DC where seen as demonstrated by FACS analysis, and numbers of integrin-positive DC in MLN were dramatically reduced. In contrast, we detected a significant subpopulation of CD103$^+$ DC within the spleen of colitic animals (Figure 4).

DISCUSSION

Antigen-presenting cells are the key to maintaining the immunological balance between active immune responses and tolerance within the intestine and DC are most likely to participate importantly in this immunological homeostasis within the gut. However, data has recently started to be available regarding intestinal DC in normal and inflamed colon. Comparing DC populations from

different tissues we were able to demonstrate that mucosal DC represent an immature phenotype as characterized by the absence of CD40, CD80 and CD86 expression. Whereas CD80 was found in low levels on the cell surface of MLN-DC, the molecule was absent on LP-DC, indicating that LP-DC represent an even more immature phenotype than MLN-DC. It is thought that DC can be divided into tolerogenic immature and immunogenic mature differentiation stages[32], as tolerance is mediated by partial- or semi-maturated DC, whereas only full DC maturation is immunogenic[1,2,33]. Therefore, our observation of phenotypically immature (or semi-mature) LP-DC within the healthy gut supports the hypothesis that intestinal DC, which sample antigens without being fully activated, induce tolerance against antigens of the regular gut flora. Additionally, we were able to show that DC from spleen and MLN secrete proinflammatory cytokines such as IFN-γ and IL-12 in response to the different strong inflammatory stimuli, CpG and LPS. In contrast, intestinal-derived $CD11c^+$ DC constitutively produced high levels of the anti-inflammatory cytokine IL-10 and did not release significant amounts of proinflammatory mediators after stimulation. As shown previously, pulmonary DC - situated within a mucosa that is similar to the intestine exposed to antigens - produce IL-10 in response to inhalative antigens and induce the development of regulatory T cells[34]. Therefore, it can be speculated that constitutive production of the anti-inflammatory cytokine IL-10 by LP-DC is also critical for the generation of regulatory T cells and the maintenance of tolerance towards luminal antigens within the normal gut.

However, during intestinal inflammation the cellular composition within the colonic lamina propria changes. As shown, gut inflammation in different murine models of colitis was accompanied by a marked infiltration of the colonic mucosa by $CD11c^+$ DC. LP-DC derived from the inflamed mucosa expressed high levels of CD80, a cell surface molecule thought to be involved with induction of TH_1 responses[35,36], and cells resembled a phenotype of mature activated DC. Additionally, these cells produced dramatically lower levels of IL-10 and INF-α, cytokines necessary for anti-inflammatory responses[37]. Our observation is in concordance with a recent study that also demonstrated expansion of colonic LP-DC during murine colitis[5,38] and other data that showed up-regulated expression of activation markers on DC in diseased mucosal tissues from patients with inflammatory bowel disease[39]. It is likely that the infiltration of the intestinal mucosa with mature DC during intestinal inflammation leads to a continuous activation of T lymphocytes and a sustained overproduction of proinflammatory mediators within the lamina propria, which perpetuates colitis.

Additionally, we were able to show that LP-DC from normal colonic mucosa and MLN-DC contain a significant subpopulation of $CD103^+$ ($\alpha_E\beta_7$) DC, whereas splenic-DC were negative for the mucosal adhesion molecule. This observation suggests the localization of specific DC subpopulations within the intestinal lamina propria. So far, it is known that intraepithelial lymphocytes express the integrin $\alpha_E\beta_7$ which interacts with epithelial E-cadherin and is thought to mediate localization of T cells within the epithelial layer[30]. Additionally, it seems to characterize a specific subgroup of lymphocytes with regulatory function[31] and a recent study was able to show that $CD103^+$ DC promote expression of the gut-homing receptor CCR9 on T cells[14,40], as well as generation of Foxp3+ T lymphocytes with TGF-β and retinoic-acid as cofactors[15]. Because in our study almost all intestinal LP-DC within the healthy mucosa express this integrin and show a tolerogenic phenotype and function, we hypothesize that CD103 mediates homing for tolerogenic DC into the intestinal mucosa or enables as adhesion molecule the crosstalk with other lamina propria cells, thereby influencing the balance between effector and regulatory T cell activity in the intestine. As we were not able to identify the $CD103^+$ LP-DC during colitis when tolerance is lost, this subgroup of DC could help to maintain the immunological balance within the normal intestinal mucosa. Surprisingly, during inflammation, $CD103^+$ DC were found within the spleen, suggesting that intestinal $CD103^+$ DC might migrate to systemic lymphatic tissues.

Overall, our results indicate that the specific localization of particular $CD103^+$ DC subpopulations within the intestinal mucosa may be an important mechanism of the immune system to determine between active immune responses and tolerance towards luminal antigens. Additionally, the constitutive secretion of anti-inflammatory cytokines by intestinal DC might regulate the homeostatic balance under healthy conditions. After induction of colitis, loss of $CD103^+$ intestinal DC and infiltration of mature DC that express the costimulatory molecule CD80 within the colonic mucosa would lead to a dysregulation of this balance. Antigen presentation *via* activated DC could be involved in the onset or/and chronification of colitis. Interrupting the activation of intestinal DC *in vivo* and promoting the preservation of presumably tolerogenic $CD103^+$ DC within the colonic mucosa may be key approaches to control the pathogenesis of inflammatory bowel disease.

COMMENTS

Background

Within the gut mucosa the immune system has the task of distinguishing between commensal bacteria and foreign antigens, to maintain tolerance or to mount an inflammatory response. Dendritic cells are very important for this process. However, even if in the last few years some important insights have been made, much still is unknown about these cells, especially about the changes they undergo during intestinal inflammation.

Research frontiers

Previous data indicate that antigen presentation, presumably *via* mucosal dendritic cells, plays a role in the pathogenesis of chronic intestinal inflammation. However, the properties of these cells during intestinal inflammation are only beginning to be explored.

Innovations and breakthroughs

Dendritic cells in normal intestinal lamina propria showed an immature phenotype and produced high levels of the anti-inflammatory cytokine IL-10 whereas

dendritic cells in the spleen and local lymph nodes secreted the proinflammatory cytokine IFN-γ. Furthermore, the studies in mouse models of colitis showed that the development of colitis was associated with a marked increase of activated dendritic cells within the inflamed colonic tissue and the loss of $CD103^+$ dendritic cells in the colonic mucosa and local lymph nodes but not in spleen, suggesting that $CD103^+$ dendritic cells could play important roles in the regulation of homeostatic balance between mucosal immunity and tolerance in the gastrointestinal tract.

Applications

Overall, these results indicate that the specific localization of particular $CD103^+$ dendritic cell subpopulations within the intestinal mucosa may be an important mechanism of the immune system in determining between active immune response and tolerance towards luminal antigens. Additionally, the constitutive secretion of anti-inflammatory cytokines by intestinal DC might regulate the homeostatic balance under healthy conditions. Interrupting the activation of intestinal dendritic cells *in vivo* and promoting the preservation of presumably tolerogenic $CD103^+$ dendritic cells within the colonic mucosa may be key approaches to control the pathogenesis of inflammatory bowel disease.

Peer review

This is a well prepared manuscript and the experiments described were well designed, controlled and executed. The loss of $CD103^+$ $CD11c^+$ DCs in progression of colitis is a unique and novel finding which seems to coincide with other groups' findings relating to regulatory T cells. Overall the results could contribute to our understanding of the immunopathogenesis of human inflammatory bowel diseases.

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ORIGINAL ARTICLE

Antibiotics and probiotics in chronic pouchitis: A comparative proteomic approach

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Abstract

AIM: To profile protein expression in mucosal biopsies from patients with chronic refractory pouchitis following antibiotic or probiotic treatment, using a comparative proteomic approach.

METHODS: Two-dimensional polyacrylamide gel electrophoresis and matrix-assisted laser desorption/ionization-time of flight mass spectrometry were used to characterize the changes related to antibiotic therapy in the protein expression profiles of biopsy samples from patients with chronic refractory pouchitis. The same proteomic approach was applied to identify differentially expressed proteins in the non-inflamed pouch before and after probiotic administration.

RESULTS: In the first set of 2D gels, 26 different proteins with at least 2-fold changes in their expression levels between the pouchitis condition and antibiotic-induced remission were identified. In the second set of analysis, the comparison between mucosal biopsy proteomes in the normal and probiotic-treated pouch resulted in 17 significantly differently expressed proteins. Of these, 8 exhibited the same pattern of deregulation as in the pouchitis/pouch remission group.

CONCLUSION: For the first time, 2D protein maps of mucosal biopsies from patients with ileal pouch-anal anastomosis were provided, and differentially expressed proteins following antibiotic/probiotic treatment were identified.

Key words: Chronic disease; Pouchitis; Antibiotics; Probiotics; Proteins; Gene expression

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INTRODUCTION

Total proctocolectomy with ileal J-pouch-anal anastomosis (IPAA) is the surgical treatment of choice for patients with refractory ulcerative colitis (UC) or UC with dysplasia. Although the surgery generally cures UC and has been shown to result in a significant improvement of health-related quality of life, complications can occur after IPAA[1].

The most common long-term complication is pouchitis, an idiopathic inflammatory disease of the ileal reservoir. The reported incidence of pouchitis is variable, largely because of differences in the type and duration of follow-up. However, studies have shown that as many as 15%-46% of patients with UC develop at least 1 episode of pouchitis within 5 years after surgery[2].

Clinically, pouchitis is characterized by variable symptoms, including increased stool frequency and fluidity, abdominal cramping, pelvic discomfort, bleeding, tenesmus, fever and weight loss, and extra-intestinal manifestations in more severe cases[3]. For an unequivocal diagnosis, endoscopic examination and histologic investigation are mandatory[4]. Pouchitis Disease Activity Index (PDAI) is the most commonly used diagnostic instrument and represents an objective and reproducible scoring system for pouchitis[5]. Active pouchitis is defined as a score $\geqslant 7$ and remission is defined as a score < 7.

The etiology and pathophysiology of pouchitis are still poorly understood. However, the fact that pouchitis almost exclusively occurs in patients with underlying UC and that it generally responds to antibacterial therapy suggests a role for the gut microbiota and a genetic predisposition[6].

The disease activity of pouchitis can be defined as remission, mild-moderate or severe based primarily on symptoms. Duration can be classified as acute (< 4 wk) or chronic ($\geqslant 4$ wk). Disease pattern can be infrequent (1-2 acute episodes), relapsing ($\geqslant 3$ acute episodes) or chronic (a treatment-responsive form requiring maintenance therapy or a treatment-resistant form). Approximately 10%-15% of patients with pouchitis experience a chronic pouchitis, either treatment-responsive or treatment-refractory, and some of them require surgical excision or exclusion of the pouch because of impairment of reservoir function and poor quality of life[7].

Treatment of pouchitis is largely empirical. Broad-spectrum antibiotics have been widely used and represent the mainstay of treatment. Small randomized trials have shown that both metronidazole and ciprofloxacin, alone, sequentially or in combination, are effective in reducing the PDAI score and achieving a significant improvement in clinical symptoms and endoscopic and histologic findings. However, metronidazole is poorly tolerated and treatment with systemically active antibiotics is not ideal from the perspective of the development of antibiotic resistance. In addition, in chronic pouchitis antibiotic-induced remission periods are often short and the condition is complicated by frequent relapses[8].

Recently, several studies have suggested that altering the microbiota in the pouch by administering probiotic bacteria can be effective in maintaining remission and reducing the incidence of flare-ups in chronic pouchitis[9,10]. Moreover, the efficacy of probiotic therapy as prophylaxis to delay the first onset of pouchitis after pouch surgery, has been demonstrated[11,12].

Comparative proteomic analysis represents an effective tool to identify proteins critical for functional pathways in normal cells and phenotype changes that occur during disease development. Since biological and functional output of cells is governed primarily by proteins, the applications of proteomic technologies are beginning to have a profound impact on understanding of the molecular mechanisms underlying several disease processes, which, in turn, will help to reduce disease-related morbidity and mortality. However, despite their extensive use in proteomic profiling of gene expression in various diseases, the applications of such technologies in inflammatory bowel diseases are still in their infancy[13] and, so far, no proteomic study has been reported in IPAA research.

In the present study, we apply 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) to define the differential protein displays of mucosal biopsy samples from patients with chronic refractory pouchitis before and after antibiotic treatment. The same proteomic approach has also been applied to identify specific changes in protein expression in the non-inflamed *vs* probiotic-administered pouch in order to provide a picture of the intestinal mucosa protein modulation by probiotics.

MATERIALS AND METHODS

Patients and biopsy collection

Six patients who underwent restorative proctocolectomy with IPAA were recruited for this study and routinely followed up by the Department of Internal Medicine and Gastroenterology, University of Bologna, Polyclinic S. Orsola. Patients were included if they had a chronic refractory pouchitis, defined as no response to at least 4 wk of standard antibiotic therapies (ciprofloxacin 1 g twice daily (*bid*) or metronidazole 400 mg 3 times daily). They were divided in 2 groups according to PDAI score at study entry and treatment received. In the first group, 3 patients with PDAI $\geqslant 7$ were orally administered with a combination of metronidazole (500 mg *bid*) and ciprofloxacin (500 mg *bid*) for 1 mo. The second group, including the other 3 patients with chronic refractory pouchitis but with a total PDAI < 7 at study entry, received VSL#3 (VSL pharmaceuticals Inc., Ft. Lauderdale, FL, USA) 2 packets *bid* for 3 mo. VSL#3 contains 450 billion viable lyophilized bacteria per packet, comprised of 4 strains of lactobacilli (*Lactobacillus acidophilus*, *L. casei*, *L. delbrueckii* subsp. *bulgaricus* and *L. plantarum*), 3 strains of bifidobacteria (*Bifidobacterium breve*, *B. infantis* and *B. longum*) and one strain of *Streptococcus thermophilus*. Mucosal biopsies were collected during pouch endoscopy before and after antibiotic/probiotic therapy.

All samples were immediately snap frozen in liquid nitrogen. The institutional ethics committee approved all protocols and all enrolled subjects gave their informed consent.

Protein extraction

Frozen mucosal biopsies (about 10-20 mg) were washed in 200 μL of cold low salt washing buffer (3 mmol/L

KCl, 1.5 mmol/L KH_2PO_4, 68 mmol/L NaCl, 9 mmol/L NaH_2PO_4), with Complete Protease Inhibitor (Roche Molecular Biochemicals, Mannheim, Germany). After centrifugation at 13000 r/min for 2 min, tissue samples were homogenized in 1 mL of lysis solution (0.11 mol/L DTT, 0.11 mol/L CHAPS, 8 mol/L urea, 2 mol/L thiourea, 35 mmol/L Tris and Complete Protease Inhibitor) using an Ultra-Turrax® homogenizer (IKA Labortechnik, Staufen, Germany). Protein extraction was performed as previously described[14]. Total protein concentration of the cell extract was calculated using the PlusOne 2D Quant Kit™ (GE Healthcare, Uppsala, Sweden). The protein extract preparation was immediately used or aliquoted and frozen at -20℃.

2D-PAGE

Samples containing 100 μg of protein were diluted to 250 μL with rehydration solution (8 mol/L urea, 2% CHAPS, 10 mmol/L DTT, 2% (v/v) ampholine, pH 3.5-9.5 (GE Healthcare) and trace bromophenol blue) and applied to Immobiline DryStrips (13 cm, pH 3-10, GE Healthcare) for 12 h rehydration at 50 V. Isoelectric focusing was performed using IPGphor apparatus (GE Healthcare) to give a total of 19 kVh. IPG strips were then reduced and alkylated[15] prior to loading onto 15% acrylamide separating gels (20 cm long, 1 mm thickness). Electrophoresis was performed at 250 V for 7 h using Protean Ⅱ xi Cell (Bio-Rad, Hercules, CA, USA). Protein spots were visualized with a MS-compatible silver-staining procedure[16].

Image analysis

Protein patterns in the gels were recorded as digitalized images using a GS-800 imaging densitometer (Bio-Rad). Spot detection, matching and the examination of differentially expressed proteins were performed by PDQuest v6.2 software (Bio-Rad). Three technical replicates were made per patient and condition and formed 1 replicate group with average normalized spot intensities. The comparison was carried out for each patient before and after antibiotic/probiotic therapy. Proteins that showed at least 2 times enhanced/decreased expression were selected for identification along with a few spots that showed a similar expression pattern in all 2D gels.

Protein identification

Protein spots with conserved expression levels throughout the gels in all patients and conditions were identified. Two identification methods were employed: comparison of our reference proteome map with Swiss-2D PAGE (http://www.expasy.ch/ch2d/) and other published 2D proteome patterns[17-21] obtained under very similar experimental conditions, and MALDI-TOF MS analysis. Since both methods provided the same identification result for each spot, we used the gel matching method to identify the differentially expressed proteins in pouchitis/antibiotic-induced remission and normal pouch/probiotic-treated pouch groups. When gel matching produced an unreliable and doubtful identification, because of excessive deviations in p*I* and M_r values across gels, MALDI-TOF MS was employed.

Protein spots were manually excised from 2D gels, washed and in-gel digested as previously reported[22]. Crude digests were concentrated and desalted using mC18 ZipTips (Millipore, Bedford, MA, USA). Peptide extracts were mixed on the MALDI-TOF target (Applied Biosystems, Foster City, CA, USA) with an equal matrix volume of 5 mg/mL α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich, St. Louis, MO, USA) saturated with 50% acetonitrile/0.2% trifluoroacetic acid, and analyzed using a Voyager-DE Pro Biospectrometry Workstation (Applied Biosystems). All mass spectra were obtained in a reflectron mode, with an accelerating voltage of 20 kV and a delayed extraction of 40 ns. Internal mass calibration with peptides arising from trypsin autoproteolysis was performed. Peptide masses were searched against Swiss-Prot, TrEMBL and NCBI non-redundant protein databases using ProFound (http://prowl.rockefeller.edu/prowl-cgi/profound.exe) and Aldente (http://expasy.org/tools/aldente) programs. Search parameters were set to allow up to one missed tryptic cleavage and a peptide mass tolerance of 50 ppm. Only protein hits with a significant probability score calculated by software and at least 3 matching peptide masses were considered.

Statistical analysis

Statistical analysis of protein expression was performed using the Student's *t*-test carried out with SigmaStat v3.5 software (Systat Software, Point Richmond, CA, USA). A P value < 0.05 was considered as statistically significant. Bibliometric analysis for co-citation was performed using Bibliosphere Pathway Edition from Genomatix (Genomatix Software, Munich, Germany).

RESULTS

Clinical outcome of antibiotic/probiotic treatment

All the enrolled subjects completed the study. In the first group of patients, after 1 mo of antibiotic therapy, clinical and endoscopic remission was achieved with a significant decrease in both PDAI and median stool frequency (data not shown). In the second group, no episodes of active pouchitis were recorded during the probiotic administration. Both treatments were well tolerated and no side effects were recorded.

Antibiotic administration-related effects on mucosal biopsy proteome in pouchitis

An example of 2D gels obtained from mucosal biopsies in pouchitis and pouch remission is provided in Figure 1A. Approximately 1200 protein spots per gel were detected within a p*I* range of 3-10 and a M_r range of 5-220 kDa. The resolution of the polypeptides showed better quality in the low molecular mass area and toward the acidic side of the gels whereas increased streaking and precipitation,

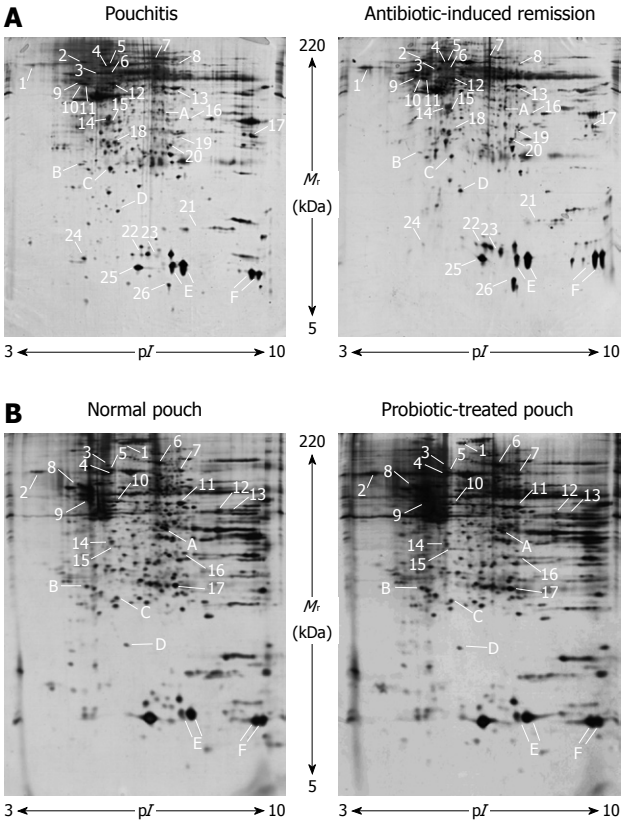


Figure 1 Representative 2D gel maps of the mucosal biopsy proteomes from a patient with chronic refractory pouchitis before (left) and after (right) antibiotic therapy (A) and from a subject with a non-inflamed pouch before (left) and after (right) probiotic administration (B). Proteins showing altered expression identified by gel matching and MALDI-TOF MS analysis are numbered and reported in Table 1. Identified spots with conserved expression levels in all patients and conditions are marked by letters and shown in Table 2.

a well known phenomenon observed in 2D-PAGE, were visible on the basic side.

For each patient, 2D patterns of mucosal biopsies collected before and after antibiotic administration were compared by PDQuest. Because of the high intrinsic variability among individuals, a stringent criterion was applied whereby only those proteins with at least 2 times increased or decreased expression and deregulation in the same way in all patients were considered. Out of 40 differentially expressed protein spots, 26 (65%) were identified, of which 15 were upregulated and 11 downregulated in antibiotic-induced remission of pouchitis (Figure 1A and Table 1). In addition, 6 protein spots with a similar expression pattern in all 2D gels were selected and identified (Figure 1 and Table 2).

The altered proteins were classified in terms of their subcellular location and biological function by information from Swiss-Prot, HPRD (Human Protein Reference Database, http://www.humanproteinpedia.org), and COGs (Cluster of Orthologous Groups of proteins, http://www.ncbi.nlm.nih.gov/COG/) (Figure 2A). The majority of the identified proteins were located in the cytoplasm (38%), mitochondria (27%) and endoplasmic reticulum (11%). Twenty-seven percent of the altered proteins play a key role in post-translational modifications and protein turnover as chaperones, 15% are involved in energy production and conversion, and 11% are related to lipid transport and metabolism.

The results of a histogram data analysis carried out on the spot quantity values determined by PDQuest are displayed in Figure 3 together with representative gel images for each protein spot in each patient and clinical condition. A statistically significant increased expression in pouch remission was detected for tubulin β-2C chain (TUBB), ATP synthase subunit β (ATP5B) and calponin-2 (CNN2) in all patients, whereas calreticulin (CALR), 60 kDa heat shock protein (HSP60), heat shock cognate 71 kDa protein (HSPA8), and intestinal (FABP2) and liver fatty acid-binding proteins (FABP1) expression patterns showed an increase with statistical significance in only 1 or 2 out of the 3 patients enrolled. For ileal lipid binding protein (FABP6) and electron transfer flavoprotein subunit α (ETFA), *P* values of 0.07 and 0.06, respectively, near the threshold of significance were obtained. Among downregulated protein spots after antibiotic treatment, statistical significance was achieved in all patients for thioredoxin domain-containing protein 5 (TXNDC5), type I cytoskeletal keratin 20 (KRT20) and cathepsin D (CTSD). Pyruvate dehydrogenase E1 component subunit β (PDHB) showed a statistically significant decreased expression in only 1 patient.

Probiotic administration-related effects on mucosal biopsy proteome in non-inflamed pouch

Representative 2D gels obtained from mucosal biopsies in normal pouch and after probiotic therapy are shown in Figure 1B, confirming the protein maps reported in Figure 1A in terms of number, M_r and p*I* of the spots.

For each of the 3 subjects enrolled, the comparison of the 2D patterns of non-inflamed mucosal biopsies before and after VSL#3 administration was performed by PDQuest as reported above. Seventeen spots, which represented 75% of total proteins recognized as differentially expressed, were identified, of which 7 were upregulated and 10 were downregulated in the probiotic-treated pouch (Figure 1B and Table 1). In addition, it was possible to identify 6 protein spots that showed a similar expression pattern in all 2D gels (Figure 1 and Table 2).

Pie charts representing the subcellular location and the functional distribution of the probiotic administration-altered proteins are reported in Figure 2B. The majority of the identified proteins were in the cytoplasm (41%), mitochondria (35%) and endoplasmic reticulum (12%). The functional classification indicated that 29% play a key role in energy production and conversion, 17% are related to post-translational modifications and protein turnover as chaperones and 12% are involved in carbohydrate transport and metabolism.

The spot quantity values determined by PDQuest are shown in the form of a histogram in Figure 4 together with representative gel images for each protein spot in each subject and condition. A statistically significant increased

Table 1 Differentially expressed proteins before and after antibiotic/probiotic administration

Spot ID	Swiss-Prot Acc. No.	Protein name	COG[1]	Subcellular location	Theoretical M_r/p*I*	Experimental M_r/p*I*	Method of identification[2]	Change in protein expression with AB/PB treatment[3]
Pouchitis/antibiotic-induced remission								
1	P27797	Calreticulin (CALR)	O	Endoplasmic reticulum	48.14/4.29	68.52/4.35	GM (Swiss-2D PAGE)	Up
2	P11021	78 kDa glucose-regulated protein (GRP78)	O	Endoplasmic reticulum	72.33/5.07	73.88/4.95	GM (Swiss-2D PAGE)	Down
3	P10809	60 kDa heat shock protein, mitochondrial precursor (HSP60)	O	Mitochondrial matrix	61.05/5.70	60.20/5.32	GM (Swiss-2D PAGE)	Up
4	P11142	Heat shock cognate 71 kDa protein (HSPA8)	O	Nucleolus	70.90/5.37	69.20/5.18	GM[20]	Up
5	P38646	Stress-70 protein, mitochondrial precursor (75 kDa glucose-regulated protein) (GRP75)	O	Mitochondrion	73.68/5.87	71.41/5.70	GM (Swiss-2D PAGE)	Down
6	Q9BU08	Putative uncharacterized protein, fragment (CCT5)	S	Undefined	59.47/5.45	60.46/5.58	GM[21]	Up
7	P02787	Serotransferrin precursor (TF)	P	Extracellular	77.05/6.81	79.49/7.09	GM (Swiss-2D PAGE)	Down
8	Q16822	Phosphoenolpyruvate carboxykinase (GTP), mitochondrial precursor (PCK2)	C	Mitochondrion	70.73/7.56	71.67/7.62	GM[19]	Up
9	P68371	Tubulin β-2C chain (TUBB)	Z	Cytoplasm	49.83/4.79	52.44/4.79	MALDI-TOF MS	Up
10	P06576	ATP synthase subunit β, mitochondrial precursor (ATP5B)	C	Mitochondrion	56.56/5.26	48.675.01	MALDI-TOF MS	Up
11	Q8NBS9	Thioredoxin domain-containing protein 5, precursor (TXNDC5)	R	Endoplasmic reticulum	47.63/5.63	49.43/5.09	GM[20]	Down
12	P35900	Keratin, type I cytoskeletal 20 (KRT20)	W	Cytoplasm	48.49/5.52	48.15/5.54	GM[19]	Down
13	P06733	α-enolase (ENO1)	G	Cytoplasm	47.17/7.01	46.80/7.57	MALDI-TOF MS	Down
14	P11177	Pyruvate dehydrogenase E1 component subunit β, mitochondrial precursor (PDHB)	C	Mitochondrion	39.25/6.20	32.96/5.64	MALDI-TOF MS	Down
15	P17707	S-adenosylmethionine decarboxylase proenzyme (AMD1)	T	Cytoplasm	38.34/5.71	31.91/5.74	MALDI-TOF MS	Up
16	P13804	Electron transfer flavoprotein subunit α, mitochondrial precursor (ETFA)	C	Mitochondrion	35.08/8.62	34.01/7.91	GM[20]	Up
17	P21796	Voltage-dependent anion-selective channel protein 1 (VDAC1)	P	Mitochondrion	30.77/8.62	30.60/9.20	GM[19]	Down
18	P07339	Cathepsin D, precursor (CTSD)	O	Lysosome	44.55/6.10	28.02/5.70	GM (Swiss-2D PAGE)	Down
19	Q99439	Calponin-2 (CNN2)	Z	Cytoplasm	33.70/6.94	29.64/7.55	MALDI-TOF MS	Up
20	P00915	Carbonic anhydrase Ⅰ (CA1)	R	Cytoplasm	28.87/6.59	27.52/7.45	GM[19]	Down
21	P62937	Peptidyl-prolyl cis-trans isomerase A (PPIA)	O	Cytoplasm	18.01/7.68	16.42/8.09	GM[19]	Up
22	P12104	Fatty acid-binding protein, intestinal (FABP2)	I	Cytoplasm	15.21/6.62	14.07/6.99	MALDI-TOF MS	Up
23	P51161	Ileal lipid binding protein (FABP6)	I	Cytoplasm	14.37/6.29	13.58/7.22	MALDI-TOF MS	Up
24	P09382	Galectin-1 (LGALS1)	W	Extracellular	14.72/5.33	13.25/5.26	MALDI-TOF MS	Down
25	P07148	Fatty acid-binding protein, liver (FABP1)	I	Cytoplasm	14.21/6.60	12.13/6.80	MALDI-TOF MS	Up
26	Q5T1C5	Protein S100-A10 (S100A10)	R	Plasma membrane	11.20/6.82	10.52/7.25	MALDI-TOF MS	Up
Non-inflamed pouch/probiotic-treated pouch								
1	P18206	Vinculin (VCL)	Z	Cytoplasm	123.80/5.50	114.44/5.81	GM (Swiss-2D PAGE)	Up
2	P27797	CALR	O	Endoplasmic reticulum	48.14/4.29	68.52/4.35	GM (Swiss-2D PAGE)	Down
3	P28331	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial precursor (NDUFS1)	C	Mitochondrion	79.47/5.89	77.54/5.52	GM[21]	Down
4	P11142	HSPA8	O	Nucleolus	70.90/5.37	69.20/5.18	GM[20]	Up
5	P38646	GRP75	O	Mitochondrion	73.68/5.87	71.41/5.70	GM (Swiss-2D PAGE)	Down
6	P02787	TF	P	Extracellular	77.05/6.81	79.49/7.09	GM (Swiss-2D PAGE)	Down

7	Q16822	PCK2	C	Mitochondrion	70.73/7.56	71.67/7.62	GM[19]	Up
8	Q71U36	Tubulin α-1A chain (TUBA1A)	Z	Cytoplasm	50.15/4.94	56.47/4.82	GM[17]	Down
9	Q8NBS9	TXNDC5	R	Endoplasmic reticulum	47.63/5.63	49.43/5.09	GM[20]	Down
10	P35900	KRT20	W	Cytoplasm	48.49/5.52	48.15/5.54	GM[19]	Down
11	P06733	ENO1	G	Cytoplasm	47.17/7.01	46.80/7.57	MALDI-TOF MS	Down
12	P12532	Creatine kinase, ubiquitous mitochondrial precursor (CKMT1B)	C	Mitochondrion	47.04/8.60	43.16/8.48	GM[20]	Down
13	P22695	Cytochrome b-c1 complex subunit 2, mitochondrial precursor (UQCRC2)	C	Mitochondrion	48.44/8.74	44.10/8.83	GM[21]	Up
14	P11177	PDHB	C	Mitochondrion	39.25/6.20	32.96/5.64	MALDI-TOF MS	Up
15	P17707	AMD1	T	Cytoplasm	38.34/5.71	31.91/5.74	MALDI-TOF MS	Up
16	P00918	Carbonic anhydrase Ⅱ (CA2)	R	Cytoplasm	29.25/6.87	30.75/7.69	MALDI-TOF MS	Down
17	P60174	Triosephosphate isomerase (TPI1)	G	Cytoplasm	26.67/6.45	26.14/7.32	MALDI-TOF MS	Up

[1]Abbreviation of cellular role categories. Categories were taken from Cluster of Orthologous Groups (COG) (http://www.ncbi.nlm.nih.gov/COG/), and the abbreviation was used to mark the categories. C: Energy production and conversion; G: Carbohydrate transport and metabolism; I: Lipid transport and metabolism; O: Posttranslational modification, protein turnover, chaperones; P: Inorganic ion transport and metabolism; R: General function prediction only; S: Function unknown; T: Signal transduction mechanisms; W: Extracellular structures; Z: Cytoskeleton; [2]GM: Gel matching; [3]AB: Antibiotic; PB: Probiotic.

Table 2 Summary of identification results of protein spots conserved in pouchitis/pouch remission and normal pouch/probiotic-treated pouch groups

Spot ID	Swiss-Prot Acc. No.	Protein name	COG[1]	Subcellular location	Theoretical *M*r/*p*I	Experimental *M*r/*p*I	Method of identification[2]
A	Q15365	Poly(rC)-binding protein 1 (PCBP1)	A	Nucleus	37.53/6.66	35.99/7.17	MALDI-TOF MS
B	Q6IBM5	Rho GDP dissociation inhibitor (GDI) α, isoform CRA_a (ARHGDIA)	T	Cytoplasm	23.21/5.03	25.73/4.99	MALDI-TOF MS
C	Q5R8R5	Glutathione S-transferase P (GSTP1)	O	Cytoplasm	23.36/5.93	24.33/5.80	MALDI-TOF MS
D	P61088	Ubiquitin-conjugating enzyme E2 N (UBE2N)	O	Nucleus	17.14/6.13	19.00/6.10	MALDI-TOF MS
E	P68871	Hemoglobin subunit β (HBB)	C	Extracellular	16.00/6.74	13.10/7.70	MALDI-TOF MS
F	Q1HDT5	Hemoglobin α 1-2 hybrid (HBA1)	C	Extracellular	15.27/9.04	12.50/9.55	MALDI-TOF MS

[1]A: RNA processing and modification. For other abbreviations see Table 1; [2]For each protein spot, the gel matching identification method was also employed. Spots A, B and D were identified by comparison with published 2D proteome patterns[17,18]; spots C, E and F by comparison with Swiss-2D PAGE.

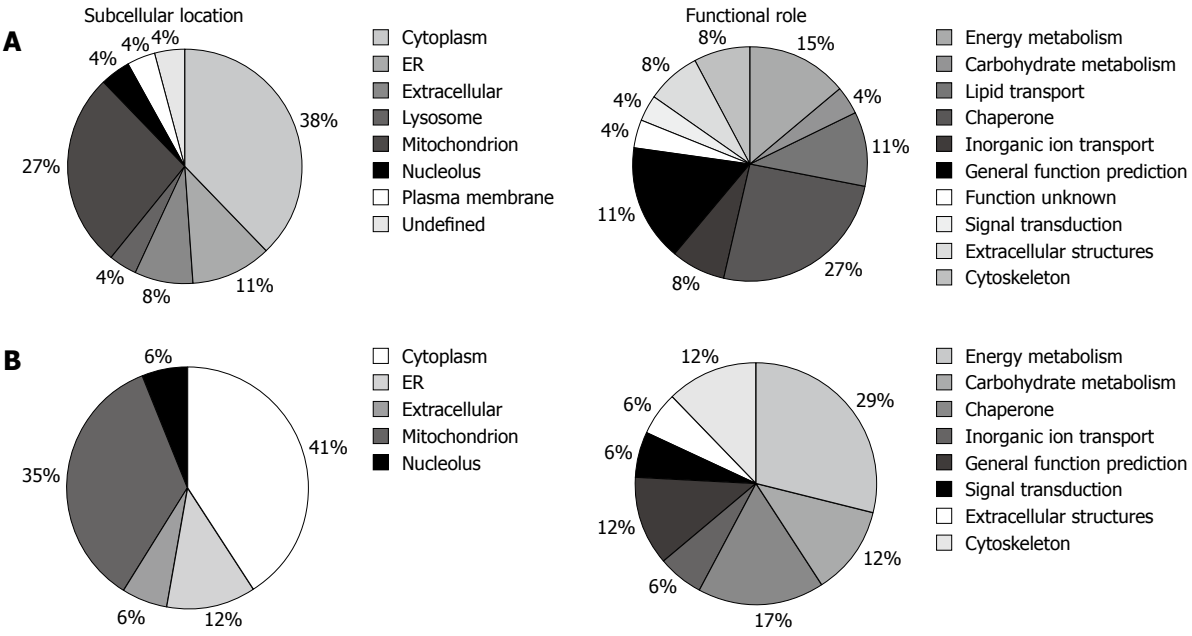
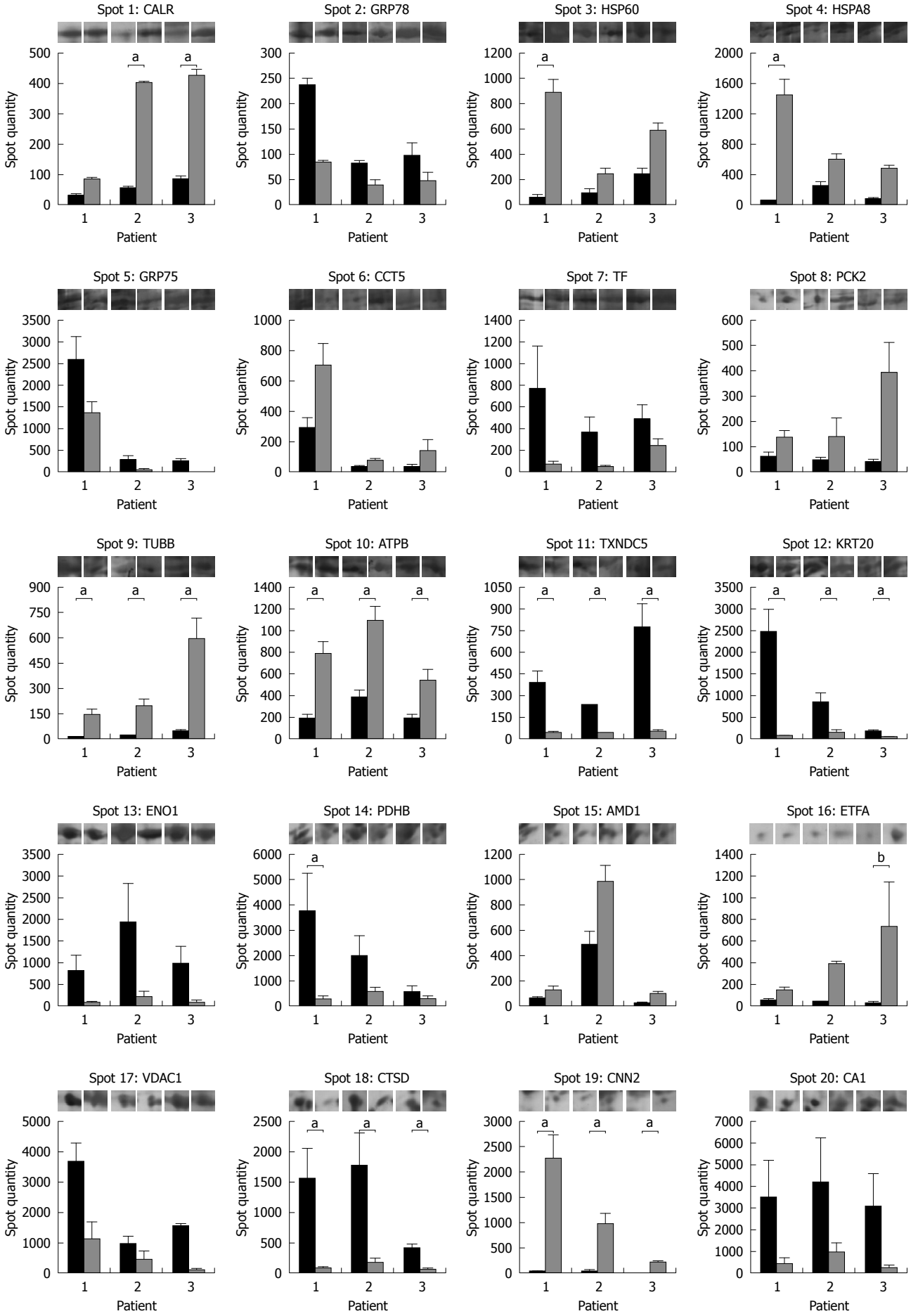


Figure 2 Pie charts representing the distribution of the differentially expressed proteins from pouchitis/pouch remission (A) and normal pouch/probiotic-treated pouch (B) group comparison, according to their subcellular location and biological function. ER: Endoplasmic reticulum.


Spot 1: CALR
Spot 2: GRP78
Spot 3: HSP60
Spot 4: HSPA8
Spot 5: GRP75
Spot 6: CCT5
Spot 7: TF
Spot 8: PCK2
Spot 9: TUBB
Spot 10: ATPB
Spot 11: TXNDC5
Spot 12: KRT20
Spot 13: ENO1
Spot 14: PDHB
Spot 15: AMD1
Spot 16: ETFA
Spot 17: VDAC1
Spot 18: CTSD
Spot 19: CNN2
Spot 20: CA1
Spot quantity
Patient
a
b

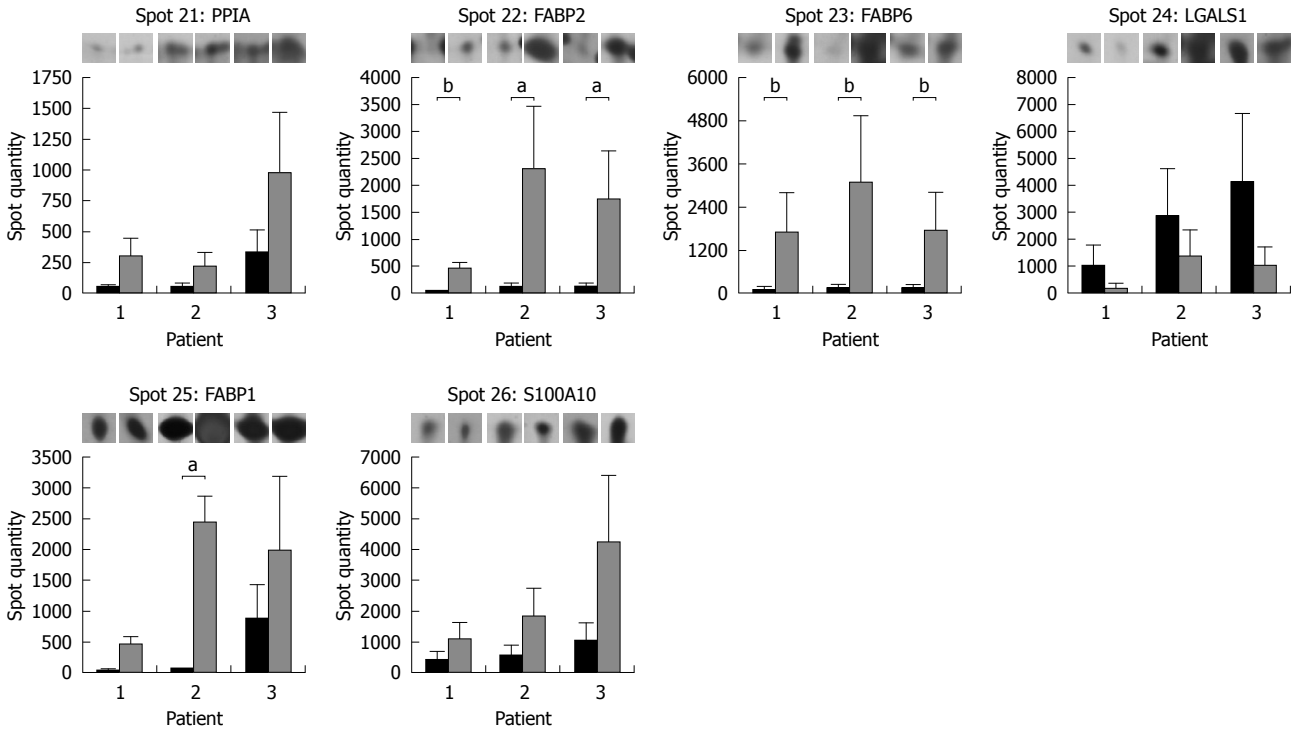


Figure 3 Protein expression histograms of the 26 differentially expressed protein spots between pouchitis (dark grey) and antibiotic-induced remission (light grey). Each bar represents the average spot quantity determined from 3 technical replicates for each patient condition by PDQuest. Representative gel images are displayed on top of each graph. [a]$P < 0.05$, [b]$P = 0.06$ for ETFA and $P = 0.07$ for FABP2 and FABP6.

expression after 3 mo of probiotic administration was detected for HSPA8 and PDHB in all the subjects enrolled. *P* values of 0.06, near the threshold of significance, were obtained for vinculin (VCL) and phosphoenolpyruvate carboxykinase (PCK2) in 3 and 2 patients, respectively. Among protein spots with downregulated expression levels after VSL#3 therapy, statistical significance was achieved in all patients for KRT20 and in only 1 for TXNDC5.

Bibliometric analysis

On the basis of literature co-citation from NCBI PubMed, a protein-protein network tree using the data-mining program Bibliosphere software was generated. As shown in Figure 5, the network tree was compiled of 28 different proteins forming 2 network clusters. Group 1 consisted of 26 highly interrelated proteins including ATP5B, carbonic anhydrase I (CA1) and Ⅱ (CA2), creatine kinase (CKMT1B), α-enolase (ENO1), PCK2, PDHB and triosephosphate isomerase (TPI1), associated with energy, carbohydrate and amino acid metabolism, as well as glycolysis/gluconeogenesis, oxidative phosphorylation and electron transport chain. The second group was formed by 2 linear co-cited proteins, NADH-ubiquinone oxidoreductase 75 kDa subunit (NDUFS1) and cytochrome b-c1 complex subunit 2 (UQCRC2), related to energy production and conversion. The residual 5 detected proteins, S-adenosylmethionine decarboxylase (AMD1), CNN2, tubulin α-1A chain (TUBA1A), TUBB and TXNDC5 were completely disconnected from the network tree.

DISCUSSION

In this study, we provided for the first time 2D protein maps of mucosal biopsy samples collected during pouch endoscopy in patients who underwent IPAA.

The comparison between mucosal biopsy proteomes in pouchitis and in antibiotic-induced remission enabled the identification of 26 different proteins with at least 2-fold changes in their expression levels. Statistical significance was achieved for ATP5B, CNN2, CTSD, KRT20, TUBB and TXNDC5. In addition, a statistically significant altered expression pattern was obtained for CALR, HSP60, HSPA8, FABP1, FABP2 and PDHB in 1 or 2 of the 3 patients enrolled.

Among the identified mitochondrial proteins, ATP5B, ETFA and PCK2 directly participate in the process of energy production. The decrease of their expression levels in the inflamed pouch suggests the decline of mitochondrial function with pouchitis onset. This assumption is consistent with a previous hypothesis that chronic intestinal inflammation represents an energy-deficiency disease with alterations in the oxidative metabolism of the epithelial cells[23]. Moreover, the low expression of FABP1, FABP2 and FABP6, involved in enhancing the uptake of fatty acids into cells and facilitating their transport to intracellular organelles, could reinforce the speculation that pouchitis-diseased enterocytes do not perform β-oxidation/oxidative phosphorylation owing to a lack of normal supply of fatty acids[24]. Combined with these results, the overexpression of ENO1 found in the inflamed pouch may reflect a shift toward anaerobic

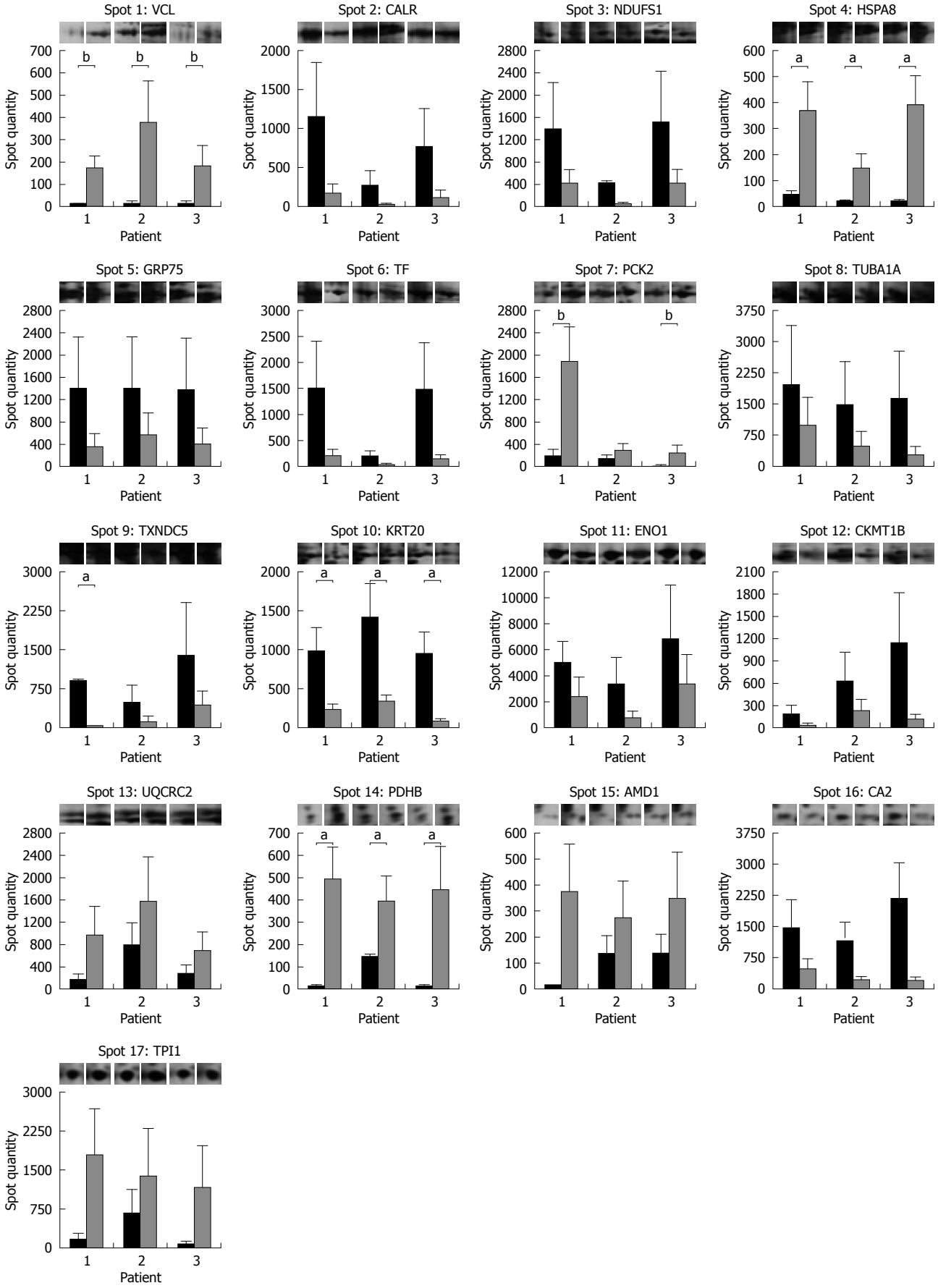


Figure 4 Protein expression histograms of the 17 differentially expressed protein spots in non-inflamed pouch before (dark grey) and after (light grey) probiotic treatment. Each bar represents the average spot quantity determined from 3 technical replicates for each subject condition by PDQuest. Representative gel images are displayed on top of each graph. [a]$P < 0.05$, [b]$P = 0.06$ for VCL and PCK2.

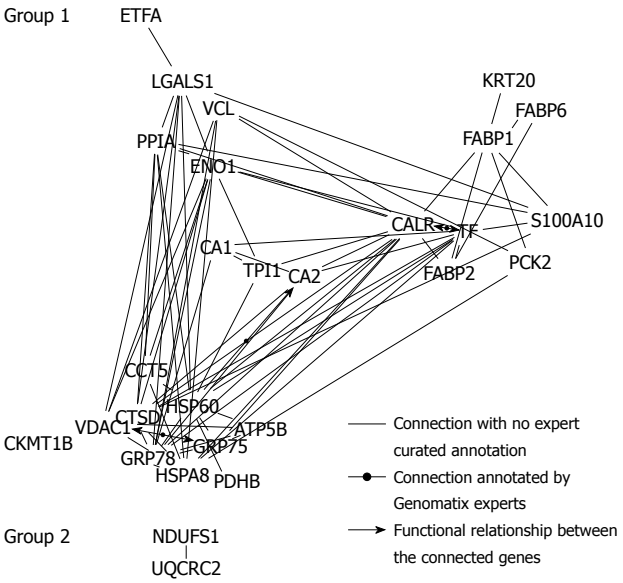


Figure 5 Bibliometric data analysis. Protein-protein network tree generation using the data-mining program Bibliosphere software.

glycolysis to overcome the decreased ATP formation by a dysfunctional oxidative phosphorylation[21].

The hypothesis of cellular stress and hypoxic conditions in chronically inflamed tissues is supported by the induction of several chaperone proteins, including 75 (GRP75) and 78 kDa glucose-related proteins (GRP78), TXNDC5, voltage-dependent anion-selective channel protein 1 (VDAC1), CTSD and peptidyl-prolyl cis-trans isomerase A (PPIA)[25-27]. In addition, we detected a statistically significant altered expression pattern for TUBB, KRT20 and CNN2, suggesting changes in cytoskeletal architecture with potential alterations in signal transduction and cellular transcription profiles[27,28].

Next, we compared mucosal biopsy proteomes in the normal pouch before and after probiotic administration and we identified 17 different proteins with significant changes in their expression levels. Interestingly, 8 of the differentially expressed proteins exhibited the same pattern of deregulation as in the pouchitis/pouch remission group. Indeed, both antibiotic and probiotic therapy resulted in downregulation of GRP75, serotransferrin (TF), TXNDC5, KRT20, ENO1 and in upregulation of HSPA8, PCK2 and AMD1, suggesting profound structural and metabolic alterations in enterocytes. In particular, TXNDC5 is a newly identified member of the thioredoxin family of endoplasmic reticulum proteins[29], and it has been proposed as a promising biomarker for cancer diagnosis[30]. Because of its important role in redox regulation[31], the altered expression profile of TXNDC5 in IPAA may be related to the increased oxidative stress with significantly lower plasma concentrations of lipophilic antioxidants and higher free radical activity measured in patients with restorative proctocolectomy compared to normal subjects[32]. Furthermore, for KRT20 and ENO1, widely applied as diagnostic markers for colon adenocarcinomas and many other tumors[33,34], as well as for PCK2 and AMD1 a differential protein profile in inflammatory bowel disease has been already reported[21,35,36].

In addition to these results, in the VSL#3-treated pouch we found a statistically significant upregulation of VCL and an altered expression pattern for TUBA1A, supporting the assumption of a positive modulation exerted by probiotics at cytoskeleton level for cell morphology and integrity[37,38]. In addition, the dysregulated expression levels of NDUFS1, CKMT1B, UQCRC2, PDHB, CA2 and TPI1, directly involved in energy metabolism, strengthen the hypothesis of significant changes in the metabolic profiles of the host associated with probiotic administration[39,40]. Nonetheless, although the manipulation of the ubiquitin/proteasome pathway and the ability to intervene with the complex host system of detoxification of potentially harmful xenobiotics and endobiotic compounds may account for some of the cytoprotective effects of probiotics[37,41,42], we did not find any significant change in glutathione S-transferase P (GSTP1) and ubiquitin-conjugating enzyme E2 N (UBE2N) protein expression levels.

The bibliometric data analysis including all the 33 differentially regulated proteins from the pouchitis/pouch remission and non-inflamed/probiotic-treated pouch group comparison generated a complex network with 26 highly interrelated proteins. As expected, the majority of clustered proteins were associated with glycolysis/gluconeogenesis, oxidative phosphorylation and electron transfer chain pathways.

In conclusion, the identified proteins, both upregulated and downregulated, may be involved in pouchitis pathophysiology and participate in disease onset or in maintenance of the non-inflamed pouch.

COMMENTS

Background

Restorative proctocolectomy with ileal pouch-anal anastomosis (IPAA) is the procedure of choice for complicated ulcerative colitis. In the long-term, up to 50% of patients develop pouchitis, an idiopathic inflammatory disease of the ileal reservoir. The management of pouchitis is largely empirical and only few small placebo-controlled clinical trials have been conducted. Although antibiotics represent the mainstay of treatment, probiotics have recently gained more attention as an effective therapeutic option for pouchitis management.

Research frontiers

The etiology and pathophysiology of pouchitis are still not entirely clear but the bulk of the evidence points towards an abnormal mucosal immune response to altered microbiota patterns. By investigating the dynamic nature of protein expression, cellular and subcellular distribution, posttranslational modifications and protein-protein interaction networks, proteomic technologies could play a major role in unraveling the mystery of immunopathogenic mechanisms of pouchitis and in discovering novel biomarkers for disease activity, diagnosis and prognosis.

Innovations and breakthroughs

The current study is the first proteomic study to be reported in IPAA research. The authors provided the 2D protein maps of mucosal biopsy samples collected during pouch endoscopy in patients with chronic refractory pouchitis. The changes in the protein expression profiles following antibiotic or probiotic treatment were characterized.

Applications

The identified proteins, upregulated or downregulated following antibiotic/

probiotic treatment, may be involved in pouchitis pathophysiology and participate in disease onset or in maintenance of the non-inflamed pouch. Future work will be focused in validating the list of proteins identified in larger patient cohorts.

Peer review

The results are well described and interesting, although the number of patients is a bit on the small side. Even though this manuscript does not give a clear understanding to the mechanistic differences, the results may aid other scientists in making a follow-up study.

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ORIGINAL ARTICLE

Is there an association between *Helicobacter pylori* in the inlet patch and globus sensation?

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Abstract

AIM: To determine the association between *Helicobacter pylori* (*H. pylori*) and globus sensation (GS) in the patients with cervical inlet patch.

METHODS: Sixty-eight patients with esophageal inlet patches were identified from 6760 consecutive patients undergoing upper gastrointestinal endoscopy prospectively. In these 68 patients with cervical inlet patches, symptoms of globus sensation (lump in the throat), hoarseness, sore throat, frequent clearing of the throat, cough, dysphagia, odynophagia of at least 3 mo duration was questioned prior to endoscopy.

RESULTS: Cervical heterotopic gastric mucosa (CHGM) was found in 68 of 6760 patients. The endoscopic prevalence of CHGM was determined to be 1%. *H. pylori* was identified in 16 (23.5%) of 68 patients with inlet patch. 53 patients were classified as CHGM Ⅱ. This group included 48 patients with globus sensation, 4 patients with chronic cough and 1 patient with hoarseness. All the patients who were *H. pylori* (+) in cervical inlet patches had globus sensation.

CONCLUSION: Often patients with CHGM have a long history of troublesome throat symptoms. We speculate that disturbances in globus sensation are like non-ulcer dyspepsia.

Key words: Barrett's esophagus; Cervical heterotopic gastric mucosa; Globus sensation; *Helicobacter pylori*; Inlet patch

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INTRODUCTION

Islands of heterotopic gastric mucosa are found throughout the gastrointestinal tract, the most common site being the cervical esophagus. Cervical heterotopic gastric mucosa (CHGM) or cervical inlet patches are commonly seen during withdrawal of the gastroscope. Patients with CHGM have various laryngeal and oropharyngeal symptoms, ranging from asymptomatic to protracted symptoms such as globus sensation and chronic cough due to acid secretion from the inlet patch[1-4].

Globus sensation (GS) is caused mainly by cervical disturbances. The usual complaint in patients with globus sensation or globus pharyngeus is the sensation of a ball or lump in the throat, generally not accompanied

by dysphagia. Globus sensation is felt medially deep in the throat during dry swallowing (empty swallow), and almost never while drinking or eating. It is not painful, and there is no obstruction of food[2,3].

Helicobacter pylori (*H. pylori*) produces chronic inflammation in the CHGM (as in non-ulcer dyspepsia). *H. pylori* infection plays a role in altered gastric perception in non-ulcer dyspepsia. *H. pylori* in CHGM may cause altered cervical perception such as globus sensation. In this prospective study, we aimed to determine the association between *H. pylori* and globus sensation in patients with CHGM.

MATERIALS AND METHODS

Subjects

Over a one-year period, between 2005 and 2006, the number of patients with cervical inlet patches from a total of 6760 consecutive patients undergoing upper gastrointestinal endoscopy at the Hospital of the Gazi University in Ankara, Turkey were identified. Patients were referred for endoscopy for a variety of reasons, primarily for evaluation of dyspepsia. In these patients, symptoms of globus sensation (lump in the throat), hoarseness, sore throat, frequent clearing of the throat, cough, dysphagia and odynophagia of at least 3 mo duration were questioned prior to endoscopy.

The investigation conformed to the principles outlined in the Declaration of Helsinki. Informed consent was obtained from all subjects and the study was approved by the ethical review committee of Gazi University in Ankara, Turkey.

Esophagogastroduodenoscopy

After an overnight fast, a routine esophagogastroduodenoscopy was performed with a standard endoscope using topical anesthesia with or without conscious sedation, depending on patient preference. Conscious sedation was performed with midazolam (2-5 mg). During all procedures the esophagus was carefully surveyed and special attention was paid to the area of the upper esophageal sphincter. This region was best examined when slowly withdrawing the endoscope, with repeated short inflations while rotating the instrument.

Heterotopic gastric mucosa was defined as patches covered with salmon-red mucosa distinguishable from surrounding greyish-pearly colored esophageal mucosa by well-defined margins (Figure 1). The size of the patches was determined by the top span of the fully open biopsy forceps. In all subjects, the distance between the patch and the frontal incisor was recorded and the patch size measured under the guidance of the open biopsy forceps.

pH monitoring

pH monitoring was performed in our laboratory in the patients with inlet patch. A 2.1-mm diameter dual-electrode antimony pH catheter (pHersaflex ambulatory catheter, MMS) was placed transnasally after an overnight fast. Recording sites were fixed on the catheter, with a distance of 15 cm to measure proximal and distal pH. The distal electrode was placed 5 cm above the manometrically defined lower esophageal sphincter. The proximal electrode was placed at 16-21 cm, which corresponded approximately with the endoscopic finding of inlet patches. The pH values from both intraesophageal electrodes were recorded continuously on an ambulatory Mark Ⅲ Digitrapper (Synectics Medical Inc.). Abnormal distal esophageal reflux and proximal reflux were defined as the percentage of esophageal total acid exposure (pH < 4) of $\geqslant 4.2\%$ and $\geqslant 1\%$, respectively[5].

Biopsies

A minimum of two biopsies were obtained from the CHGM (Figure 2) and antral gastric mucosa. The samples were taken using large cup and side-opening forceps. Pathology and/or the rapid urease test were performed to determine the presence of *H. pylori* in all patients (Figure 3). The presence of *H. pylori* was identified using hematoxylin-eosin, cresyl violet, giemsa and silver stain.

Statistical analysis

All statistical analyses were carried out using the Statistical Package for Social Sciences (SPSS) 13.0 software for Windows XP. Categorical variables were compared with the chi-squared test or Fisher's exact test, and continuous variables were compared using Student's *t*-test and univariate analysis. A P value of less than 0.05 was considered to be statistically significant.

RESULTS

Prevalence, demographic characteristics

CHGM patches were found in 68 of 6760 patients (3173 female, 3587 male). The endoscopic prevalence of CHGM was determined to be 1%. Demographic characteristics of the patients with and without patches are shown in Table 1. The female/male ratio was 1.12 in the 68 patients with patches and 0.88 in those without. There was no significant difference between the mean age of the patients with CHGM with and without *H. pylori* ($P > 0.05$). Female patients with inlet patches had higher colonization of *H. pylori* than male patients ($P < 0.05$).

Size, number, symptoms, pH monitoring

The size of the inlet patches varied between 5 and 32 mm and occupied between 10% and 30% of the circumference. Five patients had "double" patches. Symptoms are shown in Tables 1 and 2. Five patients had distal reflux and 1 patient had both proximal and distal reflux. There were no patients with only proximal reflux. The other patients were normal.

Histological characteristics

***H. pylori*:** *H. pylori* was identified in the CHGM in

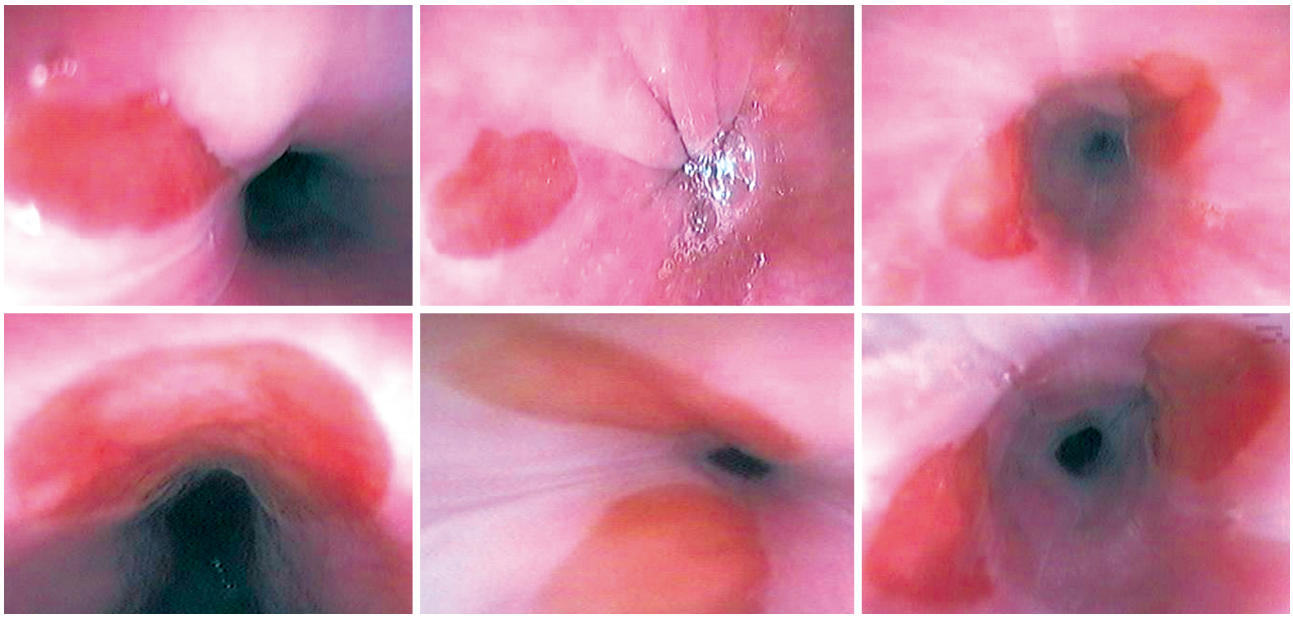

Figure 1 Various endoscopic images of inlet patches (single or double).

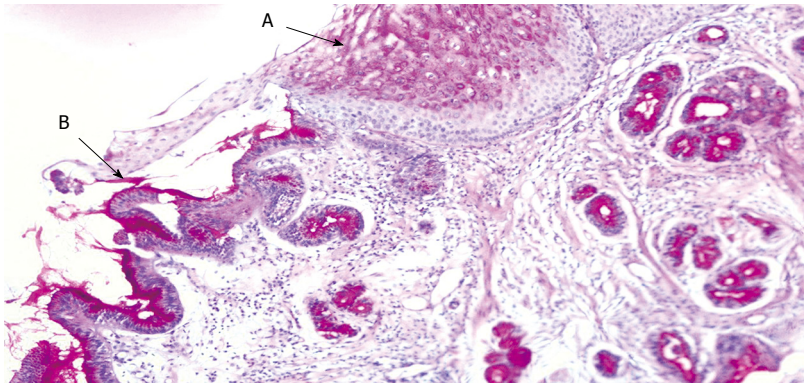

Figure 2 Biopsies. A shows heterotopic gastric mucosa, B shows squamous epithelium.

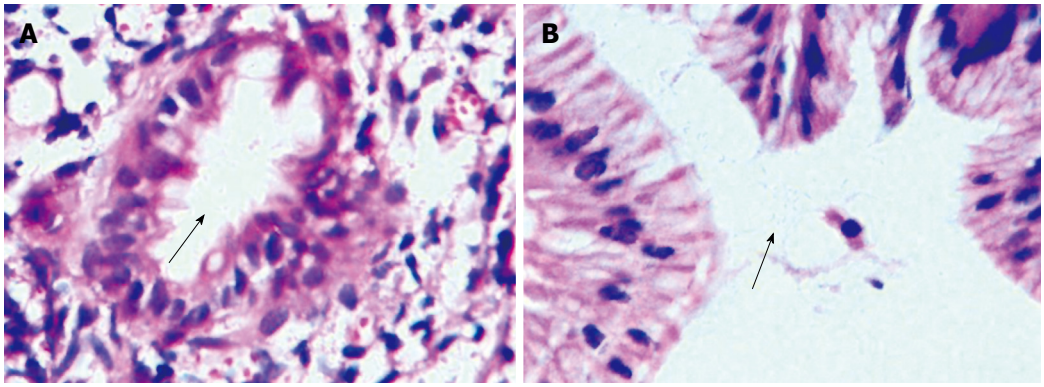

Figure 3 The presence of Hp bacilli in heterotopic gastric mucosa with HE stain (× 1000). A: Few Hp bacilli (arrow); B: Hp colonization (arrow).

23.5% of patients (16/68) (Table 3) and gastric *H. pylori* infection was positive in all (16/16) of these patients (Table 4, Figure 3). Colonization of *H. pylori* was most common in fundic-type mucosa (81.2%). All the patients who were *H. pylori* (+) in the cervical inlet patches had globus sensation ($P < 0.05$).

Mucosal type: In the inlet patch, fundic-type mucosa was the most common histologic type (44/68), followed by antral-type mucosa (15/68) (Table 3). Eight specimens of the inlet patch contained only foveolar epithelium and were therefore considered too superficial to be classified. In one patient, mucosal type was unremarkable because one specimen had complete replacement of the underlying mucosa with intestinal metaplasia.

Intestinal metaplasia: Intestinal metaplasia was identified in the inlet patch in seven patients (10.3%). One pa-

Table 1 Characteristics of the subjects

	HGM (+)	HGM (-)
n	68	6692
Sex		
Female (%)	36 (53.1)	3173 (47.4)
Male (%)	32 (46.9)	3519 (52.6)
Female/male	1.12	0.88
Age range (yr)	(17-56)	(16-90)
Mean age (± SE)	37.29 ± 1.85[a]	47.5 ± 0.3
Endoscopic Barrett's esophagus	9 (13.2)[a]	166 (2.4)
Erosive esophagitis	7 (10.3)	636 (9.5)
Globus (%)	48 (70.6)[a]	0
Dyspepsia(%)	60 (88.2)	6550 (97.8)

HGM: Heterotopic gastric mucosa. [a]$P < 0.05$.

Table 2 Clinicopathologic classification and characteristics of CHGM patches

Clinicopathologic	Number (%)	Symptoms/findings
HGM Ⅰ	12 (17.6)	Asymptomatic
HGM Ⅱ	53 (77.9)	48 GS, 4 cough, 1 hoarseness
HGM Ⅲ	2 (2. 9)	1 ulcus , 1 polyp
HGM Ⅳ	0	
HGM Ⅴ	1 (1.4)	Esophageal adenocarcinoma

CHGM: Cervical heterotopic gastric mucosa; GS: Globus sensation; Cough: Together with clearing of the throat.

tient had complete replacement of the underlying mucosa with intestinal metaplasia. Both *H. pylori* and intestinal metaplasia were observed in the inlet patch mucosa in one patient. In the remaining five patients, intestinal metaplasia was present, but no *H. pylori*. The type of mucosa was evaluated in these patients; three had antral-type and three fundic-type.

Associated lesions

Endoscopic esophagitis (7 patients), duodenal ulcer (3 patients), hiatal hernia (9 patients) and endoscopic Barrett's esophagus (9 patients) accompanied CHGM.

Clinicopathologic classification

Clinicopathologic classification was performed according to the classification reported by von Rahden *et al*[1] in patients with CHGM. (1) Asymptomatic carriers of esophageal CHGM were classified as CHGM Ⅰ. Twelve patients were classified with CHGM Ⅰ in our study. (2) Symptomatic individuals with esophageal CHGM complaining of globus sensation, cough, hoarseness or "extraesophageal manifestations" were classified as CHGM Ⅱ without morphologic changes. Fifty-three patients were classified with CHGM Ⅱ. This group included 48 patients with globus sensation, 4 patients with chronic cough and 1 patient with hoarseness. There were 48 (70.5%) patients with globus sensation out of the 68 patients with CHGM Ⅱ. Symptoms of globus sensation were obvious attractive in these patients. (3) A smaller group of patients with additional morphologic changes (inlet patch complications) were classified as CHGM Ⅲ. This group included one patient with ulcus and polyp in the inlet patch. (4) If dysplasia was present, this was classified as CHGM Ⅳ. None of the patients belonged to this category. (5) If the diagnosis was invasive cancer and originated within the inlet patch, this was classified as CHGM Ⅴ. One patient who had adenocarcinoma in the CHGM was classified as CHGM Ⅴ.

Table 3 Histological characteristics of heterotopic gastric mucosal patches

Histological characteristics	*n*
Type of patch	
Fundic	44
Antral	15
Foveolar epithelium	8
Complete IM	1
Chronic inflammation	68
IM (+), Hp (-)	6 (3 antral-type, 2 fundic-type, 1 complete IM)
IM (-), Hp (+)	15 (3 antral-type, 12 fundic-type)
IM (+), Hp (+)	1 (1 fundic-type)
IM (-), Hp (-)	46 (9 antral-type, 29 fundic-type, 8 foveolar epithelium)

IM: Intestinal metaplasia; Hp: *Helicobacter pylori*.

Table 4 Comparison of the HP (+) and Hp (-) subjects with CHGM

	Hp (+) CHGM (*n* = 16)	Hp (-) CHGM (*n* = 52)
Globus, *n* (%)	16 (100)	32 (61.5)[a]
Age (yr)	35.6 ± 2.3	38.6 ± 2.5
Female *n* (%)	11 (68.8)[a]	25 (48.1)
Male *n* (%)	5 (31.2)	27 (51.9)[a]
Fundic-type	13 (81.2)	31 (59.6)[a]
Antral-type	3 (18.8)	12 (23.1)
Intestinal metaplasia	-	1 (1.9)
Foveolar epithelium	-	8 (15.4)

[a]$P < 0.05$.

DISCUSSION

The usual endoscopic appearance of CHGM is a salmon-rose-colored mucosal patch with a sharp border or edge in the upper esophagus. The patches vary in diameter from 1 to 20 mm or more. Inlet patches are recognized endoscopically as 1 or 2 patches mostly in the lateral walls between the level of the cartilage and the fifth tracheal ring, and are seen as sharply demarcated, salmon-rose-colored oval or round patches.

The prevalence of CHGM varied between 0.29% and 2.27% in one prospective study[6]. Akbayir *et al*[7] reported a prevalence of 1.67% and Tang *et al*[8] reported a prevalence of 1.1%. In our prospective study, over a period of 1 year, 68 cases (1%) of CHGM were documented and confirmed by histology.

Microscopically, gastric mucosa containing either

cardiac, antral and potentially acid-secreting fundic mucosa can be found. In general, CHGM is uniformly of the fundic-type, containing both parietal and chief cells. Less frequently, histopathologic examination of CHGM shows an "antral pattern", defined by the absence of chief cells and only a few parietal cells[1,8,9]. In our series, fundic-type mucosa was found in 44 of 68 patients examined histologically.

H. pylori is a well known pathogenic micro-organism responsible for chronic inflammation. Ectopic gastric mucosa of the inlet patch is an ideal location for *H. pylori* colonization[10]. Borhan-Manesh *et al*[11] found *H. pylori* in the inlet patch in 35% of patients in a subset with gastric *H. pylori*. Among our 68 patients with inflamed inlet patches, 16 were positive for *H. pylori* (23.5%), and all of these 16 patients also had *H. pylori* in the antrum. Coinfection of *H. pylori* in the inlet patch and gastric antrum has also been reported by others[10]. Since the infection by *H. pylori* is through the oral route, inlet patches may be important sites of *H. pylori* infection in the upper gastrointestinal tract because of its more proximal location. The inlet patches may function as reservoirs for *H. pylori*. Inlet patch colonization by *H. pylori* can occur during ingestion of food, and the presence of gastric *H. pylori* may play a role in the development of inlet patches. We searched but did not find any follow-up studies on antibiotic therapy for *H. pylori* and its impact on infection of inlet patches in the literature. We believe that the elimination of *H. pylori* in both the inlet patch and antrum is very important in the treatment of patients with coinfection.

In this study, the female/male ratio in the *H. pylori* (+) CHGM group was higher than that in the *H. pylori* (-) CHGM group. Females had higher inlet patch colonization with *H. pylori* than males ($P < 0.05$). The mechanism of *H. pylori* colonization in the inlet patch is unclear.

A clinicopathological classification of CHGM as proposed by von Rahden *et al*[1] was carried out on all 68 patients, 53 of whom were classified as CHGM Ⅱ; 48 had globus sensation, 4 has cough, one had hoarseness. Theoretically, laryngeal and oropharyngeal symptoms should be common due to acid secretion from the inlet patch. Several studies have reported cases of esophageal inlet patch presenting with various laryngeal and oropharyngeal symptoms, ranging from asymptomatic to protracted symptoms such as chronic cough and globus sensation[4,12-14]. In addition, CHGM can cause stricture, ulcer, perforation, web or polyp in the esophagus because of the its capability to secrete acid[15,16]. In our two patients with CHGM Ⅲ, one had a hyperplastic polyp and one had an ulcus.

There are also reports that CHGM is associated with an increased risk for Barrett's esophagus, suggesting a possible link. Traditionally, Barrett's esophagus is considered a distinct entity from esophageal inlet patch. Barrett's esophagus is an acquired precancerous lesion and the cell origin probably involves multipotential undifferentiated cells. Inlet patch is considered to be congenital. Up to half of all patients with cervical inlet patch have concurrent Barrett's esophagus in some reports[17]. They have the same mucin core protein expression and cytokeratin pattern, suggesting a pathogenetic link between these two diseases[18]. Similarly, in the current study, the presence of CHGM in the upper esophagus was common and closely related to Barrett's esophagus (13.2%) compared with those without inlet patches ($P < 0.05$). Gastro-esophageal diseases in patients with cervical inlet patch was not statistically significant when compared with those without patches ($P > 0.05$). We speculated that the acid secretion from the inlet patches did not contribute to the pathogenesis of Barrett's esophagus. However, the patients with inlet patches were inherently predisposed to developing columnar metaplasia in the distal esophagus.

The usual complaint in GS is that of a ball or lump in the throat generally not accompanied by dysphagia. This sensation is often more pronounced when taking an "empty swallow". In our study, all the patients with *H. pylori* in cervical inlet patches had globus sensation.

CHGM should be looked for, particularly in patients with GS. *H. pylori* positivity in the CHGM correlated with GS symptoms in our study. There were no reports on a link between *H. pylori* positivity in patients with CHGM and "globus sensation" or "globus pharyngeus" in our review of the English literature. Therefore, we were not able to compare our data.

H. pylori infection plays a role in causing symptoms in patients fulfilling the criteria for non-ulcer dyspepsia. There is agreement that *H. pylori* infection causes changes in gastric physiology. In addition, *H. pylori* infection plays a role in altered gastric perception in non-ulcer dyspepsia. We speculate that the disturbances in globus sensation are like non-ulcer dyspepsia. *H. pylori* produces chronic inflammation in the CHGM (as in non-ulcer dyspepsia). It could be speculated that globus sensation is a non-ulcer dyspepsia of CHGM. *H. pylori* is a potential cause of GS in patients with CHGM.

There are no follow-up studies on antibiotic therapy for *H. pylori* and its impact on infection of inlet patches in the literature. Additional studies are needed to understand the fundamental mechanisms leading to globus sensation in CHGM. These patients might benefit from *H. pylori* eradication therapy to alleviate this potentially aggravating factor. Based on these important findings, we expect to see more studies on inlet patches in the near future.

COMMENTS

Background

Cervical heterotopic gastric mucosa (CHGM) or cervical inlet patches are commonly seen on the cervical esophagus during withdrawal of the gastroscope. Patients with inlet patches have various laryngeal and oropharyngeal symptoms, ranging from asymptomatic to protracted symptoms such as globus sensation and chronic cough due to acid secretion from the inlet patch. Globus sensation has a largely unknown etiology.

Research frontiers

All the patients who were *Helicobacter pylori* (*H. pylori*) (+) in the cervical inlet patches had globus sensation. *H. pylori* produces chronic inflammation in the CHGM (as in non-ulcer dyspepsia). It could be speculated that globus sensa-

tion is a non-ulcer dyspepsia of CHGM. *H. pylori* is a potential cause of GS in patients with CHGM.

Innovations and breakthroughs

The authors showed a causal association between *H. pylori* infection and the symptoms of globus sensation in these patients Inlet patches may function as reservoirs for *H. pylori*. Inlet patch colonization by *H. pylori* can occur during ingestion of food and the presence of gastric *H. pylori* might play a role in the development of inlet patches.

Applications

Patients might benefit from *H. pylori* eradication therapy to alleviate this potentially aggravating factor. Based on these important findings, we expect to see more studies on inlet patches in the near future.

Terminology

CHGM in the cervical esophagus appears to result from incomplete replacement of the original columnar epithelium by stratified squamous epithelium in the embryonic period. Islands of heterotopic gastric mucosa are found throughout the gastrointestinal tract, the most common site being the cervical esophagus. Macroscopically, visible islands of CHGM, referred as "inlet patches" are often detected during endoscopic examination.

Peer review

The manuscript presented interesting data on heterotopic gastric mucosa on the cervical esophagus. They proved the link between presence of inlet patch and globus sensation in these patients. Interestingly, they showed a causal association between *H. pylori* infection and the symptom of globus sensation in these patients.

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Atrial natriuretic peptide signal pathway upregulated in stomach of streptozotocin-induced diabetic mice

Zhang-Xun Qiu, Bing Mei, Yi-Song Wu, Xu Huang, Zuo-Yu Wang, Yan-Fei Han, Hong-Li Lu, Young-Chul Kim, Wen-Xie Xu

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Abstract

AIM: To investigate atrial natriuretic peptide (ANP) secretion from gastric mucosa and the relationship between the ANP/natriuretic peptide receptor type A (NPR-A) pathway and diabetic gastroparesis.

METHODS: Male imprinting control region (ICR) mice (4 wk old) were divided into two groups: control mice, and streptozotocin-induced diabetic mice. Eight weeks after injection, spontaneous gastric contraction was recorded by using physiography in control and streptozotocin-induced diabetic mice. The ANP-positive cells in gastric mucosa and among dispersed gastric epithelial cells were detected by using immunohistochemistry and flow cytometry, respectively. ANP and natriuretic peptide receptor type A (*NPR-A*) gene expression in gastric tissue was observed by using the reverse transcriptase polymerase chain reaction.

RESULTS: The frequency of spontaneous gastric contraction was reduced from 12.9 ± 0.8 cycles/min in the control group to 8.4 ± 0.6 cycles/min in the diabetic mice (n = 8, P < 0.05). However, the amplitude of contraction was not significantly affected in the diabetic group. The depletion of interstitial cells of Cajal in the gastric muscle layer was observed in the diabetic mice. ANP-positive cells were distributed in the gastric mucosal layer and the density index of ANP-positive cells was increased from 20.9 ± 2.2 cells/field in control mice to 51.8 ± 2.9 cells/field in diabetic mice (n = 8, P < 0.05). The percentage of ANP-positive cells among the dispersed gastric epithelial cells was increased from 10.0% ± 0.9% in the control mice to 41.2% ± 1.0% in the diabetic mice (n = 3, P < 0.05). ANP and *NPR-A* genes were both expressed in mouse stomach, and the expression was significantly increased in the diabetic mice.

CONCLUSION: These results suggest that the ANP/NPR-A signaling pathway is upregulated in streptozotocin-induced diabetic mice, and contributes to the development of diabetic gastroparesis.

Key words: Diabetes mellitus; Atrial natriuretic peptide; Gastric mucosa; Gastroparesis

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INTRODUCTION

Gastroparesis is a chronic complication of diabetes, also called delayed gastric emptying, occurs in > 50% of patients with long-standing diabetes[1]. Symptoms of gastroparesis include heartburn, pain in the upper abdomen, vomiting, nausea, and early feeling of fullness, but the worst effect of gastroparesis is that it can make diabetes worse by making blood glucose control more difficult[2]. Besides, there are deterioration in glycemic control and incapacitating symptoms such as malnutrition, water and electrolyte imbalance, and aspiration. However, the pathophysiology of diabetic gastropathy and gastroparesis, such as the mechanism of impaired fundic, pyloric relaxation and impaired electrical pacemaking, are still not established[3,4]. It is generally believed that diabetic gastropathy and gastroparesis may be caused by visceral autonomic neuropathy, hyperglycemia, and degeneration of smooth muscle. Hyperglycemia itself can cause antral hypomotility, gastric dysrhythmia, and delayed gastric emptying in some patients[5]. Several physiological studies have reported that dysfunction of gastric smooth muscle in diabetes is associated with neural factors and intracellular signaling pathways[6,7].

Atrial natriuretic peptide (ANP) was isolated from the atrium by de Bold *et al*[8] in 1981. From then on, brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP), dendroapsis natriuretic peptide (DNP), micrurus natriuretic peptide (MNP), and ventricular natriuretic peptide (VNP) have been found in succession. Three types of single transmembrane natriuretic peptide receptors (NPRs) for ANP, BNP and CNP have been identified[9,10], namely, NPR type A (NPR-A), type B (NPR-B), and type C (NPR-C). NPR-A and NPR-B have the membrane-bound particulate guanylate cyclase (pGC), which can catalyze the formation of cGMP from GTP[8-11]. NPR-A preferentially binds to ANP and BNP, but has a low affinity for CNP. NPR-B has a much higher affinity for CNP than either ANP or BNP[12]. Besides the heart, ANP is also distributed in other organs, for example, ANP can be secreted by gastric mucosa[13-15]. It is well known that ANP and other family members exert natriuretic-diuretic effects, vasorelaxation, and other functions including: decreasing blood pressure, and controlling electrolyte homeostasis. Some studies have demonstrated that the natriuretic peptide family plays an inhibitory role in regulating gastrointestinal motility, for example, in the chicken rectum[3], rat tenia coli[16] and guinea-pig cecum[17].

Our previous study also indicated that CNP relaxes gastric circular and longitudinal smooth muscles in human, rat and guinea-pig stomach, and NPRs are distributed in rat gastric smooth muscle layer[18-20]. Recently, we have also reported that the CNP-induced relaxation and the production of cGMP of gastric smooth muscle are potentiated in streptozotocin (STZ)-induced diabetic rats. As well as the activity of pGC, the expression of *NPR-B* gene in gastric smooth muscle is upregulated in STZ-induced diabetic rats[21]. These results suggest that the CNP/(NPR-B)/pGC/cGMP signaling pathway is involved in the pathogenesis of diabetes. Our previous studies have confirmed that ANP-synthesizing cells exist in different regions of the gastric mucosa in rats[15], therefore, ANP can be considered an endogenous natriuretic peptide of gastric mucosa. However, it is not clear whether the ANP/NPR-A signaling pathway is involved in the pathogenesis of diabetic gastroparesis.

In the present study, we investigated the relationship between the ANP signaling pathway and STZ-induced diabetic gastroparesis to confirm whether ANP contributes to the development of gastroparesis. The present study focused on whether the amount of ANP secretion from gastric mucosa was enhanced and the expression of the *NPR-A* gene in gastric smooth muscle was upregulated in a mouse model of STZ-induced diabetic gastroparesis.

MATERIALS AND METHODS

Drugs

STZ, TRIzol Reagent and chemicals were purchased from Sigma. C-kit antibody, ANP antibody and pronase were purchased from Santa Cruz Biotechnology and Roche. Other chemicals were purchased from Sangon Biological Company.

STZ-induced diabetic mouse model

Male imprinting control region (ICR) mice (4 wk old) were purchased from the Experimental Animal Center of Shanghai Jiaotong University School of Medicine. A total of 80 mice were divided into two groups: control group and STZ-induced diabetic group. STZ-induced diabetes was created as follows: the mice were fasted overnight and intraperitoneally administered STZ solution (Sigma-Aldrich, St. Louis, MO, USA). STZ was diluted in 0.1 mol/L citrate buffer (pH = 4.0) and used at a dose of 200 mg/kg. Control mice were intraperitoneally administered with the same volume of 0.1 mol/L citrate buffer. The glucose concentration of blood was determined with One-touch Apparatus (Johnson & Johnson Medical Company). STZ-induced diabetic mice were confirmed by measuring glucose concentration from tail blood after fasting, and diabetes was defined when the blood glucose level was > 16 mmol/L. All experimental protocols included in this study were approved by the local Animal Care Committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the Science and Technology Commission of P.R.C. (STCC Publication No. 2, revised 1988).

Gastric motility in mouse intact stomach

Eight weeks after treatment with STZ, the animals

were euthanized by lethal dose of intraperitoneal pentobarbital sodium (50 mg/kg). The abdomen of each mouse was opened along the midline, and the intact stomach was removed and placed in pre-oxygenated Krebs solution (containing in 118.1 mmol/L NaCl, 4.7 mmol/L KCl, 1.0 mmol/L KH_2PO_4, 1.0 mmol/L $MgSO_4$, 25.0 mmol/L $NaHCO_3$, 2.5 mmol/L $CaCl_2$, and 11.1 mmol/L glucose), which was equilibrated with 95% oxygen and 5% CO_2. The connective tissue was removed and the pylorus was connected to a pressure transducer (Chengdu Equipment Factory, China) with a thin glass tubule, and the gastric cardia was tied with thin string. The stomach was incubated in a 15-mL organ bath filled with Krebs solution and gassed with 95% O_2 and 5% CO_2 at 37℃. The sensitivity of the pressure transducer was adjusted to an appropriate value and we recorded gastric motility by using the SMUP-E biological signal processing system (Chengdu Equipment Factory). The stomach was allowed to incubate for at least 60 min before the experiments were started, and we eliminated error by injecting an equal volume of Kreb's solution into the stomach.

Immunohistochemistry

The mice were euthanized by lethal dose of intraperitoneal pentobarbital sodium (50 mg/kg), the stomach was removed and washed with saline, and then fixed in 4% paraformaldehyde (4℃, 24 h). The fixed tissue was washed with running water (room temperature, 2 h), immersed with 95% and 100% alcohol (room temperature, 2 × 2 h), xylene (2 × 20 min) and paraffin (68.5℃, 30/40/50 min). Sections of 6 μm thickness were cut and deparaffinized in xylene (4 × 30 min). The specimens were hydrated in graded concentrations of ethanol, and washed three times in PBS, and incubated in the blocking reagent for 30 min. The samples were incubated with polyclonal antibody against c-Kit protein (sc-5535; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:100 dilution). c-Kit protein was used as a marker of interstitial cells of Cajal (ICCs)[22,23] and with polyclonal antibody against ANP (sc-20158; Santa Cruz Biotechnology; 1:100 dilution) for 24 h at 4℃, followed by 3 h incubation in Rho-anti-rabbit and horseradish peroxidase-anti-rabbit second antibody, respectively. The negative control group was omitted by incubating with primary antibody against ANP. The slice was visualized and photographed under a fluorescence microscope (Olympus IX71, Tokyo, Japan).

Flow cytometry analysis of gastric epithelial cell

Eight weeks after injection with STZ, the mice were euthanized by lethal dose of intraperitoneal pentobarbital sodium (50 mg/kg). The stomach was removed and washed with saline. After the stomachs were inverted to make mucosal-side-out stomachs, the stomachs were rinsed with saline, and pronase solution was injected into the stomach. The pronase was diluted into 1 mg/mL with MA solution (0.5 mmol/L NaH_2PO_4, 1 mmol/L Na_2HPO_4, 20 mmol/L $NaHCO_3$, 80 mmol/L NaCl, 5 mmol/L KCl, 50 mmol/L HEPES, 11 mmol/L glucose, 20 g/L BSA, 2 mmol/L EDTA; pH = 7.4). The stomach sacks were incubated in the oxygenated MA solution for 3 × 30 min, at 37℃ equilibrated with 95% oxygen and 5% CO_2, followed by gently stirring for 1 h in MB solution (0.5 mmol/L NaH_2PO_4, 1 mmol/L Na_2HPO_4, 20 mmol/L $NaHCO_3$, 80 mmol/L NaCl, 5 mmol/L KCl, 50 mmol/L HEPES, 11 mmol/L glucose, 10 g/L BSA, 1 mmol/L $CaCl_2$, 1.5 mmol/L $MgCl_2$; pH = 7.4). MB solution that contained the gastric mucosal cells was collected, and filtered through a 200-mesh cellular sieve. The solution sample was centrifuged at 1500 × *g* for 5 min. The centrifuged sample was re-suspended with MC solution (0.5 mmol/L NaH_2PO_4, 1 mmol/L Na_2HPO_4, 20 mmol/L $NaHCO_3$, 80 mmol/L NaCl, 5 mmol/L KCl, 50 mmol/L HEPES, 11 mmol/L glucose, 1 mmol/L $CaCl_2$, 1.5 mmol/L $MgCl_2$, 1 mmol/L dithiothreitol; pH = 7.4) and centrifuged at 1500 × *g* for 5 min again. The density of the cell suspension was adjusted to 3 × 10^6/mL. The cells were fixed with 75% cold alcohol (-20℃, 24 h), and washed with PBS, followed by 0.2% Triton X-100 for 10 min. The antigen was blocked by 10% goat serum diluted in PBS, and incubated with antibody against ANP (sc-20158; Santa Cruz Biotechnology; 1:50 dilution) overnight at 4℃. The cells were stained with FITC-conjugated goat anti-rabbit IgG and examined by flow cytometry (Becton Dickinson). Using Cellquest software, 10^4 cells were analyzed per sample.

Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of ANP, and NPR-A gene expression

The whole gastric tissue was quickly removed from the mouse. Total RNA was isolated from the tissue as recommended by the manufacturer of TRIzol Reagent (Sigma). RNA concentration was determined by absorbance reading at 260/280 nm. Reverse transcription was performed with a volume of 20 μL mixture that contained 11 μL mRNA, and 1 μL oligo dt_{18}, which was incubated at 70℃ for 5 min, and 4 μL 5 × reaction buffer, 2 μL dNTP, 1 μL RNase inhibitor, 1 μL M-MLV RT was added to the mixture, followed by incubation at 42℃ for 1 h. The enzyme was inactivated by heating at 70℃ for 10 min. cDNA samples were used for analyzing specific cDNA of ANP and NPR-A. One microliter of cDNA was added to 19 μL PCR reaction mixture that contained: 7 μL nuclease-free water, 10 μL 2 × reaction buffer, 1 μL sense primer, and 1 μL anti-sense primer. The following conditions were used for PCR amplification: for GAPDH, 95℃ for 4 min; 95℃ for 30 s; followed by 40 cycles at 52.9℃ for 1 min; 72℃ for 30 s; 72℃ for 7 min; for ANP, 40 cycles at 54.6℃ for 30 s; for NPR-A, 60℃ for 30 s. RT-PCR was performed on an iCycler™ Thermal Cycler (Bio-Rad, Hercules, CA, USA) using the Access RT-PCR System (Promega, Madison, WI, USA). Specific primers for murine GAPDH, ANP and NPR-A were synthesized by Sangon Biological Company (Shanghai, China). The primer sequences were as follows: GAPDH (sense) 5'TCAACGGCACAGTCAAGG3', GAPDH (antisense) 5'ACCAGTGGATGCAGGGAT3'; ANP

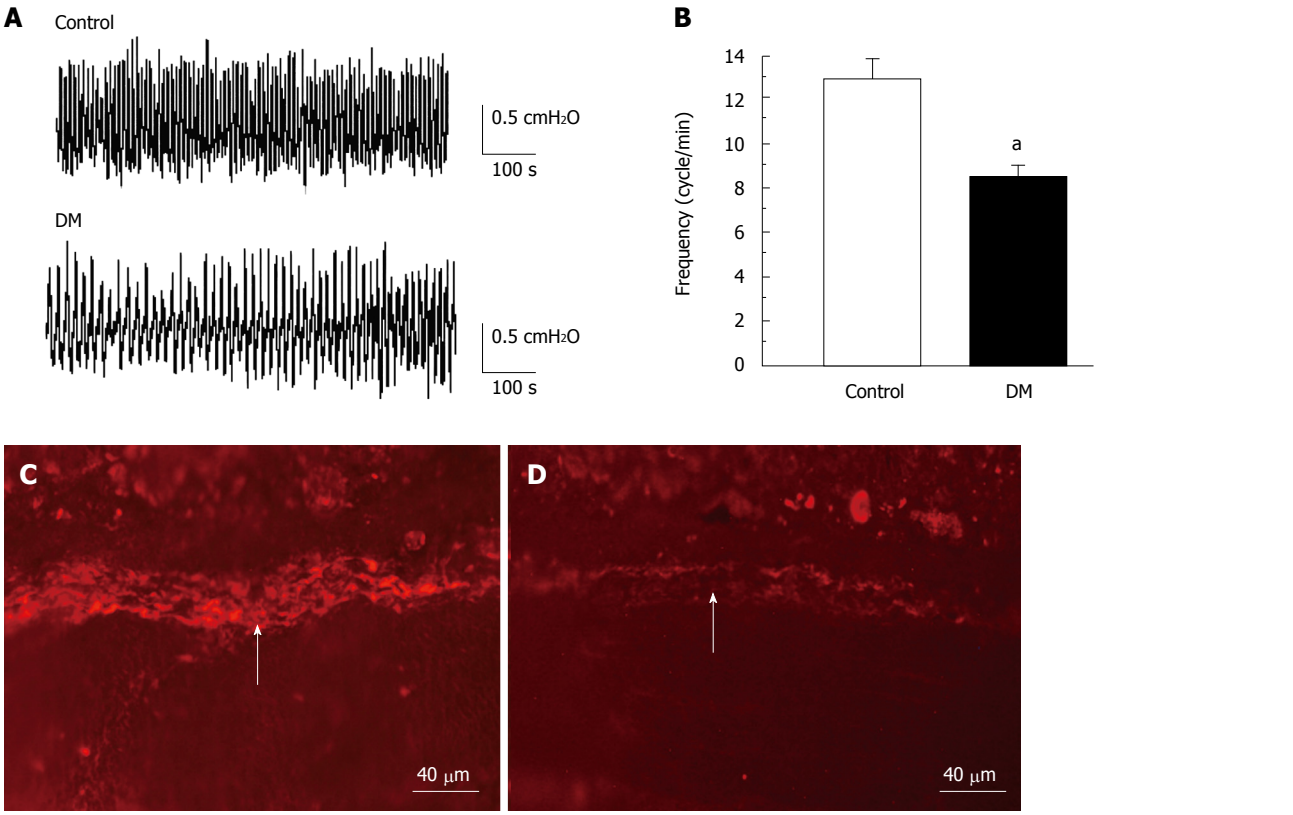

Figure 1 Comparision of gastric contractility and expression of ICCs in normal and STZ-induced diabetic mice. A: Representative traces of spontaneous gastric contraction in control and STZ-induced diabetic mice; B: Summary of amplitudes and frequencies of gastric motility in control and STZ-induced diabetic groups (n = 8; ^{a}P < 0.05 *vs* control group); C and D: Expression of c-Kit-positive cells in the muscle layer. Arrow indicates the positive staining area. Expression of c-Kit was more obvious in STZ-induced diabetic mice than in the normal controls. Scale bar = 40 μm.

(sense) 5'TCCTTCTCCATCACCCTG3', ANP (antisense) 5'CCTAGAGCACTGCCGTCT3'; NPRA (sense) 5'AGACGATGGGCAGGATAG3', NPRA (antisense) 5'GGATGTCAGGAGGTGGGT3'. The PCR products were size-fractionated by 1% agarose gel electrophoresis, and visualized under ultraviolet light with 0.5% ethidium bromide staining. GAPDH, ANP and NPR-A cDNAs were quantified by Gel Doc XR System and Quantity One software (Bio-Rad). Gene expression for ANP and NPR-A was normalized to that for GAPDH.

Statistical analysis

The data was expressed as the mean ± SE. We evaluated differences between the treatment groups using Student's t test. Differences were considered to be significant at P < 0.05. The density index referred to the number of ANP-positive cell per field of vision.

RESULTS

Change in plasma glucose concentration

We detected changes in blood glucose concentration. Mice were used at 8 wk after injection of STZ. At the time of the study, most STZ-treated mice exhibited hyperglycemia; their average blood glucose concentration was 26.7 ± 1.3 mmol/L (n = 32), which was significantly higher than that of the non-diabetic control mice (6.9 ± 0.6 mmol/L, n = 32; P < 0.01).

Gastric motility and ICC expression in STZ-induced diabetic mice

To determine whether diabetic gastropathy occurred, the amplitude and frequency of spontaneous gastric contraction were observed in normal and STZ-induced diabetic mice. The frequency of spontaneous gastric contraction decreased significantly from 12.9 ± 0.8 cycles/min in the control group to 8.4 ± 0.6 cycles/min in diabetic mice (n = 8, P < 0.05; Figure 1A and B). However, the amplitude of contraction was not changed in the diabetic group. Spontaneous rhythmic contraction of gastrointestinal smooth muscle is triggered by ICCs, which are also mediators of neuromuscular transmission in the gastrointestinal tract. Depletion of ICCs contributes to dysfunction of gastrointestinal motility in patients and animal models[24]. ICCs distributed in the gastric smooth muscle layer were observed by immunochemistry. c-Kit protein, an ICC marker, was detected in the inter-muscular layer of normal and diabetic mice (Figure 1C and D), but the expression was significantly decreased in the diabetic group (n = 6; Figure 1D). The results suggested that the STZ-induced diabetic mice exhibited gastric dysfunction, and the reduced frequency of gastric motility in diabetic mice might have been related to ICC depletion.

ANP secretion from gastric mucosa

Besides the heart, the ANP family is also distributed in other organs, and some studies have found that ANP

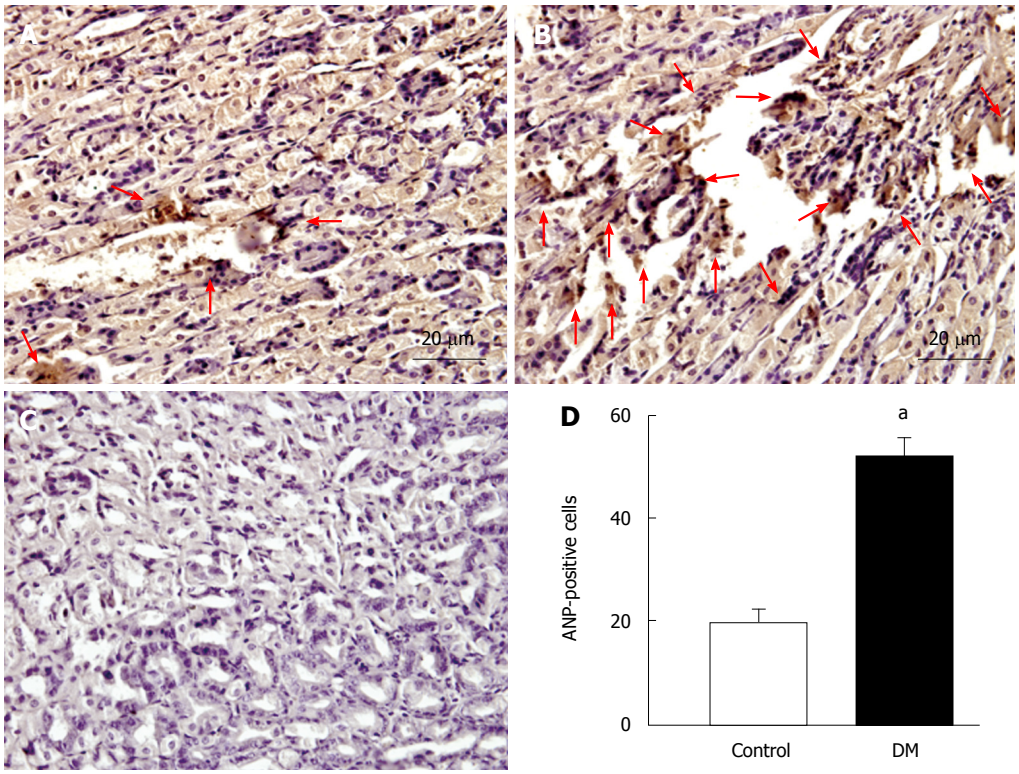

Figure 2 Immunohistochemical analysis of atrial natriuretic peptide (ANP) in mouse gastric tissue. The photographs show ANP expression in gastric mucosa in the control group (A), STZ-induced diabetic group (B), and negative control group (C); The paranuclear cytoplasmic stained cells that display an intense dark brown color were ANP-positive-staining cells in the mucosa. The red arrows indicate ANP-positive cells; B shows that the number of ANP-positive cells increased in the STZ-induced diabetic group; D shows the density index that referred to the number of ANP-positive cells per field of vision (n = 8, ^{a}P < 0.05 *vs* control group). Scale bar = 20 μm.

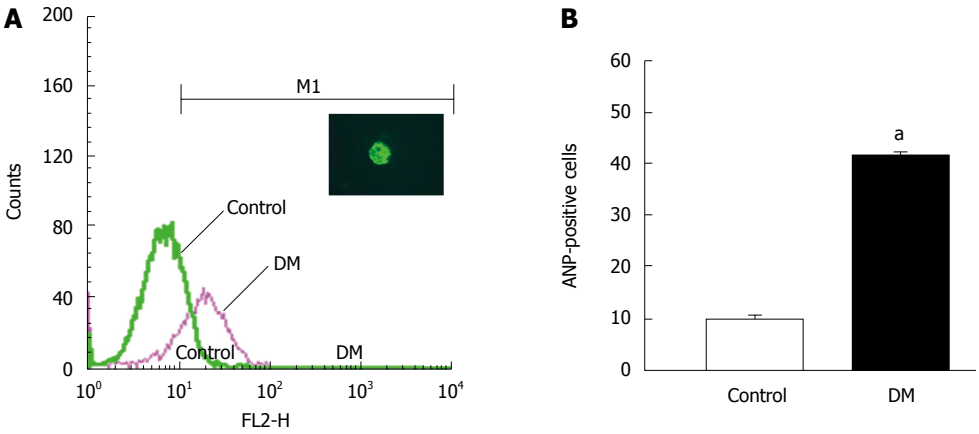

Figure 3 Flow cytometric analysis of ANP-positive cells among gastric mucosal cells. The percentage of ANP-positive cells was determined by flow cytometry with FITC-conjugated goat anti-rabbit IgG. A: Relative fluorescence intensity of gastric mucosal cells in the control and diabetic groups. M1 indicates the proportions of ANP-positive cells that displayed positive FITC labeling. The inserted fluorescent image is a single ANP-positive cell that had a positive fluorescent signal in its cytoplasm (× 400); B: ANP-positive cells among the gastric mucosal cells (10^4 cells per sample) in control and STZ-induced diabetic mice (n = 3, ^{a}P < 0.05 *vs* control).

can be secreted from gastric mucosa[13-15]. To determine whether ANP secretion from gastric mucosa is enhanced in STZ-induced diabetic mice, ANP-positive cells in the gastric mucosa were observed in the control (Figure 2A) and STZ-induced diabetic (Figure 2B) mice. The number of ANP-positive cells was increased significantly in the gastric mucosa of diabetic mice, and the density index was enhanced from 20.9 ± 2.2 cells/field of vision in the controls to 51.8 ± 2.9 cells/field of vision in the diabetic group (n = 8, P < 0.05; Figure 2D). We further analyzed the number of ANP-positive cells in gastric epithelial cells by flow cytometry. The relative fluorescence intensity of the diabetic group was higher than that of the control group (Figure 3A). The number of ANP-positive cells was increased significantly in the diabetic group. The percentage of ANP-positive cells among dispersed epithelial cells was enhanced from 10.0% ± 0.9% in the control group to 41.2% ± 1.0% in the

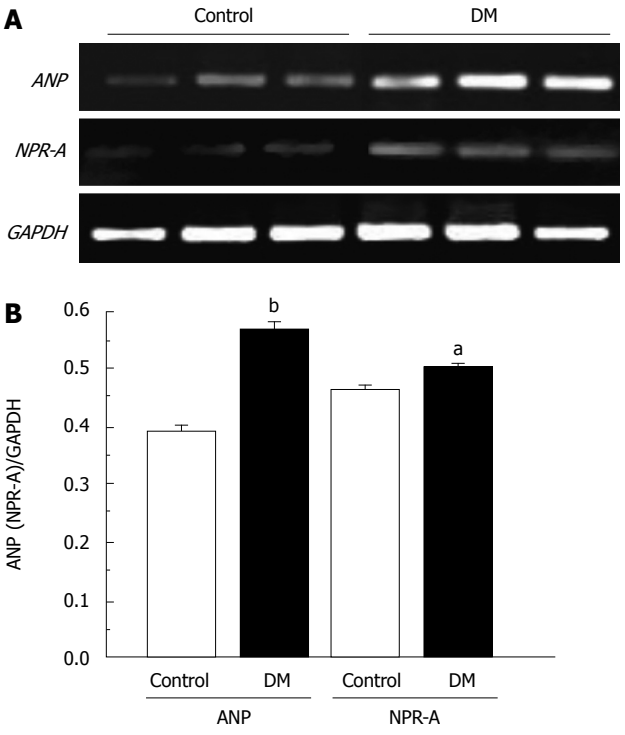

Figure 4 RT-PCR of gastric *ANP* and *NPR-A* genes in control and STZ-induced diabetic mice. A: Results of agarose gel electrophoresis, including *ANP*, *NPR-A* and *GAPDH* genes. The density signal was normalized to that of GAPDH. The mean value of the density was obtained from three separate experiments; B: Expression of *ANP* and *NPR-A* genes was increased in STZ-induced diabetic mice (n = 3; $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control).

diabetic group (n = 3, $P < 0.05$; Figure 3B). The results suggested that ANP secretion from gastric mucosa was potentiated in the stomach of STZ-induced diabetic mice.

Expression of ANP and NPR-A gene

The number of ANP-positive cells was increased in the STZ-induced diabetic group, therefore, we investigated whether *ANP* and *NPR-A* gene expression was also increased. *ANP* and *NPR-A* gene expression was observed in gastric tissue by RT-PCR. *ANP* and *NPR-A* gene expression was increased in the diabetic group (n = 3; Figure 4A). The signal was normalized to that of GAPDH. The ratio of ANP/GAPDH and NPR-A/GAPDH was 0.54 ± 0.02 and 0.5 ± 0.01 and 0.39 ± 0.01 and 0.46 ± 0.02 in the STZ-induced diabetic and control group, respectively (n = 3, $P < 0.05$ and $P < 0.01$; Figure 4B).

DISCUSSION

Diabetic gastroparesis is defined as slow gastric emptying in the absence of mechanical obstruction, and constipation is considered to be the most significant clinical manifestation. It occurs in 30%-60% of diabetic patients observed at tertiary referral centers[25]. Many investigators view these changes as the consequences of irreversible autonomic neuropathy, which affects the vagus and sympathetic nerves primarily[2,5]. However, the cause of gastroparesis is multifactorial and also involves enteric neurons, mucosal endocrine cells, smooth muscle cells and ICCs[4]. The main conclusion drawn from the present study is that the ANP/NPR-A signaling pathway is upregulated and may contribute to the development of gastroparesis in STZ-induced diabetic mice, and then lead to gastric motility dysfunction.

In non-obese diabetic (NOD) mice, a well-established model of human type I diabetes mellitus, gastric emptying of solids is delayed after 6-8 wk of untreated diabetes[23]. In the present study, we established an STZ-induced diabetic model and gastric motility was observed *in vitro* after 8 wk of untreated diabetes. The frequency of spontaneous contraction in the STZ-induced diabetic group was decreased significantly, however, the amplitude of contraction was not significantly different between these two groups. The results suggest that diabetic gastropathy mostly exhibits slow rhythm and turbulence, however, the amplitude of spontaneous contraction is not affected significantly in the early stage of diabetes. ICCs play a key role in gastric motility, and damage to the ICC networks may contribute to the development of gastropathy and gastroparesis[26]. In the present study, we confirmed whether the number of ICCs in the gastric smooth muscle layer was changed, by using immunochemistry in the STZ-induced diabetic mice. c-Kit expression in the muscle layer was observed in normal and diabetic mice, however, c-Kit expression was decreased in diabetic mice. The results suggest that gastric dysfunction in STZ-induced diabetic mice may be related to ICC depletion.

Atrial myocytes are the main source of ANP, but ANP is also found in other tissues, such as the gastrointestinal tract[27]. ANP and its receptor NPR-A have been detected in the gastric antrum[28]. NPR-A has the same membrane-bound guanylate cyclase activity as NPR-B, which catalyzes the formation of cGMP from GTP[9,10]. Some studies have demonstrated that ANP has an inhibitory effect on the regulation of gastrointestinal motility; for example, in chicken rectum[16], rat tenia coli[17] and guinea-pig cecum[3]. Our previous study also has indicated that NPs relax gastric circular and longitudinal smooth muscles in human, rat and guinea-pig stomach, and NPRs are distributed in the rat gastric smooth muscle layer[18,19]. Another previous study has indicated that ANP release is augmented in the atrium of STZ-induced diabetic rats[29]. Recently, we have found that the expression of NPR-B gene is increased and NP-dependent guanylate cyclase/cGMP signaling is upregulated in STZ-induced diabetic rats[20,21]. ANP is also secreted from the gastric mucosa[15], therefore, ANP can be considered endogenous to the gastric mucosa. However, the relationship between the ANP/NPR-A signaling pathway and STZ-induced diabetic gastroparesis is not clear. In the present study, we confirmed that the expression of ANP in gastric mucosa was increased significantly in STZ-induced diabetic mice. We demonstrated that the percentage of ANP-positive

cells was also enhanced significantly among the dispersed gastric epithelial cells, and the expression of the *ANP* gene in gastric tissue was upregulated in STZ-induced diabetic mice. These results suggest that ANP secretion from gastric mucosa is increased significantly in STZ-induced diabetes. The amount of ANP secretion is increased in diabetic mice, therefore, we investigated whether expression of NPR-A in the stomach was upregulated in STZ-induced diabetic mice. We demonstrated that expression of the *NPR-A* gene was also increased significantly in gastric tissue of diabetic mice. The results suggest that the ANP/NPR-A signaling pathway is upregulated in the stomach of STZ-induced diabetic mice.

Our previous study has demonstrated that the CNP/NPR-B signaling pathway is upregulated in STZ-induced diabetic rats[20,21], while the present study indicates that the ANP/NPR-A signaling pathway is also upregulated in STZ-induced diabetic mice. Previous studies have indicated that diabetes may also affect expression of the *ANP* gene; for example, *ANP* gene expression in heart and kidney are increased in STZ-induced diabetic rats[11,30] and plasma concentration of pro-ANP is increased in comparison with control rats[31]. Christoffersen *et al*[32] also have reported that diabetic mice show an increase in *NPR-B* gene expression in the heart, and have suggested that increased NPR-B signaling affects development of diabetic cardiomyopathy. The NP/NPR signaling pathway has an inhibitory effect on gastrointestinal motility, therefore, upregulation of this signaling pathway may be involved in development of gastroparesis in STZ-induced diabetic mice.

In summary, gastroenteropathy causes considerable morbidity in patients with diabetes mellitus and it has become a major healthcare burden. Current treatments are mainly symptomatic and frequently ineffective. Development of new therapeutic options is hampered because of poor understanding of the underlying pathological mechanisms. Our study demonstrates that the ANP/NPR-A signaling pathway is upregulated. The results suggest that ANP/NPR-A signaling is involved in the development of gastroparesis in STZ-induced diabetic mice.

COMMENTS

Background

Gastroparesis is a gastrointestinal complication of diabetes, which is also called delayed gastric emptying. The mechanism of gastroparesis is not clear, but a recent study has reported that the C-type natriuretic peptide-natriuretic peptide receptor B (CNP/NPR-B) pathway is upregulated in the stomach of diabetic rats. Atrial natriuretic peptide (ANP) has an inhibitory effect on motility of the gastrointestinal tract, but whether secretion of ANP is increased in the stomach of diabetic mice has not been reported.

Research frontiers

NPs are located in several organs besides the heart. Recently, the authors have found that ANP and its receptor (NPR-A) are also present in the mouse stomach, and the CNP/cGMP pathway in diabetic gastroparesis was investigated in STZ-induced diabetic rats. This study was designed to investigate whether ANP secretion is altered in the stomach of STZ-induced diabetic mice.

Innovations and breakthroughs

ANP with an inhibitory effect on gastrointestinal motility is present in the stomach, and previous studies have focused on the relationship between the CNP/NPR-B pathway and gastric motility. This is believed to be the first study to investigate ANP secretion and NPR-A in the stomach of STZ-induced diabetic mice. The results suggest that ANP secretion and expression of NPR-A mRNA are both increased in the stomach of STZ-induced diabetic mice. This may be involved in stomach motility dysfunction in STZ-induced diabetic mice.

Applications

The dysfunction of stomach motility that occurs in diabetic gastroparesis may be related to ANP secretion and upregulation of NPR-A. This may contribute to the treatment and preventive intervention of diabetic gastroparesis in the future.

Terminology

Gastroparesis also called delayed gastric emptying, and usually occurs in diabetes. Symptoms of gastroparesis include heartburn, pain in the upper abdomen, vomiting, nausea, and early feeling of fullness.

Peer review

This study deals with questions about the mechanisms involved in gastroparesis in diabetes.

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BRIEF ARTICLE

Double balloon enteroscopy in children: Diagnosis, treatment, and safety

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Abstract

AIM: To assess the feasibility and utility of double balloon enteroscopy (DBE) in the management of small bowel diseases in children.

METHODS: Fourteen patients (10 males) with a median age of 12.9 years (range 8.1-16.7) underwent DBE; 5 for Peutz-Jeghers syndrome (PJ syndrome), 2 for chronic abdominal pain, 4 for obscure gastrointestinal (GI) bleeding, 2 with angiomatous malformations (1 blue rubber bleb nevus syndrome) having persistent GI bleeding, and 1 with Cowden's syndrome with multiple polyps and previous intussusception. Eleven procedures were performed under general anesthesia and 3 with deep sedation.

RESULTS: The entire small bowel was examined in 6 patients, and a length between 200 cm and 320 cm distal to pylorus in the remaining 8. Seven patients had both antegrade (trans-oral) and retrograde (trans-anal and *via* ileostomy) examinations. One patient underwent DBE with planned laparoscopic assistance. The remaining 6 had trans-oral examination only. The median examination time was 118 min (range 95-195). No complications were encountered. Polyps were detected and successfully removed in all 5 patients with PJ syndrome, in a patient with tubulo-villous adenoma of the duodenum, in a patient with significant anemia and occult bleeding, and in a patient with Cowden's syndrome. A diagnosis was made in a patient with multiple angiomata not amenable to endotherapy, and in 1 with a discrete angioma which was treated with argon plasma coagulation. The source of bleeding was identified in a further patient with varices. DBE was normal or revealed minor mucosal friability in the remaining 3 patients. Hence a diagnostic yield of 11/14 with therapeutic success in 9/14 was achieved.

CONCLUSION: Double balloon enteroscopy can be a useful diagnostic and therapeutic tool for small bowel disease in children, allowing endo-therapeutic intervention beyond the reach of the conventional endoscope.

Key words: Double balloon enteroscopy; Gastrointestinal; Peutz Jeghers syndrome; Wireless video capsule endoscopy; Children

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INTRODUCTION

The advent of flexible fiberoptic endoscopes transformed

the diagnosis and management of gastrointestinal (GI) disorders in adults and children, allowing direct visualization with targeted mucosal biopsies. Furthermore, endo-therapeutic procedures have now been possible throughout the upper GI tract and ileo-colon. However, the small bowel distal to the ligament of Trietz is inaccessible to conventional GI endoscopes. Recently, push enteroscopy, allowing the therapeutic endoscopist access up to 70-100 cm beyond the pylorus[1-4], intra-operative enteroscopy techniques which are relatively invasive[5,6], and wireless video capsule endoscopy (WCE) which affords excellent diagnostic yield combined with lack of morbidity but is non-therapeutic[7-9], have been performed.

Double balloon enteroscopy (DBE) is a more recent modality which enables high resolution endoscopic imaging of the entire small bowel, allowing interventional endo-therapy (e.g. non-variceal hemostasis, snare polypectomy and pneumatic balloon stricture dilatation)[10-12]. It is clear that this technology could allow treatment of lesions, possibly identified by WCE or other less invasive investigations such as magnetic resonance imaging (MRI) enteroclysis, in parts of the small bowel inaccessible to standard endoscopy, and hence may preclude the need for formal surgical approaches in such children. We present the first pediatric-only experience of DBE, although 2 predominantly adult series have included a few children with an age range up to 20 years and no specification as to those under 16 years[13,14].

MATERIALS AND METHODS

We prospectively collected the following data on all consecutively enrolled children between January 2004 and December 2007. All had undergone upper endoscopy, ileo-colonoscopy, and most had had WCE. Various imaging techniques had been employed but none had undergone virtual CT. The double balloon enteroscopy system (Fujinon; Fujinon Inc., Japan) (Figure 1) consists of a high resolution video enteroscope (EN-450P5/20) with a flexible over-tube (TS-12140), as well described elsewhere. The enteroscope has a working length of 200 cm and an outer diameter of 8.5 mm. The flexible over-tube has a length of 140 cm and outer diameter of 12 mm. Two enteroscopes are available, currently with 2.2 mm and 2.8 mm working channels, allowing therapeutic intervention. The enteroscopes and over-tube have balloons fitted at the distal tip of each, which are sequentially inflated and deflated with air from a pressure controlled pump system with a maximum inflatable pressure of 45 mmHg.

Specifics of the procedure are not provided here as these have been well documented elsewhere and do not differ in pediatric practice compared to that in adults. Patients received bowel preparation as for colonoscopy in anticipation of a trans-anal approach. For bowel preparation, Senokot 1-2 mg/kg (max 30 mg) and sodium picosulphate 2.5-10 g (depending on age) were given on the evening prior to the day of procedure with sodium picosulphate repeated on the morning of the procedure.

The new double balloon enteroscopy (DBE) system features the following components
An EN-450P5/20 video enteroscope
A 400 (VP-402, XL-402) processor
A TS-12140 overtube
BS-1 balloons
A PB-10 balloon controller

Figure 1 Double balloon enteroscope system configuration.

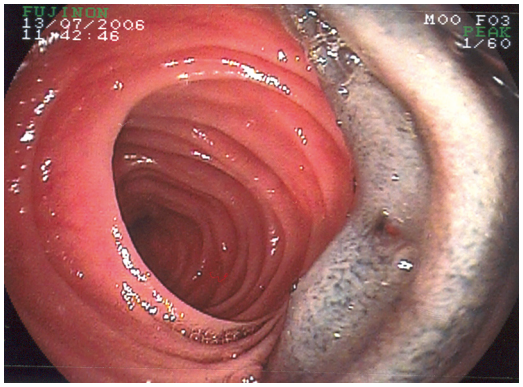

Figure 2 Double balloon tattoo.

The preference was for general anesthesia but moderate conscious sedation was employed in the older patients in one centre. If the terminal ileum (TI) was not reached then the most distal part of the small bowel negotiated was "tattooed" in the sub-mucosal plane with an endo-needle (Figure 2). The DBE could then be repeated *via* the trans-anal route and retrograde movement from the TI proximally was attempted to attain the marked area. No external compression, fluoroscopy, or other aides were necessary or useful in aiding intubation. No anti-spasmodics were employed, and air rather than carbon dioxide was insufflated. If lesions were encountered it was usual practice to treat as they were encountered rather than on withdrawal in case the lesions were not then found again. Potential adverse events such as pancreatitis, perforation, or bowel damage due to traction or torsion around the mesentery have been reported in the adult literature. Two of the authors (Thomson M and Jaacobs M) performed all of the DBE having attained competence in DBE in adult patients first. Training and learning curves for this procedure are, it is estimated by the procedurists, similar to that encountered in ileo-colonoscopy, and clearly it is not yet apparent in pediatric practice how many DBE procedures are necessary in order to attain a high degree of competence.

Patients

Fourteen patients (10 males), median age 12.9 years (range 8.1-16.7), median weight 39.6 kg (range 24.1-67.3)

Table 1 Investigations performed on patients prior to DBE

1	WCE: ?small sessile polyp in mid-small bowel
2	Intra-operative enteroscopy with polypectomy MRI enteroclysis: normal
3	OGD: polyps in stomach and duodenum MRI: 3 big polyps in small bowel
4	WCE: multiple polyps in mid-small bowel
5	WCE: possible polyp in ileum
6	WCE: ?polyps seen in small bowel, ?intermittent intussusception Colonoscopy: polyp in rectum
7	WCE: normal, Abdominal Ultrasound: enlarged spleen
8	WCE: no source of bleeding
9	OGD, WCE: lymphonodular hyperplasia in duodenum of little clinical significance
10	OGD, WCE: no positive findings
11	OGD, Ileo-colonoscopy, WCE: multiple blue rubber bleb nevus lesions throughout bowel
12	WCE: angioma in small bowel
13	WCE: polyp in mid-small bowel
14	WCE: multiple polyps seen throughout the small bowel including a lymphangitic polyp

DBE: Double balloon enteroscopy; WCE: Wireless video capsule endoscopy; OGD: Gastroduodenoscopy.

underwent DBE. Indications: patients 1-5 for Peutz-Jeghers syndrome (PJS); patients 6-7 for recurrent abdominal pain; patients 8, 9, 10 and 13 for obscure GI bleeding. Patients 11 and 12 had, respectively; blue rubber bleb nevus syndrome and an angioma identified by WCE, and had transfusion-dependent persistent GI bleeding. Patient 14 had Cowden's syndrome with previous episode of intussusceptions. Thirteen patients had undergone WCE and 1 MRI enteroclysis (Table 1).

Eleven patients received general anesthesia and 3 procedures were performed under sedation with fentanyl and midazolam. Thirteen patients had trans-oral DBE, of whom 6 patients also had trans-anal, and 1 patient had trans-stomal DBE through an ileostomy. One patient underwent intra-operative DBE.

RESULTS

The results of this investigation suggest that DBE is both useful and feasible in children with small bowel disease. The entire small bowel was examined in 6 patients, either trans-oral alone or with both trans-oral and trans-anal DBE. When TI was not attained, trans-oral progression was assessed as approximately 200 to 320 cm beyond the pylorus (Table 2), based on the assumption that each set of maneuvers to advance the enteroscope traversed around 30 cm of bowel with diminishing distance the more attempts at advancement were made. No fluoroscopy was used hence these estimates of distance attained are presumptive. The median examination time was 118 min (range 95-195). No complications of DBE were encountered, and mild post-procedure abdominal discomfort, as occurs secondary to bowel insufflation in some ileo-colonoscopies, was temporary and controlled easily with simple analgesia.

Patients 1-5 were known to have PJS. Patient 1 had not undergone WCE, and both trans-oral and trans-anal DBE allowed the whole small bowel to be visualized. No polyps were found in the small bowel; however a rectal polyp was resected which confirmed PJS on histology. Patient 2 had undergone previous intra-operative enteroscopy with polypectomy, and DBE revealed a presumably new small polyp in the jejunum which was removed. Patient 3 underwent laparoscopic-assisted DBE and 7 polyps were removed. The postoperative period was complicated by pelvic abscess requiring surgical drainage, but no intestinal perforation had occurred, hence the reason for the laparoscopic complication was unclear. Patient 4 had multiple sessile polyps in the jejunum and patient 5 had one PJS polyp removed from the mid jejunum. It is therefore suggested that PJS patients undergo WCE prior to DBE and if no polyps are found, then no DBE should take place.

Patients 6 and 7 had recurrent abdominal pain as the main presenting complaint with multiple negative investigations. Patient 6 had a family history of PJS and WCE had suggested a polyp in the mid-ileum. However, trans-oral (200 cm post-pylorus) and trans-anal (35 cm proximal to ileo-cecal valve) DBE failed to identify a polyp. Patient 7 presented with recurrent abdominal pain over 3.5 years (repeated upper GI endoscopy and ileocolonoscopy were inconclusive), and WCE had suggested proximal jejunal polyps. Trans-oral DBE demonstrated thickened folds in the proximal jejunum, which on histology proved to be a tubulo-villous adenoma. Surveillance enteroscopy after 1 year identified progression to intra-mucosal carcinoma. Surgical excision of the affected bowel and pancreas has proved curative. Clearly, this finding is very uncommon and the literature does not suggest an incidence in this age group. Of course, it is not suggested that all patients with recurrent abdominal pain undergo DBE. Clear clinical indication and warning signs such as a family history of polyp syndromes, in spite of negative WCE, are reasonable pointers towards DBE.

Patients 8-13 were investigated for GI bleeding. Patient 8 was transfusion-dependent and DBE was non-contributory since esophago-gastric varices were identified which could also have been identified and treated by standard upper GI endoscopy, although prior to transfer to our unit this procedure had not identified the varices. Mid-small bowel varices were not found at DBE. Patient 9 was investigated for obscure GI bleeding and trans-oral DBE did not reveal a bleeding site. Subsequently a Meckel's scan, initially negative, was repeated, found to be positive and surgical resection occurred. Had trans-anal DBE been performed this may have identified the Meckel's diverticulum, but this was not attempted due to a technical failure of the system and remains conjectural. The technical failure was due to the distal balloon bursting and is not considered as a dangerous adverse event.

Patient 10 had intestinal aganglionosis, an ileostomy and a gastrostomy, and presented with a 3-year history

Table 2 Details of indications, approach and findings in patients undergoing DBE

Patient No.	Age/Sex	Indication	Approach	Complete /incomplete	Findings
1	13/M	PJS	Oral + anal	Complete	Rectal polyp
2	12/M	PJS	Oral	320 cm[1]	Small polyp in jejunum
3	16/M	PJS	Intra-operative	Complete	3 small polyps removed endoscopically and 3 large polyps removed surgically
4	11/M	PJS	Oral	250 cm[1]	Multiple polyps in mid-small bowel
5	9/M	PJS	Oral	Incomplete	Mid-small bowel polyp
6	10/F	Chronic abdominal pain	Oral + anal	Up to 200 cm[1] trans-orally, 35 cm TI proximal to ICV trans-anally	Normal
7	16/M	Chronic abdominal pain with family history of colorectal carcinoma	Oral + anal	Complete	Tubulo-villous adenoma in duodenum; Lymphoid aggregates in ileum
8	11/M	Upper GI bleeds/possible vascular malformation	Oral	300 cm[1]	Grade 1 esophageal varices; no source found in small bowel
9	16/M	Occult bleeding	Oral	200 cm[1]	No source found
10	8/M	Occult bleeding	Oral + *via* ileal stoma	Complete	Increased friable mucosa throughout the small bowel
11	12/F	Blue rubber bleb syndrome with persistant GI bleeding	Oral + anal	200[1] cm trans-orally, 50 cm proximal to ICV trans-anally	Numerous angiomas throughout small bowel not amenable to therapy
12	9/F	Angioma	Oral	Incomplete	Angioma identified: APC applied
13	16/M	Occult bleeding with significant anemia	Oral + anal	Complete	Polyp 40 cm from TI: removed
14	12/F	Cowden's syndrome	Oral + anal	Complete	Multiple polyps: 2 snare polypectomies; Meckel's diverticulum found

[1]Post-pylorus distance achieved. PJS: Peutz-Jeghers syndrome; ICV: Ileo-caecal valve; APC: Argon plasma coagulation.

of transfusion-dependent obscure GI bleeding. DBE identified very friable small bowel mucosa with contact bleeding, but no histological diagnosis was concluded with normal biopsies obtained. Patient 11 had transfusion-dependent recurrent GI bleeding due to multiple lesions consistent with blue rubber bleb nevus syndrome, identified in the colon at colonoscopy, and throughout the small bowel at DBE (Figure 3). Argon plasma coagulation (APC) was used in order to ablate some of the lesions and transfusion requirement diminished. The extensive nature of the lesions precluded definitive surgery and further DBE is planned, but has not occurred to date, therefore post-APC images are not available. However, transfusion dependency in both patients 10 and 11 had ceased.

Patient 12 had angioma detected in the mid-small bowel on WCE. This was identified with DBE, and APC was applied. Patient 13 presented with occult bleeding and significant anemia, and a polyp was detected in the small bowel on WCE. At DBE, a 4 mm polypoid structure was found (Figure 4A) and removed (Figure 4B). Patient 14 had Cowden's syndrome with a history of intussusception. DBE revealed presence of multiple sessile polyps and polypectomies were performed on 2 polyps. Incidentally, a Meckel's diverticulum was found (Figure 5) in this patient.

No patients referred and considered for DBE were rejected, i.e. there seems no reason not to consider this minimally invasive approach. No complications occurred in the 13 patients who had DBE alone without intra-operative assistance. Significant post-procedure abdominal pain was not encountered, and only paracetamol was needed to counter minor abdominal discomfort, except in the individual who had undergone laparoscopy. All patients were in-patients although it is anticipated that day case procedures are viable. No evidence of pancreatitis, perforation or bowel damage was encountered, and the intra-abdominal abscess could have been the result of a micro-perforation rather than bowel damage due to the laparoscope, although the authors consider this unlikely. All patients were allowed home with no evidence of significant complications or discomfort within 24 h of the DBE being performed. All of those who did not undergo polypectomy were allowed home on the same day as the procedure. The longest duration at 195 min has to be considered as a long endoscopic procedure, but this has to be counter-balanced by the relative lack of invasiveness of the technique.

DISCUSSION

Flexible GI endoscopy is sufficient for diagnostic and therapeutic procedures in the vast majority of pediatric cases, and in adult patients with obscure GI bleeding this procedure is known to determine the source in up to 90% of cases. However, in the small number of cases where the pathology is confined to the small bowel beyond the reach of conventional endoscopy, WCE and DBE have been recently employed. In our series the entire small bowel was examined in 6/14 patients in whom trans-oral and trans-anal approaches were combined. One cannot claim that DBE diagnosed the disease in this series of patients, but it can be considered that it had a very important role in treatment. In all patients with PJ syndrome polyps were detected. Prior to the advent of these technologies, modalities such

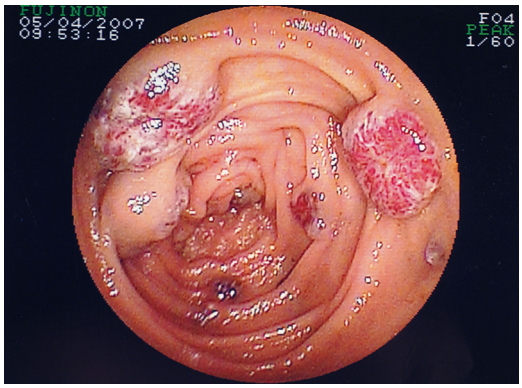

Figure 3 Multiple angiomas in small bowel.

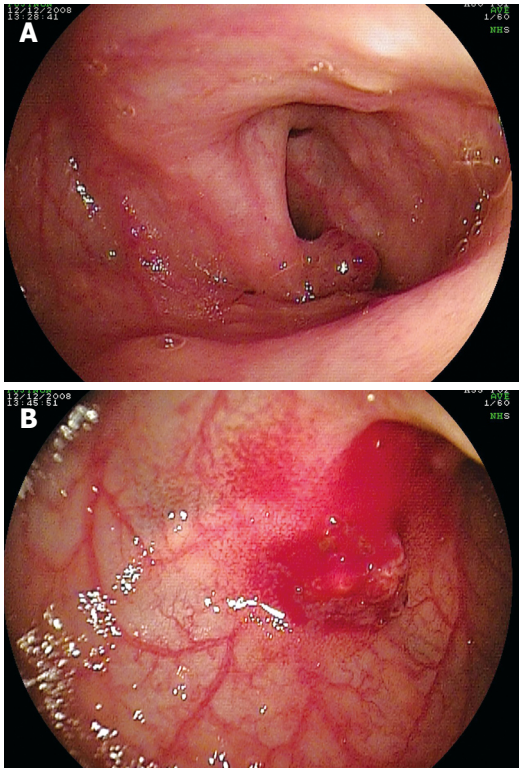

Figure 4 Polyp detected (A) and removed (B).

as push enteroscopy (PE) have had limited pediatric exposure due to safety concerns. In children, 80% of all mucosal lesions identified and biopsied by PE, and 20% of therapeutic procedures performed, were beyond the reach of a standard GI endoscope[13]. Small bowel series, angiography, scintigraphy and enteroclysis have been used with variable results in the evaluation of adult patients with obscure GI bleeding[15,16]. WCE has recently attained the position of investigation of first choice for such diagnoses, while intra-operative enteroscopy, despite its invasive quality, has been the mainstay in the subsequent treatment of obscure GI bleeding in children and adults[5,17,18].

Wireless capsule endoscopy (WCE) has been compared favorably with intra-operative enteroscopy for

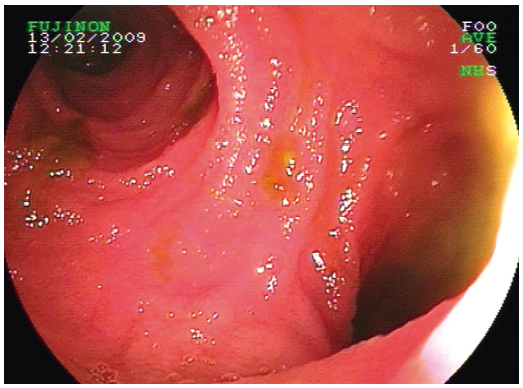

Figure 5 Meckel's diverticulum.

the diagnosis of obscure bleeding in adults, with 95% sensitivity and 75% specificity[19]. WCE has been found to be diagnostically superior to PE[20,21] and barium follow through/CT scan in obscure GI bleeding, and has been recently evaluated in children[22]. WCE is, however, non-therapeutic by its nature, and since the imperative in pediatric gastroenterology is the drive to diagnosis by mucosal histology, this is a shortcoming of WCE.

Trans-oral and, if necessary, subsequent trans-anal DBE allow therapeutic interventions such as polypectomy, hemostasis, balloon dilatation and placement of stents for the whole of the small bowel[23]. In a prospective comparative study between WCE and DBE in patients with obscure GI bleeding, the diagnostic rate was 80% for WCE and 60% for DBE; however, 51% of the patients had therapeutic intervention using argon plasma, underlining the therapeutic utility of DBE[24]. In a recent large retrospective analysis of 152 patients undergoing DBE for obscure GI bleeding in adults, 75% had the potential source of bleeding detected and, for 83% of patients, management was changed as a direct result of DBE[25]. Yamamoto has described full small intestinal examination in 86% of adults using DBE[23]. DBE in this series of children had a diagnostic yield of 11/14, and therapeutic success in 9/14 was achieved. Clearly if a regional or national small bowel diagnostic and therapeutic centre is contemplated then the duality of WCE and DBE is mandatory to achieve the goals of diagnosis and treatment without operative intervention, which should be the goal of a pediatric endoscopic centre of excellence. Hence, with the results of our prospective DBE study a case could be made for the discontinuation of push enteroscopy in the investigation and treatment of children with suspected small bowel pathology.

Complications have been reported in the literature with DBE, including intestinal perforation[26,27], pancreatitis[28] and paralytic ileus[29]. However, the only complication in our group of children occurred secondary to surgical intervention in the child who underwent intra-operative DBE.

Training remains an issue with no clear resolution attempted by this small series which included only two

experienced endoscopists.

In conclusion, double balloon enteroscopy is a useful diagnostic and therapeutic tool for the investigation of small bowel disease. It is useful in conjunction with WCE for optimizing diagnostic potential in the small bowel and offering a therapeutic option. It is also of benefit in situations where diagnosis has not been reached by other investigative modalities, and particularly in those lesions amenable for therapeutic intervention endoscopically, but not reachable by the conventional endoscope.

COMMENTS

Background

The small bowel distal to the ligament of Trietz is inaccessible to conventional gastrointestinal (GI) endoscopes. Several techniques such as push enteroscopy, intra-operative enteroscopy techniques and wireless video capsule endoscopy have been developed. All these procedures have some limitations. Double balloon enteroscopy (DBE) is a recently developed tool which enables high resolution endoscopic imaging of the entire small bowel and allows interventional endo-therapy.

Research frontiers

Small bowel is a particular area of the GI tract difficult to be imaged completely by conventional endoscope. DBE enables high resolution endoscopic imaging of the entire small bowel. In addition to diagnosis, DBE permits interventional endo-therapy.

Innovations and breakthroughs

DBE has found application in the investigation of obscure GI bleeding in adults. This is the first study to assess the usefulness, safety and diagnostic potential of DBE in children.

Applications

DBE is a valuable diagnostic and therapeutic tool for the investigation of small bowel disease both in adults and in children. DBE is useful in conjunction with other investigative modalities in optimizing diagnostic potential in the small bowel and offering a therapeutic option.

Peer review

This is a good introductory study on DBE in children.

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BRIEF ARTICLE

Z-line examination by the PillCam™ SB: Prospective comparison of three ingestion protocols

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Abstract

AIM: To evaluate the Z-line visualization by the PillCam™ SB2 using three different ingestion protocols.

METHODS: Ninety consecutive patients undergoing small bowel capsule endoscopy (SBCE) between January and May 2008 were included in the study. They swallowed the capsule in the standing (Group A = 30), supine (Group B = 30) and right supine positions (Group C = 30). Baseline patient characteristics, difficulties in capsule ingestion, esophageal transit times (ETT) and Z-line visualization were noted.

RESULTS: No significant differences were found between the groups with regard to baseline patient characteristics, ingestion difficulties and complete SB examinations ($P > 0.05$). At least 1 frame of the Z-line was detected in 15.8%, 46.7% and 90% of patients in groups A, B and C, respectively ($P < 0.001$). The average number of Z-line images was 0.21 ± 0.53, 3.23 ± 6.59 and 5.53 ± 7.55 and the mean % of the Z-line detected was 71.3, 25.1 and 8.3, in groups A, B and C, respectively (both $P < 0.001$). ETT times were longer in the supine group followed by the right supine and the standing groups (median of 237 s *vs* 64 s and 39 s, respectively; $P < 0.001$).

CONCLUSION: Z-line visualization in patients undergoing SBCE can be accurately achieved in most cases when the capsule is swallowed in the right supine position.

Key words: Barrett; Capsule endoscopy; Esophagus; Gastroesophageal reflux disease; Ingestion; Varices; Z-line

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INTRODUCTION

Small bowel examination has recently become possible because of emerging procedures such as capsule endoscopy. As demonstrated by previous studies, capsule endoscopy is an accurate, easy and safe method which allows examination of the entire small bowel in most cases[1-3]. Moreover, capsule endoscopy has been demonstrated to be more effective than small bowel follow-through and push-enteroscopy for small bowel exami-

nation[4-8]. Nevertheless, images and lesions outside the small bowel (i.e. esophagus, stomach and colon) can also be detected by the capsule[9-11]. These images or lesions are sometimes missed by conventional endoscopy[12,13] which means that non-small bowel segments of capsule videos should be carefully reviewed by physicians. As the small bowel capsule is usually swallowed in the standing position, the esophageal transit time becomes very short resulting in few images taken in the esophagus. Recent advances in capsule designs have demonstrated that an accurate examination of the esophagus is feasible[14-18]. In fact, the esophageal capsule is swallowed by the patient in the supine or right supine positions in order to increase esophageal transit time allowing the capsule to take more images in the esophagus. However, the esophageal capsule battery lasts 20 min on average, which means that only upper gastrointestinal segments, usually including the esophagus and stomach, can be examined. Since the small bowel capsule has longer battery time, the esophagus in addition to the stomach, small bowel and colon, could be explored. Whether esophageal mucosa can be accurately explored by the small bowel capsule in the supine and right supine positions has not been previously studied. The aims of this study were to evaluate and compare the Z-line visualization by the PillCam™ SB in patients undergoing small bowel capsule endoscopy using three different ingestion protocols: standing, supine and right supine positions.

MATERIALS AND METHODS

Patients

This study was conducted at a single hospital between January and May 2008. All patients who were not contraindicated to undergo capsule endoscopy, despite procedure indications, were suitable for inclusion in the study. Exclusion criteria were: age < 18 years, swallow and or esophageal motility disorders and previous prokinetic drugs administration. Patients were randomized, by means of computer-generated random numbers, to swallow the capsule in one of the three different positions: standing (Group A), supine (Group B) and right supine position (Group C).

Capsule endoscopy

All capsule procedures were performed with the PillCam™ SB2 (Given Imaging Ltd; Yoqneam, Israel). Two CE-experienced gastroenterologists (Fernandez-Urien I and Borobio E) reviewed the videos helped by the latest version of the program RAPID® 5.1.

Ingestion protocols

All patients underwent capsule endoscopy after an 8-h fast. Prokinetics, laxatives or simethicone were not used, and all patients were asked to drink 100 mL of water before capsule ingestion in order to clear the esophagus of secretions. They were also kindly asked not to talk during the ingestion procedure.

Standing position (Figure 1A): Patients from Group A were asked to swallow the capsule in the standing position with a small amount of water if required (no more than 20 mL).

Supine position (Figure 1B): Patients from Group B were asked to swallow the capsule in the supine position with a small amount of water if required (no more than 20 mL). They remained in this position for two min and then they were raised to an inclination of 30 degrees for 2 min and 60 degrees for additional 1 min in order to facilitate the transit of the capsule through the esophagus. Then, all patients were asked to drink a small sip of water (10 mL), allowed to sit upright and then asked again to drink 10 mL of water (in order to ensure complete esophageal examinations).

Right supine position (Figure 1C): Patients from Group C were asked to swallow the capsule in the right supine position with a small amount of water if required (no more than 20 mL). They remained in this position for 7 min and then were asked to drink small sips of water (10 mL) every 30 s helped by a flexible straw in order to ensure that the capsule reached the distal part of the esophagus. After that, all patients were allowed to sit upright and asked to drink 10 mL of water (in order to ensure complete esophageal examinations).

Variables analyzed

Baseline patient characteristics, difficulties in capsule ingestion, esophageal transit times (from mouth to stomach) and Z-line visualization were prospectively noted. Difficulties in capsule ingestion were classified as follows: easy when the capsule was swallowed before 1 min and without nausea, difficult when the capsule was swallowed after 1 min and/or with nausea, and impossible when the capsule was not swallowed by the patient. The Z-line visualization was measured on screen using a 4-quadrant scale (Figure 2).

Sample size

Sample size estimation is not possible in the absence of data regarding the incidence of Z-line visualization with the PillCam™ SB2 ingested in the supine and right supine positions. However, assuming an incidence of Z-line visualization of 10% in the standing position, 35% in the supine position and 70% in the right supine position, 30 patients would be required in each group to detect significant differences (with α level set at 0.05 and β at 95%).

Statistical analysis

Data from quantitative variables which did not follow a Gaussian distribution are presented as median and interquartile range (IQR) and compared using the Kruskal-Wallis and the Mann-Whitney tests. Those data from quantitative variables which followed a Gaussian distribution are presented as mean and standard deviation

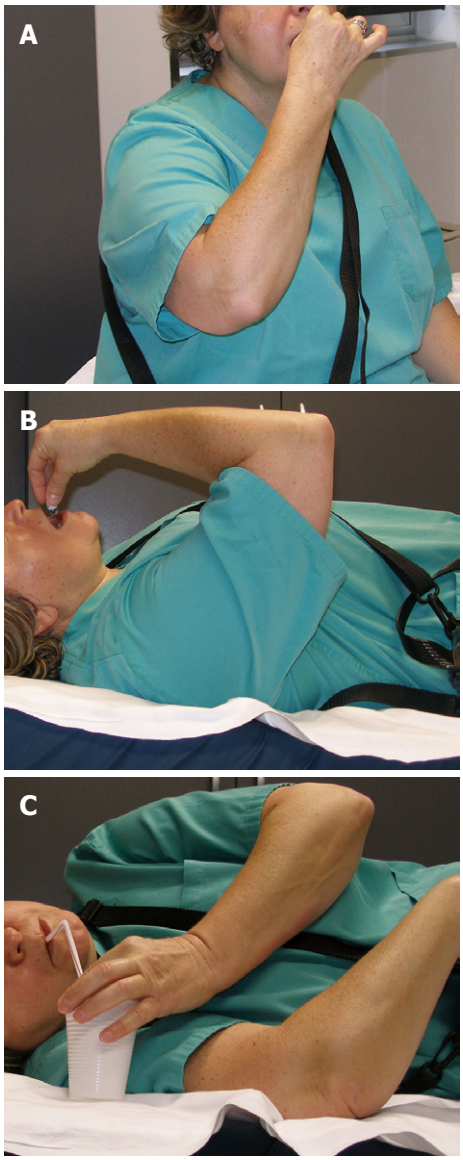

Figure 1 Capsule ingestion in the standing position (A: group A), supine position (B: group B) and right supine position (C: group C).

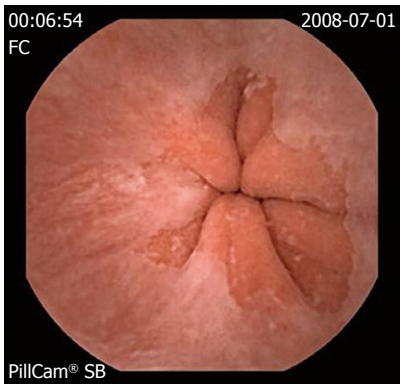

Figure 2 Z-line visualization by the PillCam™ SB.

and compared using ANOVA and Tamhane tests (for post-hoc comparisons, if needed). Qualitative variables (presented as simple proportions) and proportions are compared using the Pearson-Fischer χ^2 tests. Statistical analysis was performed with SPSS version 15.0 (SPSS Inc; Chicago, Ill, USA). Values of $P < 0.05$ were considered to be statistically significant.

Table 1 Baseline patient characteristics

	Group A (standing)	Group B (supine)	Group C (right supine)	*P* value
n	30	30	30	(-)
Age[1] (yr)	58.6 ± 19.3	56.8 ± 19.1	51.8 ± 18.3	NS
Gender (M:F)[2]	14:16	16:14	17:13	NS
BMI[1] (kg/m^2)	25.6 ± 2.8	25.2 ± 2.8	25.6 ± 5.8	NS
Indication (%)[2]	OGIB 73.1	OGIB 60	OGIB 66.7	NS
Outpatient (%)[2]	63.2	70	80	NS

[1]ANOVA test; [2]Pearson-Fischer χ^2 test. OGIB: Obscure gastrointestinal bleeding; NS: Not significant.

Table 2 Capsule ingestion *n* (%)

	Group A (standing)	Group B (supine)	Group C (right supine)	*P* value
Easy[1]	29 (96.6)	28 (93.3)	28 (93.3)	NS
Difficult[1]	1 (3.3)	2 (6.6)	2 (6.6)	NS
Impossible[1]	0 (0)	0 (0)	0 (0)	NS

[1]Pearson-Fischer χ^2 test.

RESULTS

Baseline patient characteristics

Baseline patient characteristics are shown in Table 1. There were no statistically significant differences in age, gender, body mass index (BMI), procedure indication and outpatient setting between groups A, B and C ($P > 0.05$).

Capsule ingestion

Capsule ingestion was possible in all patients (results of capsule ingestion are summarized in Table 2). Capsule ingestion was easy in more than 90% of the patients in all positions. No significant differences were observed between groups A, B and C ($P > 0.05$).

Esophageal transit time

Esophageal transit times were significantly longer in the supine group followed by the right supine and the standing groups [median (IQR) of 237 s (80-474), 64 s (40-108) and 39 s (24-55), respectively; $P < 0.001$]. Post-hoc comparisons showed that the differences group by group were also statistically significant ($P < 0.05$) (Table 3).

Z-line visualization

Table 4 shows the results concerning Z-line visualization in the standing, supine and right supine positions. At least one image of the Z-line was detected by the capsule in 15.8%, 37.3% and 90% of patients when the capsule was swallowed in the standing, supine and right supine positions, respectively ($P < 0.001$). Post-hoc comparisons showed that the differences found group by group were also significant ($P < 0.05$). The Z-line was detected by the

Table 3 Capsule transit times (s)

	Group A (standing)	Group B (supine)	Group C (right supine)	*P* value
Median (IQR)	39 (24-55)	237 (80-474)	64 (40-108)	< 0.001[1]
	Standing *vs* supine	Standing *vs* right supine	Supine *vs* right supine	
Transit times	*P* < 0.001[2]	*P* = 0.024[2]	*P* < 0.001[2]	(-)

[1]Kruskal-Wallis test; [2]Mann-Whitney test. IQR: Interquartile range.

Table 4 Z-line visualization

	Group A (standing)	Group B (supine)	Group C (right supine)	*P* value
%	4/30 (15.8%)	11/30 (37.3%)	27/30 (90%)	< 0.001[1]
Mean % Z-line	8.68 ± 22.96	25.16 ± 34.52	71.33 ± 33.47	< 0.001[2]
Mean frames	0.21 ± 0.53	3.23 ± 6.59	5.53 ± 7.55	0.017[2]
	Standing *vs* supine	Standing *vs* right supine	Supine *vs* right supine	
% patients	*P* = 0.027[3]	*P* < 0.001[3]	*P* < 0.001[3]	(-)
Mean % Z-line	NS[4]	*P* < 0.001[4]	*P* < 0.001[4]	(-)
Mean frames	NS[4]	*P* = 0.002[4]	NS[4]	(-)

[1]Pearson-Fischer χ^2 test; [2]ANOVA test; [3]Pearson-Fischer χ^2 test; [4]Tamhane test (post-hoc comparisons).

capsule in 5.5 ± 7.5, 3.2 ± 6.5 and 0.2 ± 0.5 frames per procedure in the right supine, supine and standing groups. Although these differences were significant ($P < 0.05$), post-hoc comparisons showed that only the differences between the standing and the right supine groups were significant ($P < 0.05$). The mean % of Z-line detected by the capsule was 71.3% in the right supine group, 25.1% in the supine group and 8.6% in the standing group. These differences were also significant ($P < 0.001$) but post-hoc comparisons group by group demonstrated that the differences between the standing and the supine group were not significant ($P > 0.05$).

Complete small bowel examinations

The cecum was reached by the capsule in 89.5%, 86.2% and 96.7% of cases in the standing, supine and right supine positions, respectively. These differences were not statistically significant ($P > 0.05$).

DISCUSSION

Wireless capsule endoscopy has opened a new era for small bowel examination. In fact, more than 500 000 capsule procedures have been performed worldwide. Capsule endoscopy offers excellent images of the small bowel but also from the esophagus, stomach and colon in most cases. As demonstrated by some previous studies[12,13], non-small bowel lesions detected by capsule endoscopy are sometimes missed by conventional endoscopy which means that non-small bowel segments of capsule videos should be carefully reviewed by physicians. However, esophageal examination with the PillCam™ SB has been demonstrated not to be feasible in the standing position[19] but possible in the supine and right supine positions as shown with the PillCam™ ESO capsule[14-18]. Esophageal images taken by the capsule when it is swallowed in the standing position are not usually enough in terms of number and quality. Since the first small bowel examinations, capsule endoscopy has been performed in the standing position in most institutions and the reason for this seems to be simple, to reach the duodenum as soon as possible to ensure complete small bowel examinations. Currently, the rate of complete examinations is up to 80% in published series[20] and it depends on factors such as previous abdominal surgery, patient hospitalization and diabetes. Although there are no references in the literature, it seems that capsule ingestion in the standing position does not improve the rate of complete examinations. Moreover, there is a recent study which concludes that the right supine position after capsule ingestion improves the rate of complete examinations[21]. Thus, there are no specific reasons to perform small bowel capsule endoscopy in the standing position.

The main objective of our study which was to analyze the Z-line visualization with the PillCam™ SB in the supine and right supine positions has not been previously analyzed. This new modality of the small bowel capsule endoscopy procedure could optimize the capsule resources without affecting small bowel examinations and patients' tolerability. In fact, we did not find significant differences in the rate of complete small bowel examinations and patients' swallowing difficulties despite their positions during capsule ingestion. Capsule ingestion in the right supine position was significantly more effective for Z-line visualization than the standing and supine positions. On the one hand, our results showed that the Z-line was detected in most patients who swallowed the capsule in the right position. On the other hand, the frequency and the quality of Z-line images taken by the capsule were greater in the right supine position than in the standing and supine positions. Although in some patients it was not completely visualized by the capsule in the right supine position, the Z-line was detected more than 5 times per procedure on average. Therefore, it seems reasonable to affirm that the Z-line was almost completely visualized in most cases. These results are consistent with those previously obtained by esophageal capsule endoscopy[22,23].

Surprisingly, esophageal transit times which were significantly longer in the supine group did not affect the Z-line visualization. More time in the esophagus did not mean more and better images from the Z-line. A reasonable explanation for this may be the position of the His angle at the gastroesophageal junction. While the capsule remains too long in the mid and distal esophagus but far away from the Z-line when is swallowed in the supine position, it rapidly reaches the distal esophagus but is kept by the His angle over the Z-line for several seconds in the right supine position. Therefore, the right supine position seems to be anatomically optimal for Z-line examination. Moreover, a previous study by Gralnek *et al*[22] in healthy volunteers using the PillCam™ ESO, tested

several ingestion procedures including standing, supine, right supine and left supine positions, concluding that the right supine position was the best approach to explore the distal esophagus.

Several studies have previously evaluated the feasibility of capsule endoscopy in the evaluation of the esophagus, however, the majority of them employed the PillCam™ ESO. The PillCam ESO and the ESO2 offer excellent images of more than 75% of the Z-line in most patients[22,23]. However, to our knowledge, there is only one study which has evaluated the role of the small bowel capsule for esophageal examinations[19]. In that study, an adequate assessment of the Z-line (50% and 100% of the circumference) was achieved in 10.4% and 0% of patients in the standing position and in 12.5% and 37.5% of patients in the supine position. Therefore, the authors concluded that esophageal examinations using small bowel capsule endoscopy was not feasible. Our results in patients who swallowed the capsule in the standing and supine positions are consistent with those obtained in that study, however, those authors did not include the right supine position as an additional comparative arm.

In this situation, the main question is: should all patients undergoing capsule endoscopy, despite indications, swallow the capsule in the right supine position? The answer is probably yes, because this alternative is easy to perform, is not uncomfortable for the patient, is not time consuming for physicians and the most importantly, it offers excellent images of the Z-line in most cases. However, the PillCam™ SB has to demonstrate that it is accurate in detecting esophageal lesions such as gastroesophageal reflux disease (GERD) lesions or varices. Other capsule prototypes such as the PillCam ESO capsule have demonstrated a high diagnostic accuracy for detecting GERD lesions, Barrett's esophagus and esophageal varices[14-16]. Nevertheless, it has to be taken into account that this capsule prototype takes 14 images per second and the PillCam SB, only 2 per second. Therefore, future studies in patients with suspected esophageal diseases should be performed. If favourable results are obtained, then this alternative should be used in all capsule procedures including small bowel, colon and of course, esophageal examinations.

COMMENTS

Background

Capsule endoscopy has become a very important tool for small bowel examination. However, images from other parts of the gastrointestinal (GI) tract, can also be detected by the capsule. These images or lesions are sometimes missed by conventional endoscopy, which means that images from the esophagus, stomach and colon should be carefully reviewed.

Research frontiers

Esophageal examination is not feasible if the capsule is ingested in the standing position as shown by previous studies. With recent prototypes designed for esophageal examination, new ingestion protocols have been evaluated. The supine and right supine positions have been demonstrated to be good positions to achieve a good esophageal examination. Whether the small bowel capsule is capable of examining the esophagus in these positions has not been previously studied.

Innovations and breakthroughs

This study demonstrates that the PillCam SB can accurately explore the Z-line when it is ingested in the supine and right supine positions.

Applications

Esophageal examination could be of interest in those patients who undergo capsule endoscopy of the small bowel. Missed lesions in the esophagus by conventional endoscopy could be detected by the capsule if it is ingested in the right supine position.

Peer review

This study demonstrate that Z-line examination is those patients undergoing small bowel capsule endoscopy is feasible if the capsule is ingested in the right supine position.

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BRIEF ARTICLE

Systematic review of randomised controlled trials: Probiotics for functional constipation

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Abstract

AIM: To systematically evaluate and update evidence on the efficacy and safety of probiotic supplementation for the treatment of constipation.

METHODS: The MEDLINE, EMBASE, CINAHL, and Cochrane Library databases were searched in May 2009 for randomised controlled trials (RCTs) performed in paediatric or adult populations related to the study aim.

RESULTS: We included five RCTs with a total of 377 subjects (194 in the experimental group and 183 in the control group). The participants were adults (three RCTs, n = 266) and children (two RCTs, n = 111) with constipation. In adults, data suggests a favourable effect of treatment with *Bifidobacterium lactis* DN-173 010, *Lactobacillus casei* Shirota, and *Escherichia coli* Nissle 1917 on defecation frequency and stool consistency. In children, *L. casei rhamnosus* Lcr35, but not *L. rhamnosus* GG, showed a beneficial effect.

CONCLUSION: Until more data are available, we believe the use of probiotics for the treatment of constipation condition should be considered investigational.

Key words: Randomised controlled trials; Constipation; Probiotics; Adults; Children

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INTRODUCTION

Constipation is a common condition affecting children and adults[1,2]. In the vast majority of cases, no underlying organic cause is found and functional constipation is diagnosed[3,4]. The standard treatment consists of disimpaction and the administration of laxatives to achieve a normal bowel habit of passing a soft stool without pain. Even though traditional treatment is well established and safe, for many patients it does not provide satisfying improvement, prompting interest in other therapeutic strategies[5].

Currently, probiotics, defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host[6], are increasingly being used in the management of constipation. Those most widely studied are organisms within the genera *Bifidobacterium*

and *Lactobacillus*. There are several reasons why probiotics might have therapeutic potential for the treatment of constipation. Firstly, there are data demonstrating differences in the intestinal microbiota between healthy individuals and patients with chronic constipation[7,8]. The key features are an increased number of clostridia and bifidobacteria, with different species of clostridia and enterobacteriaceae being frequently isolated. A number of key questions remain to be answered, principally, what is the origin of this dysbiosis? Is dysbiosis a secondary manifestation of constipation, or is it a factor contributing to constipation? Secondly, studies involving the administration of *B. lactis* DN-173 010 have shown improved colonic transit times, both in a healthy population[9] and in constipated patients[10]. Finally, probiotics lower the pH in the colon. This reduction in pH is due to the bacterial production of short-chain fatty acids (butyric acid, propionic acid, and lactic acid). A lower pH enhances peristalsis in the colon[8] and, subsequently, might decrease the colonic transit time.

In view of the uncertainty regarding the use of probiotics for the treatment of constipation, we decided to systematically review and update data from randomised controlled trials (RCTs) on the efficacy and safety of using probiotics for the treatment of constipation in both paediatric and adult populations. If the probiotics were effective, another aim was to determine what strain(s) of probiotic microorganisms is the most effective.

MATERIALS AND METHODS

The guidelines from the Cochrane Collaboration for undertaking and reporting the results of this systematic review were followed[11]. Briefly, we searched three electronic bibliographic databases (MEDLINE, EMBASE, and CINAHL) and the Cochrane Library. Every database was searched from inception to May 2009. Additionally, the reference lists from identified studies and key review articles assessing the effects of probiotics on the treatment of constipation were searched. While no language restrictions were applied, in practice the search was restricted to English-language papers, papers written in languages known to the reviewers, or those with English-language abstracts. The review was restricted to RCTs only carried out in paediatric or adult populations. Participants in the experimental groups received any well-defined probiotic at any dosage regimen for at least several days; those in the control group received placebo or no intervention. The search strategy included the use of a validated filter for identifying RCTs, which was combined with a topic-specific search strategy. In brief, the search terms were: *constipation* AND *probiotic*, Lactobacillus, L. GG, L. acidophilus, L. rhamnosus, L. plantarum, L. casei, L. gasseri, L. reuteri, L. lactis, Bifidobacterium, B. breve, B. longum, B. infantis, B. adolescentis, B. lactis, Bacillus, Clostridium butyricum, Streptococcus thermophilus, Escherichia coli, Propionibacterium freundendsreichii, Enterococcus SF68, Enterococcus faecalis, Saccharomyces boulardi,* and *VSL#3*. The primary clinical outcome measure was treatment success (as defined by the investigators). In addition, a priori it was decided to extract other data reported by the investigators if clinically relevant to the current review and/or adverse effects. All of the published studies that met our eligibility criteria were assessed for methodological quality, with the following strategies associated with good-quality studies: adequate generation of allocation sequences; concealment of allocation; blinding of investigators, participants, outcome assessors, and data analysts; intention-to-treat analysis (yes or no); and comprehensive follow-up ($\geqslant$ 80%).

Data extraction was performed using standard data-extraction forms. For dichotomous outcomes, the total number of participants and the number of participants who experienced the event were extracted. For continuous outcomes, the total number of participants and the means and standard deviations were extracted. If feasible, the data were entered into Review Manager (RevMan) (Computer program. Version 5.0. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2007) for analysis.

Statistical methods

As the studies we identified were not sufficiently similar and of sufficient quality we did not perform a meta-analysis. The binary measure for individual studies is reported as the risk ratio (RR) between the experimental and control groups with 95% confidence intervals (95% CI). The mean difference (MD) between the treatment and control groups was selected to represent the difference in continuous outcomes (with 95% CI).

A priori defined subgroup analyses were planned based on factors that could potentially influence the magnitude of the treatment effect, such as the probiotic strain or study population (children, adults); however, these analyses were not performed due to the limited data available.

RESULTS

By means of our systematic search, we identified six trials[12-17]. One was a protocol of an ongoing study[15], so it was not included. Thus, eventually five trials, including a total of 377 subjects (194 in the experimental group and 183 in the control group), met our predefined inclusion criteria. The characteristics of the included studies are presented in Table 1. The list of excluded trials (n = 22) is available upon request. The most usual reason for exclusion of a study was that the study was not randomised, the study was carried out in healthy volunteers, or the intervention was treatment with a symbiotic, not a probiotic alone. In addition, some studies were published in Japanese with no English abstract, and thus, were not accessible to the reviewers, even for initial screening.

All of the trials were full peer-reviewed publications.

Table 1 Characteristics of included trials

Study ID	Probiotic	Design	Allocation concealment/ Blinding/Intention-to-treat analysis/Description of withdrawals or dropouts	Exp/cont (age, yr)	Definition of constipation	Duration of intervention	Intervention (daily dose)	Placebo
Studies in adults								
Mollenbrink *et al*[13] 1994 (Germany)	*E. coli* Nissle 1917	RCT, crossover	Unclear/Yes/No/Yes	35/35 (18-60)	< 2 BM per week	4 + 4 wk	25×10^9 CFU	Placebo
Koebnick *et al*[12] 2003 (Germany)	*L. casei* Shirota	RCT, parallel	Unclear/Yes/Yes/Yes	35/35 (18-70)	Not provided	4 wk	6.5×10^9 CFU, probiotic beverage	Placebo
Yang *et al*[14] 2008 (China)	*B. lactis* DN-173 010	RCT, parallel	Unclear/No/Yes/Yes	63/63 (25-65, only women)	< 3 BM per week; increased stool hardness; non-organic constipation and habitual constipation	2 wk	Fermented milk containing 1.25 $\times 10^{10}$ CFU of probiotic plus yoghurt strains	Placebo (acidified milk without any ferments or probiotics)
Studies in children								
Banaszkiewicz *et al*[17] 2005 (Poland)	*L. rhamnosus* GG	RCT, parallel (computer-generated randomisation list)	Yes/Yes/Yes/Yes	43/41 (2-16)	< 3 BM per week during 14 days for at least 12 wk	12 wk	Lactulose plus LGG 2×10^9 CFU	Lactulose plus placebo
Bu *et al*[16] 2007 (Taiwan)	*L. casei* rhamnosus Lcr35	RCT, parallel (computer-generated randomisation list)	Yes/Yes/Yes/Yes	18/9 (< 10)	< 3 BM per week for > 2 mo plus at least one of the criteria: anal fissures with bleeding due to constipation, faecal soiling, or passage of large and hard stool	4 wk	8×10^8 CFU	Placebo (starch)

RCT: Randomised controlled trials; CFU: Colony-forming units; BM: Bowel movements.

Four of the included studies were RCTs with a parallel design, and the remaining included RCT had a crossover design. All were placebo-controlled trials. The participants were adults (three RCTs, $n = 266$) and children (two RCTs, $n = 111$) with constipation defined as stated in Table 2. The following different probiotic strains were tested: *Bifidobacterium lactis* DN-173 010, *E. coli* Nissle 1917, *Lactobacillus casei rhamnosus* Lcr35, *L. casei* Shirota, and *L. rhamnosus* GG. One RCT assessed the effectiveness of using *L. rhamnosus* GG as an adjunct to lactulose therapy compared with treatment with lactulose alone[17]. The durations of the interventions in the parallel-design studies were two weeks in one study, four weeks in two studies, and 12 wk in one study. The duration of the intervention in the crossover design study was eight weeks. The doses of the probiotic used ranged from 8×10^8 to 25×10^9 colony-forming units (CFU)/d.

The methodological quality of the trials varied. While all were randomised trials, the randomisation method was described and adequate in only two RCTs[16,17]. Except for one study[14], double blinding was applied in the remaining RCTs. An adequate description of the intention-to-treat analysis was provided in all but one study[13]. The withdrawals and dropouts were described adequately in all of the studies, and all included an adequate number (i.e. $\geqslant 80\%$) of participants in the final analysis.

Study description

RCTs in adults: The study by Mollenbrink and Bruckschen[13] was a single-centre, randomised, double-blind, crossover trial that investigated the efficacy of treating 70 constipated patients with *E. coli* Nissle 1917 or placebo. After four weeks of treatment, there was a significant difference in the mean number of stools per week in the *E. coli* group compared with the placebo group (4.9 ± 1.5 *vs* 2.6 ± 1.0, respectively, MD 2.3 stools per week, 95% CI 1.7 to 2.9), which also remained significant at eight weeks (6 ± 1.3 *vs* 1.9 ± 1.5, respectively, MD 4.1, 95% CI 3.2 to 5). This study also revealed a significant difference between the probiotic and the control group in the incidence of hard stools (2/34 *vs* 16/30, respectively, RR 0.1, 95% CI 0.03 to 0.4). Both the effectiveness and tolerance of the treatment, as assessed both by a physician and the patients, were significantly better in

Table 2 The summary of study outcomes

Study ID	Probiotic	Outcomes
Studies in adults		
Mollenbrink *et al*[13] 1994 (Germany)	*E. coli* Nissle 1917[1]	Number of stools per week [week 4: 4.9 ± 1.5 *vs* 2.6 ± 1.0, MD 2.3 (95% CI 1.7 to 2.9); week 8: MD 4.1 (95% CI 3.2 to 5)] Hard stools [2/34 *vs* 16/30, RR 0.1 (95% CI 0.03 to 0.4) ($P < 0.001$)] Effectiveness of a probiotic compared to placebo assessed by physicians: 55.9% *vs* 6.7% Effectiveness of a probiotic compared to placebo assessed by patients: 52.9% *vs* 6.7% ($P < 0.001$) Tolerance of a probiotic compared to placebo assessed by physicians: 58.85% *vs* 26.7% ($P = 0.01$) Tolerance of a probiotic compared to placebo assessed by patients: 50% *vs* 26.7% ($P = 0.03$)
Koebnick *et al*[12] 2003 (Germany)	*L. casei* Shirota[2]	Occurrence of moderate and severe constipation ($P < 0.001$) Degree of constipation ($P = 0.003$) Defecation frequency ($P = 0.004$) Occurrence of hard stools ($P < 0.001$) Degree of stool consistency ($P < 0.001$) Occurrence of flatulence (NS) Degree of flatulence (NS) Occurrence of bloating (NS) Degree of bloating (NS)
Yang *et al*[14] 2008 (China)	*B. lactis* DN-173 010[1]	Stool frequency (*n*/wk) [week 1: 3.5 ± 1.5 *vs* 2.5 ± 0.9, MD 1 (95% CI 0.6 to 1.4); week 2: 4.1 ± 1.7 *vs* 2.6 ± 1.0; MD 1.5 (95% CI 0.7 to 1.6)] Defection condition scores [week 1: 1.1 ± 0.9 *vs* 1.6 ± 1.1, MD -0.5 (95% CI -0.85 to -0.18); week 2: 0.8 ± 1.0 *vs* 1.6 ± 1.1; MD -0.8 (95% CI -1.14 to -0.44)] Grade Ⅰ (0 points)-normal defecation Grade Ⅱ (1 point)-only bearing down and uncomfortable sensation Grade Ⅲ (2 points)-obvious bearing down and uncomfortable sensation, or frequent defecation with difficult and little defecation, seldom abdominal pain or anal burning sensation Grade Ⅳ (3 points)-often abdominal pain or anal burning sensation to influence defecation Stool consistency scores (according to classification method of Bristol) [week 1: 1.0 ± 0.8 *vs* 1.4 ± 1.0, MD -0.4 (95% CI -0.73 to -0.12); week 2: 0.6 ± 0.8 *vs* 1.3 ± 1.0, MD -0.7 (95% CI -1 to -0.4)] Grade Ⅰ (0 points)-like sausage or snake, smooth and soft; like sausage, with fissure on the surface Grade Ⅱ (1 point)-sausage-shaped, with lumps; noncohesive lumps, with coarse edges Grade Ⅲ (2 points)-separating hard lumps, like fruit kernel (difficult discharge)
Studies in children		
Banaszkiewicz *et al*[17] 2005 (Poland)	*L. rhamnosus* GG[2]	Treatment success (≥ 3 spontaneous BMs per week with no episodes of faecal soiling) (NS) Number of BMs per week (NS) Number of episodes of faecal soling per week (NS) Straining at defecation frequency per week (NS)
Bu *et al*[16] 2007 (Taiwan)	*L. casei* rhamnosus Lcr35[1]	Treatment success (≥ 3 spontaneous BMs per week with no episodes of faecal soiling in the fourth week) (14/18 *vs* 1/9, RR 7, 95% CI 1.1 to 45; $P = 0.01$) Defecation frequency (times/d) (0.57 ± 0.17 *vs* 0.37 ± 0.1; MD 0.2, 95% CI 0.1 to 0.3)($P = 0.03$) Hard stool (%) (22.4 ± 14.7 *vs* 75.5 ± 6.1; MD -53% (95% CI -63 to -43) (($P = 0.01$) Abdominal pain (times) (1.9 ± 1.6 *vs* 6.7 ± 3.3; MD -4.8, 95% CI -6.6 to -3) ($P = 0.03$) Use of glycerin enema (times) (1.6 ± 1.9 *vs* 4.0 ± 2.1; MD -2.4, 95% CI -4 to -0.8) ($P = 0.04$) Use of lactulose (times) (4.4 ± 3.6 *vs* 6.2 ± 3.8; MD -1.8, 95% CI -4.7 to 1.1) ($P = 0.66$) Faecal soiling (times) (2.1 ± 3.8 *vs* 2.7 ± 1.4, MD -0.6 (95% CI -3.2 to 2) ($P = 0.95$) Change of appetite (0.7 ± 0.8 *vs* 0.7 ± 0.6; MD 0, 95% CI -0.6 to 0.6) ($P = 0.81$)

[1]Mean values are presented for the experimental group and control group, respectively; [2]Comparisons of experimental and control group. NS: Not significant.

those in the *E. coli* group. The authors concluded that *E. coli* Nissle 1917 is successful in the therapy of idiopathic chronic constipation.

The study by Koebnick *et al*[12] was a single-centre, double-blind, placebo-controlled, randomised trial involving 70 patients with symptoms of chronic constipation. All of the patients received either a probiotic beverage containing *L. casei* Shirota or placebo for four weeks. Patients completed a questionnaire related to their gastrointestinal symptoms, well-being, and stool habits, and underwent a medical examination weekly. The severity of constipation, flatulence, and bloating was divided into four categories (severe, moderately severe, mild, and no symptoms). Compared to the placebo group, those randomised to the *L. casei* Shirota group experienced a significant improvement in the self-reported severity of constipation and stool consistency. That is, they experienced significant reductions in the occurrence of moderate and severe constipation ($P < 0.001$), the degree of constipation ($P = 0.003$), and the occurrence of hard stools ($P < 0.001$), and increased their defecation frequency ($P = 0.004$). However, the occurrence and degree of flatulence or bloating sensation did not significantly differ between the groups.

In the most recent study, Yang *et al*[14] administered a fermented milk product containing *B. lactis* DN-173 010 and some yoghurt strains (*S. thermophilus* and *L. bulgaricus* (1.2×10^9 CFU/pot 100 g) (experimental group) or an acidified milk containing non-living bacteria but no *B. lactis* DN-173 010 or yoghurt strains (control group) for two weeks to constipated women. Comparison of the experi-

mental group with the control group revealed a significantly higher stool frequency after one week of product administration (3.5 ± 1.5 *vs* 2.5 ± 0.9, respectively, MD 1.0 stool per week, 95% CI 0.6 to 1.4) and at two weeks (4.1 ± 1.7 *vs* 2.6 ± 1.0, respectively, MD 1.5 stool per week, 95% CI 0.7 to 1.6). The extent of defecation difficulty was assessed as 0-3 point defecation condition scores. In brief, 0 points indicates normal defecation, while 3 points indicates often abdominal pain or anal burning sensation to influence defecation. (Table 2 for complete categorisation of defecation condition scores). Both at one and two weeks after product consumption, there was a significant improvement in the defecation condition scores in the experimental group compared with the control group: 1.1 ± 0.9 *vs* 1.6 ± 1.1, respectively (MD -0.5, 95% CI -0.85 to -0.18) at 1 wk and 0.8 ± 1.0 *vs* 1.6 ± 1.1, respectively (MD -0.79, 95% CI -1.14 to -0.44) at 2 wk. The stool consistency score was determined according to the Bristol Stool Scale. In brief, 0 points indicates stools like a sausage or a snake, smooth and soft, while 2 points indicates separating hard lumps, like fruit kernel (difficult discharge) (Table 2). The stool consistency scores were significantly improved in the *B. lactis* DN-173 010 group compared to the control group at 1 wk (1.0 ± 0.8 *vs* 1.4 ± 1.0, respectively, MD -0.4, 95% CI -0.73 to -0.12) and at 2 wk (0.6 ± 0.8 *vs* 1.3 ± 1.0, respectively, MD -0.7, 95% CI -1 to -0.4). There were no significant differences between groups in food intake and safety parameters. The researchers concluded that the administration of a fermented milk product containing *B. lactis* DN-173 010 has a beneficial effect on stool frequency, defecation conditions, and stool consistency in adult women with constipation.

RCTs in children: Only two RCTs have addressed the use of probiotics in the treatment of constipation in children. In the study by *Banaszkiewicz* and *Szajewska*[17], 84 children (aged: 2-16 years) with constipation (< 3 spontaneous bowel movements per week for at least 12 wk) were enrolled in a double-blind, randomised, placebo-controlled trial in which they received 1 mL/kg per day of 70% lactulose plus 10^9 CFU of *L. rhamnosus* GG (experimental group, n = 43) or a lactulose-containing placebo (control group, n = 41) orally twice daily for 12 wk. The primary outcome measure was treatment success; all analyses were performed on an intention-to-treat basis. Treatment success was defined as ⩾ 3 spontaneous bowel movements per week with no episodes of faecal soiling. Treatment success was similar in the control and experimental groups at 12 wk [28/41 (68%) *vs* 31/43 (72%), respectively; P = 0.7] and at 24 wk [27/41 (65%) *vs* 27/43 (64%), respectively; P = 1.0]. The groups also did not differ in their mean number of spontaneous bowel movements per week or episodes of faecal soiling per week at four, eight, and 12 wk. Adverse events and overall tolerance did not differ between groups. It was concluded that *L. rhamnosus* GG, as dosed in this study, was not an effective adjunct to lactulose in treating constipation in children.

The study by Bu *et al*[16] evaluated the effect of treating children with chronic constipation with *L. casei rhamnosus* Lcr35 compared to magnesium oxide (MgO) or placebo; however, only the latter comparison is valid for this systematic review. For those treated with the probiotic (n = 18) compared with placebo (n = 9), the trial showed an increase in the treatment success defined as ⩾ 3 spontaneous defecations per week with no episodes of faecal soiling (14/18 *vs* 1/9, respectively, RR 7, 95% CI 1.1 to 45), an increase in the defecation frequency (times/d) (0.57 ± 0.17 *vs* 0.37 ± 0.10, respectively, MD 0.2, 95% CI 0.1 to 0.3), a reduction in abdominal pain (frequency) (1.9 ± 1.6 *vs* 6.7 ± 3.3, respectively, MD -4.8, 95% CI -6.6 to -3), a reduction in the use of glycerin enemas during the four weeks of therapy (frequency) (1.6 ± 1.9 *vs* 4.0 ± 2.1, respectively, MD -2.4, 95% CI -4 to -0.8), and a decrease in the percentage of hard stools in the total number of defecations (22.4 ± 14.7 *vs* 75.5 ± 6.1, respectively, MD -53%, 95% CI -63 to -43). However, there was no difference between groups in the use of lactulose or the number of episodes of faecal soiling. No change in appetite was observed. However, the sample size was too small to draw any meaningful conclusion.

Adverse events

The probiotics were well tolerated, and no adverse events associated with this supplementation were reported in any of the trials.

DISCUSSION

Principal findings

The objective of this review was to provide some resolution to the uncertainty regarding the use of probiotics for the treatment of functional constipation in paediatric and adult populations. The main finding of the review is that there is very limited evidence available from controlled trials to evaluate with certainty the effect of probiotic administration on constipation. Data published to date suggest that adults with constipation might benefit from ingestion of *B. lactis* DN-173 010, *L. casei* Shirota, and *E. coli* Nissle 1917, which were shown to increase defecation frequency and improve stool consistency. However, in some cases, even if there was a significant difference in results, their clinical relevance is unclear. For example, compared with placebo, *B. lactis* DN-173 010 increased only by one the number of stools per week. In children, the administration of *L. rhamnosus* GG was not effective, while the administration of *L. casei rhamnosus* Lcr35 augmented the number of stools and reduced the number of hard stools. Again, although the results were statistically significant, the overall effects were clinically modest. All of the conclusions are based on single studies, some of which had a very small number of participants and methodological limitations; thus, the conclusions should be interpreted with great caution. Repeat studies with the probiotic strains that have been proven effective are needed. A paucity of data did not allow us to con-

clude whether any particular probiotic is more effective than another.

Previous reports

Previously, one systematic review[18], co-authored by one of the authors of the current review, aimed at determining the effect of probiotics on constipation was performed. This systematic review, published in 2005 (search date: January 2004), identified two RCTs with a total of 140 adult participants. It was concluded that the administration of two probiotic strains (*E. coli* Nissle 1917, *L. casei* Shirota) significantly improved stool frequency and consistency, with no difference in the degree of bloating or flatulence; no adverse effects were reported. Our updated results include results from more RCTs, thus, more precisely define the effects of using probiotics for the treatment of constipation.

Evidence from non-RCTs suggests that at least some probiotics may be effective. For example, in children with constipation defined according to the Rome Ⅲ criteria, the administration of *Bifidobacteria (B. bifidum, B. infantis,* and *B. longum)* and *Lactobacilli (L. casei, L. plantarum,* and *L. rhamnosus)* to 20 children aged 4-16 years resulted in an increased frequency of bowel movements, a decreased number of faecal incontinence episodes, and reduced abdominal pain, although there was no change in stool consistency[19]. In adults, preliminary data from a non-RCT revealed that the administration of *L. rhamnosus* and *Propionibacterium freudenreichii* resulted in a small, but significant, increase in defecation frequency[20]. However, this result was only true if the probiotics were administered together and not if only a single strain was given.

Mechanism of action

Mechanisms by which probiotics might work in the treatment of constipation have been briefly discussed in the Introduction section. Clearly, they are not well understood. Perhaps the best mechanism documented is the mechanism by which *B. animalis* DN-173 010 exerts its effects. In healthy subjects, several RCTs have evaluated the effect of *B. animalis* DN-173010 on colonic transit times. One double-blind RCT conducted in 72 healthy adults (aged 21-42 years) used radio-opaque pellets to measure colonic transit times. This study revealed a statistically significant reduction in the total colonic transit time of 21% (men: $P < 0.03$, women: $P < 0.05$) and a reduction in the sigmoid transit time of 39% ($P = 0.02$), particularly in women, with probiotic treatment. However, the beneficial effect was limited to the subjects who received living *B. animalis* DN-173 010 and was not observed in those who received heat-treated *B. animalis* DN-173 010[21]. Another double-blind RCT performed in 36 healthy women (aged 18-45 years) revealed significantly shorter total colonic and sigmoid colonic transit times ($P < 0.05$) following ingestion of 375 g/d of a fermented milk containing yoghurt cultures plus *B. animalis* DN-173 010 for 10 d, compared with the transit times observed with ingestion of the control probiotic-free product[22]. Two further non-blinded RCTs were carried out in healthy elderly subjects who were divided into groups according to their different baseline colonic transit times. Both studies demonstrated a reduction in transit times in all of the groups compared with baseline with consumption of fermented milk containing *B. animalis* DN-173 010[23,24]. Further studies are needed to confirm these findings.

Strengths and limitations

The advantage of any systematic review is the low risk of subjective data selection. Study searches, assessment, and data synthesis were all based on predefined criteria and were performed with the use of well-established repetitive tools by two reviewers independently. Nevertheless, our analysis has some limitations. First, we cannot fully exclude publication bias, i.e. publication or non-publication of data depending on the results, with negative findings being less likely to be published irrespective of the methodological quality. As studies involving the administration of probiotics are often supported by the manufacturers, the possibility remains that negative results remain unpublished. No sufficiently effective strategy of identifying such studies has been developed. Second, even though no language limitation was imposed, in practice it was not feasible to assess data from reports written in Japanese. Third, any systematic review is only as good as the constituent studies. Only some of the trials included in our analysis seemed methodologically sound. Potential limitations included unclear or inadequate allocation concealment, no intention-to-treat analysis, and no blinding. Fourth, some trials included a small sample size. Finally, the effects of probiotics are strain specific as well as population specific. While a systematic review or a meta-analysis on probiotics does provide valid information, caution should be exercised in not over interpreting the results of a meta-analysis, particularly when all probiotics have been evaluated together.

Safety issues

In general, the safety profile of probiotics seems to be good. In the included trials, no adverse effects were noted. The safety issue is important, as based on the available literature there is concern that the use of probiotics in at-risk populations may result in harmful events. Most complications have occurred in immunocompromised subjects or in patients with other life-threatening illnesses, who were managed in intensive care units and treated with probiotics.

In summary, this systematic review demonstrates that the data published to date do not yet provide sufficient scientific evidence to support a general recommendation about the use of probiotics in the treatment of functional constipation. Until such data are available, we believe that the use of probiotics for this condition should be considered investigational. Also, we believe that our demonstration of clinical uncertainty about this issue is

an important finding. As pointed out by Alderson and Roberts[25], clinical uncertainty is a prerequisite for the large-scale RCTs needed to evaluate the influence of such interventions; it also helps to clarify available treatment options and stimulate new and better research.

COMMENTS

Background

Probiotics are increasingly being used in pediatric population. However, there is still uncertainty regarding the use of probiotics for the treatment of constipation.

Research frontiers

Until more data are available, the use of probiotics for the treatment of constipation condition should be considered investigational. The large-scale RCTs are needed to evaluate the effect of specific probiotic strain(s) for the treatment of constipation.

Innovations and breakthroughs

The updated results include results from more RCTs; thus, more precisely define the effects of using probiotics for the treatment of constipation.

Applications

Until such data are available, the use of probiotics for this condition should be considered investigational.

Peer review

This manuscript describes a systematic review of randomised controlled trials that evaluated the efficiency of probiotics in the treatment of functional constipation.

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BRIEF ARTICLE

Incidental findings at MRI-enterography in patients with suspected or known Crohn's disease

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Abstract

AIM: To determine the frequency and clinical impact of incidental findings detected with magnetic resonance imaging (MRI)-enterography in patients with suspected or known Crohn's disease (CD).

METHODS: Incidental findings were defined as unexpected lesions outside the small intestine, not previously known or suspected at the time of referral, and not related to inflammatory bowel disease. Through a systematic review of medical charts we analyzed the clinical impact of incidental findings, and compared the MRI findings with subsequent diagnostic procedures.

RESULTS: A total of 283 patients were included in the analysis, and MRI detected active CD in 31%, fistula in 1.4% and abscess in 0.7%. Extra-intestinal findings not related to CD were recorded in 72 patients (25%), of which 58 patients (20%) had 74 previously unknown lesions. Important or incompletely characterized findings were detected in 17 patients (6.0%). Incidental findings led to 12 further interventions in 9 patients (3.2%) revealing previously unknown pathological conditions in 5 (1.8%). One patient (0.4%) underwent surgery and one patient was diagnosed with a malignant disease. MRI detected incidental colonic lesions in 16 patients of which additional work-up in 4 revealed normal anatomy. Two patients (0.7%) benefitted from the additional examinations, whereas incidental findings led to unnecessary examinations in 9 (3.2%).

CONCLUSION: In a minority of patients with suspected or known CD, important incidental findings are diagnosed at MRI-enterography. However, a substantial number of patients experience unnecessary morbidity because of additional examinations of benign or normal conditions.

Key words: Magnetic resonance imaging; Incidental findings; Crohn's disease; Small intestine

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INTRODUCTION

In recent years magnetic resonance imaging (MRI) has been increasingly used for the assessment of small

bowel Crohn's disease (CD). MRI has a high diagnostic accuracy[1-12] and reproducibility[12], both with enteroclysis and the oral contrast method (enterography), for evaluating CD. Unlike conventional enteroclysis, MRI enables visualization of disease extension beyond the intestinal wall, i.e. abscesses and fistulas. In comparison with enteroclysis, MRI detects additional extra-intestinal lesions in 24%-58% of patients[1,3]. However, some extra-intestinal findings are unexpected and not related to CD, and are often referred to as incidental findings. The ability to detect incidental findings presents a clinical dilemma. On one hand, modern imaging techniques may detect early extra-intestinal malignant disease or disease requiring clinical intervention, thereby reducing morbidity and mortality. On the other hand, incidental findings may lead to further diagnostic work-up or surgery of benign lesions causing increased morbidity.

Only one previous study has analyzed the frequency of incidental findings in MRI-enteroclysis. Herfarth *et al*[13] found extra-intestinal lesions in 57% of 710 patients with suspected or known inflammatory bowel disease. Lesions of major clinical importance were detected in 12% of patients, of which the majority consisted of extra-intestinal manifestations of CD (abscesses). Findings were classified as tumor, metastasis or mass in 1.3% of patients. Ajaj *et al*[14] performed MRI-colonography in 375 patients with suspected colonic diseases and detected extra-colonic lesions in 69%, with 12% requiring additional examinations Approximately half of the extra-colonic lesions were previously unknown. These results emphasize that extra-intestinal findings are common when performing MRI of the abdomen. A significant proportion of incidental findings are clinically important and have an impact on clinical decision-making. However, these studies did not include the results of subsequent diagnostic work-up to reveal the benefit from detection of incidental findings.

The purpose of this study was to determine the frequency and clinical impact of incidental findings detected at MRI-enterography in patients with known or suspected CD.

MATERIALS AND METHODS

This retrospective study was conducted in the Department of Radiology, Vejle Hospital part of Lillebaelt Hospital, Denmark. The Department introduced MRI-enterography in December 2003, and a study period from December 2003 to November 2007 was chosen, allowing a minimum of 1 year follow-up after MRI. All MRI-enterographies performed in the study period were identified in the hospital's computerized radiology information system, and radiology reports were printed out. Through a systematic review of medical charts we analyzed the clinical impact of incidental findings and compared the MRI findings with subsequent diagnostic procedures. All reports were reviewed independently by the first author.

Criteria for inclusion and exclusion

MRI-enterographies performed in patients with suspected or known CD having symptoms consistent with disease activity or complications were included in the study. The subsequent analysis focused on incidental findings defined as unexpected findings outside the small intestine not previously known or suspected at the time of referral and not related to inflammatory bowel disease. Hence, extra-intestinal manifestations of CD (abscesses and fistulas) were not regarded as incidental findings.

Examinations performed on indications other than CD, repeated MRI-enterographies, and examination failures because of technical malfunctions or patient discomfort were excluded. In order to minimize selection bias, the study population was restricted to patients with no previous MRI-enterographies. The likelihood of previously unknown findings outside the small intestine is substantially reduced in repeated scans during a short study period. Therefore, in cases of 2 or more examinations performed, only the first MRI scan was included.

A total 354 patients underwent MRI-enterography. Twenty-nine scans were performed on indications other than inflammatory bowel disease, and additionally 2 scans were excluded because of failure to perform the examination. Both patients were unwilling to ingest the enteral contrast. A total of 40 scans in 29 patients were excluded because of repeated MRI-enterographies in the study period. Hence, a total of 283 MRI-enterography examinations in 283 patients were included in the analysis.

A clinical impact was defined as one or more subsequent interventions, i.e. additional diagnostic work-up, medical and/or surgical treatment, solely caused by the incidental finding at MRI-enterography. The clinical impact was assessed by analyzing the number of patients with subsequent clinical interventions, and the number and type of interventions performed in each patient. Incidental findings were classified as true or false positive on the basis of the diagnostic work-up and as beneficial or unnecessary for the patient. Data were collected from radiological reports, medical charts, laboratory data and the results of subsequent diagnostic procedures. Information was collected from the hospital's computerized medical charts and radiology information system. In patients referred from other hospitals, referrals and medical charts were collected from the department in charge of treatment.

Ethics

The study was approved by the local ethics committee of Southern Denmark and the Danish Data Protection Agency. In a few patients diagnostic work-up was performed at other hospitals, and prior to collecting these data, patients gave informed consent.

Imaging technique

Scans were carried out with an Intera 1.5T MRI system with a 5 element Syn-body coil (Philips Medical Systems, Eindhoven, The Netherlands). The evening before the examination, patients were instructed to eat a light meal

Table 1 Indications and results of 283 MRI investigations of the small intestine

	Clinical indication for MRI	*n*	Total
Suspected CD	Diagnostic MRI in patients with suspected CD not confirmed at endoscopy	156	156
Known CD	Extension of newly diagnosed CD detected at endoscopy	17	
	Evaluation of disease activity and extension or suspected complications of known CD	110	127
Total			283
Results of MRI-enterographies	CD in the small intestine	87	31%
	Stenosis	38	13%
	Entero-enteric fistula	4	1.4%
	Intra-abdominal abscess	2	0.7%
	Suspected IBD in the colon	35	12%

MRI: Magnetic resonance imaging; CD: Crohn's disease; IBD: Inflammatory bowel disease.

and fast overnight. They were allowed to drink water prior to the examination. Patients received 1000 mL water mixed with psyllium husk fiber ingested gradually over one hour. Patients were examined in the supine position. The protocol contained the sequences Cor T1 (TR/TE, 7/3.4; flip angel 15; slice thickness 4 mm; 208 matrix; FOV 375), Cor T2 (B-FFE; TR/TE, 4.1/2.0 ms; flip angle, 60; slice thickness 5 mm; 224 matrix; FOV 400), Cor SPIR (TR/TE, 3000/125 ms; flip angel 90; slice thickness 7 mm; 256 matrix; FOV 400) and axial T1W (TR/TE, 7/3.4; flip angel 15; slice thickness 4 mm; 208 matrix; FOV 375) with discontinuous breath-hold before and after contrast. Gadodiamide 0.1 mmol/kg (GE Healthcare, Medical Diagnostics, Oslo, Norway) was given intravenously, and hyoscinbutylbromide 20 mg (Buscopan, Boehringer Ingelheim, Basel, Switzerland) was administered to reduce peristalsis during the procedure. All images were evaluated using an Impax PACS workstation (Agfa, Mortsel, Belgium) with 2 Coronis monitors (1600 × 1200 pixels) (Megapixels Diagnostic Display System, Barco, Kortijk, Belgium). Radiologists performing the studies were all specialist doctors with experience in abdominal MRI techniques.

Classification of scans

MRI-enterographies were classified according to the most important incidental finding. Lesions were assessed as proposed by Zalis *et al*[15] for computed tomography (CT) colonography. E0 is an examination in which technical factors severely limit evaluation, e.g. because of artifacts. E1 denotes a normal examination or variants in anatomy that are not expected to affect the patient's health status. E2 refers to examinations with clinically unimportant extra-intestinal findings. E3 denotes incompletely characterized findings that are likely to be benign and E4 refers to examinations with potentially important extra-intestinal findings. Classification of scans was performed by the first author on the basis of the radiological reports and prior to analyzing the clinical impact of incidental findings. The co-authors subsequently evaluated the classification of incidental findings, and agreement was attained. Incidental findings located in the colon were analyzed separately.

Statistical analysis

Data were analyzed using descriptive statistics. Difference in means was calculated using the Wilcoxon rank-sum test and *P*-values less than 0.05 were considered significant.

RESULTS

Of the 283 patients included in the study, 193 (68%) were female. The mean age of the study population was 38.7 years (range 9.9-84.9 years). The indication for MRI was suspected CD in 156, and newly diagnosed or known CD in 127. MRI examinations revealed active CD in 31%, fistula in 1.4%, and abscess in 0.7% of patients (Table 1). There was no difference in mean age between patients with known and suspected CD ($P = 0.9$).

Extra-intestinal incidental findings

Extra-intestinal findings were recorded in 72 patients, of which 58 patients (20%) had previously unknown findings. Forty-one scans were classified E2, 11 were E3, and 5 were E4. In 225 scans no or previously known extra-intestinal lesions were recorded. In one examination the radiologist suspected multi-cystic ovaries, but evaluation of extra-intestinal organs was significantly compromised. The examination was classified E0, even though the finding led to further diagnostic work-up.

Seventy four incidental findings were detected in 58 patients (Table 2). In 43 patients only one finding was recorded, 14 patients had 2 findings, and 3 findings were recorded in one patient. The most frequent findings were benign cysts in the kidneys, ovaries and liver requiring no further work-up ($n = 39$). In 12 patients (4.2%) incompletely characterized extra-intestinal findings (E3) were detected. Of these, 2 patients had a large bladder suggesting previously unknown lower urinary tract disease, and one scan revealed a large hepatic cyst with a diameter of 15 cm displacing the right kidney. Potentially important findings (E4) were recorded in 5 patients (1.8%). Three patients had an undetermined mass or a cystic lesion in conjunction to the ovaries and pelvis wall, and further work-up was recommended. One scan revealed a focal hepatic lesion (atypical hemangioma), and one patient was diagnosed with an abdominal aortic

Table 2 Previously unknown extra-intestinal findings in 58 patients

		Finding	*n*
E0	Female genitals	Suspected multi-cystic ovaries	1
E2	Liver	Hepatic cysts	3
		Gallstones	7
	Kidney	Renal cysts	19
		Renal anatomical variants	3
		Reduced kidney size	1
		Metallic artifact in the kidney	1
	Female genitals	Leiomyomas in the uterus	4
		Ovarian cysts	14
	Miscellaneous	Atrophy of the abdominal musculature after surgery	1
		Small amounts of free abdominal fluid	3
			56
E3	Liver	Large hepatic cyst with displacement of the right kidney	1
	Urinary tract	Bilateral nephropathy with reduced kidney size[1]	2
		Large bladder	2
	Female genitals	Free fluid in the pelvis and suspected leiomyoma of the uterus	1
		Two lobulated and cystic lesions in the pelvis	1
	Miscellaneous	Splenomegaly	1
		Ascites	1
		Bilateral hip joint effusion	1
		Lymphadenopathy in the mesentery	1
		Spondylosis and spinal stenosis	1
			12
E4	Focal hepatic lesion (atypical hepatic hemangioma)		1
	Unexplained mass in conjunction to the ovaries		3
	Abdominal aortic aneurysm		1
			5
Total			74

[1]Both patients had a normal S-creatinine at the time of MRI-enterography with intravenous contrast.

aneurysm (Figure 1).

Significantly more scans were classified E3 and E4 in patients with suspected CD (15 out of 156) than known CD (one out of 127) suggesting that incidental findings necessitating further diagnostic work-up are more common in this group of patients ($P = 0.001$). Except for bilateral hip joint effusion, all E3 and E4 findings were detected in patients with suspected CD, and one patient had 2 E3 findings (Table 2).

Clinical impact of extra-intestinal findings

Extra-intestinal findings resulted in 12 clinical interventions in 9 patients (3.2%). The interventions consisted of ultrasound examination in one, ultrasound-guided biopsy in one, contrast-enhanced ultrasound and biopsy in one, CT-scan in one, gynecological examination including transvaginal ultrasound in 5, surgery in one and biochemical tests in one (Table 3). Succeeding work-up resulted in 5 true positive extra-intestinal findings and 3 false positive findings. One patient with bilateral hip joint effusion failed to attend the follow-up ultrasound examination.

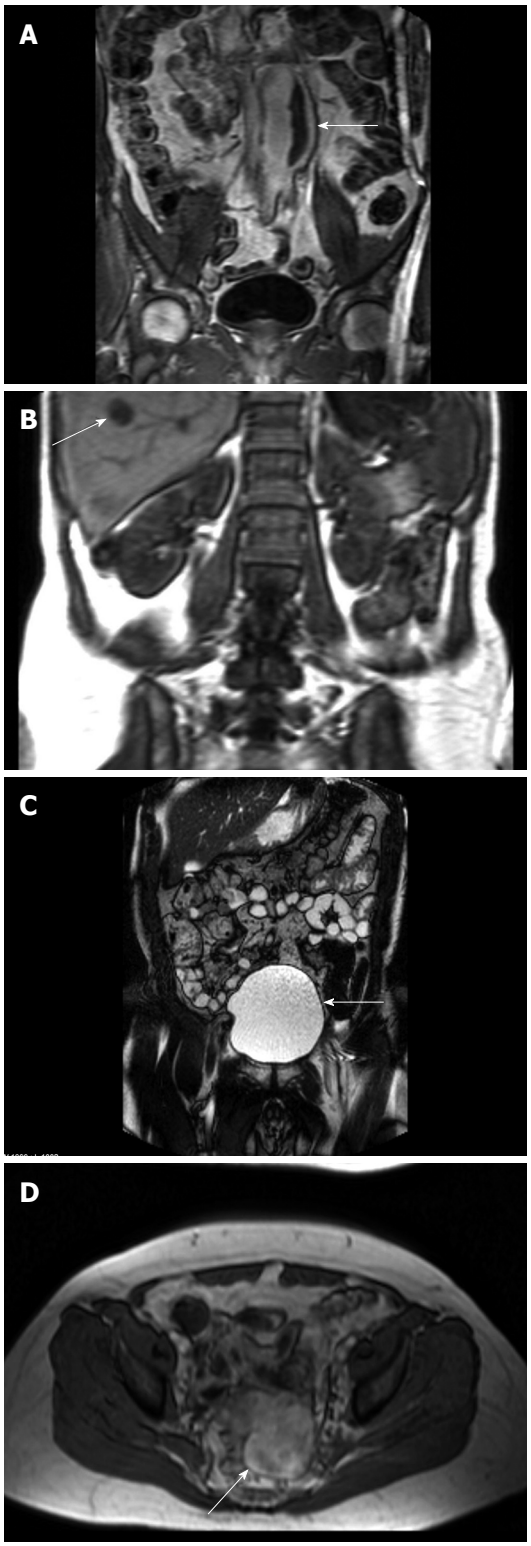

Figure 1 Incidental findings at MRI-enterography. A: Abdominal aortic aneurysm (arrow). CT scan confirmed the aneurysm and ruled out rupture; B: Atypical hepatic hemangioma (arrow). The results of ultrasound-guided biopsy were benign; C: Large bladder leading to diagnostic work-up and diagnosis of prostate cancer (arrow); D: A lesion with a diameter of 6 cm in the small pelvis (arrow) was confirmed with transvaginal ultrasound. Surgery showed a torquated leiomyoma in the top of the uterus.

In a patient with suspected CD, MRI showed an enlarged bladder. The patient was referred for fur-

Table 3 Previously unknown extra-intestinal findings leading to further examinations and the result of diagnostic work-up

	Extra-intestinal finding	Clinical intervention	Result of diagnostic work-up
5 true positive findings	Abdominal aortic aneurysm (E4)	CT-scan of the aorta	Abdominal aortic aneurism without rupture
	Focal hepatic lesion (E4)	Contrast-enhanced US and biopsy (atypical hemangioma)	Hemangioma
	Two unexplained masses in conjunction to the ovaries (E4)	GE and transvaginal US Surgery	Leiomyomas
	Free fluid in the pelvis and suspected leiomyoma of the uterus (E3)	GE and transvaginal US	Leiomyoma
	Large bladder (E3)	Transrectal US and biopsy Abdominal CT scan Biochemistry (PSA)	Prostate cancer
3 false positive findings	4.5 cm cystic lesion with an excrescens associated with the cyst wall (E4)	GE and transvaginal US	Normal
	2.9 cm solid lesion in the pelvis and displacement of the uterus (E4)	GE and transvaginal US	Normal
	Multicystic ovaries (E0)	GE and transvaginal US	Normal
Results not available	Bilateral hip joint effusion (E3)	Referred for US examination	The patient did not attend the examination

US: Ultrasound; GE: Gynecological examination; PSA: Prostate-specific antigen; CT: Computed tomography.

Table 4 Sixteen incidental findings located in the colon and their clinical impact

Finding	*n*	Clinical intervention	Result of diagnostic work-up
Suspected benign neoplasia (3 cm large polyp)	1	Colonoscopy and CT-colonography	Normal
Coprostasis	7	-	-
Indeterminate thickening of the cecum mucosa	2	Colonoscopy	Normal
Displacement of the cecum	3	-	-
Diverticulosis	2	-	-
Suspected malignant neoplasia	1	Colonoscopy and abdominal CT scan	Normal

ther urological examinations, and was subsequently diagnosed with a previously unknown prostate cancer. In another patient, MRI revealed a 6 cm wide and 9 cm long abdominal aortic aneurysm. CT scan of the aorta confirmed the aneurysm, and ruled out rupture. Five patients diagnosed with one or more lesions associated with the female genitals had further diagnostic work-up. In one patient, MRI showed a 6 cm large lesion in the small pelvis, and the finding was confirmed with transvaginal ultrasound. The patient underwent surgery, which showed a 5 cm × 4 cm × 5 cm torquated leiomyoma in the top of the uterus and 2 smaller leiomyomas in the anterior wall of the uterus. The surgeon performed a hysterectomy.

Incidental findings in the colon

MRI revealed incidental findings located in the colon and not related to inflammatory bowel disease in 16 patients (5.7%, Table 4), of whom 5 also had an extra-intestinal finding (E2 in all). In 12 patients, colonic findings were of minor or no clinical relevance. Four patients underwent additional examinations because of mucosal changes not characteristic of inflammatory bowel disease. The examinations revealed no pathological conditions.

DISCUSSION

Few studies have dealt with incidental findings in abdominal MRI. In a recent retrospective study, Herfarth *et al*[13] analyzed extra-intestinal findings in MRI-enteroclysis. In 710 patients with suspected or known inflammatory bowel disease 57% had extra-intestinal lesions and 12% of the observed lesions were of major clinical importance. In 5 patients (0.7%) extra-intestinal findings were suspicious of previously unknown malignant disease. However, findings of major importance were mainly abscesses related to CD, and comparison with the present study is difficult because of different study designs.

Extensive work has been done on extra-intestinal findings in CT-colonography. Results are summarized in a comprehensive review from 2005 including 17 studies. In total 40% of patients were recorded to have extra-colonic abnormalities, 14% had further diagnostic work-up and extra-colonic cancers were detected in 2.7%[16]. The cancer detection rate was reported in 5 studies and varied from 0.4% to 4.6% with the highest rates in the elderly.

In the present study, MRI-enterography revealed incidental findings located outside the small intestine and not related to CD in 25% of patients resulting in additional examinations in 5%. Additional investigations

confirmed abnormal lesions in 1.8%, and one patient had a malignant disease. Two patients benefitted from the additional examinations (aortic aneurysm and prostate cancer), whereas incidental findings led to unnecessary examinations in 9 patients. Detection of extra-intestinal manifestations of CD was rare (1.8%).

Incompletely characterized or clinically important findings were more common in patients with suspected than known CD, suggesting that findings necessitating additional work-up are more frequent in this group of patients. Because of the retrospective nature of this study, and the small number of patients referred for additional examinations, it was not possible to elucidate further on this assumption or whether incidental findings could explain the patients' symptoms. A prospective study would clarify this issue.

Comparing studies can be troublesome because of differences in population characteristics, classification systems, examination protocols and study designs. In the present study we used an MRI technique with intravenous contrast in a young population with a low risk of malignant disease. Compared to the study by Ajaj *et al*[14] we detected fewer extra-intestinal lesions, and the frequency of malignant disease was much higher when performing MRI-colonography. In an overall comparison with CT studies we also found a lower frequency of extra-intestinal findings and a lower rate of additional work-up. These discrepancies probably arise from differences in age, prior morbidity and the risk of malignant disease in the study populations.

MRI-enterography is a relatively new modality for evaluating CD in the small intestine. Ileo-colonoscopy, CT-enterography, capsule endoscopy, abdominal ultrasound and small bowel enteroscopy are alternative examinations. Choosing between modalities relies on several factors. Primarily a modality with a high sensitivity and specificity for luminal abnormalities as well as pathology in the bowel wall and extra-intestinal manifestations of CD is essential. Also other aspects of the investigations should be considered: risk of complications (aspiration, capsule retention, radiation exposure, *etc.*), patient discomfort, complexity of the examinations, accessibility, costs, and finally the impact of incidental findings. In the present study, the detection rate of clinically significant lesions outside the small intestine was low. In contrast, incidental findings led to unnecessary examinations in a substantial number of patients. Hence, in comparison with other modalities the detection rate of important incidental lesions was too low to be an argument in itself for performing MRI-enterography in patients with suspected or known CD.

Our study was limited by its retrospective design. Radiological reports were not performed with the focus on incidental findings, and underestimation of clinically unimportant findings are likely. The study population contained a preponderance of women (ratio 2:1), which is reflected by the frequency of incidental findings in the female genitalia. The second most common finding was ovarian cysts, and lesions in the female genitals were common in all classification groups. It is well established that CD is more common in females (1.2-1.5:1) and in specialized centers for inflammatory bowel diseases the prevalence of women with irritable bowel syndrome is up to 4 times as high as that of men[17,18].

In conclusion, incidental findings were common in patients with known and suspected CD having MRI for evaluation of small intestinal disease. Additional examinations revealed important disease in only a minority of patients. However, a substantial number of patients experienced unnecessary morbidity because of the additional examinations of benign or normal conditions. The detection rate of important incidental lesions not related to CD was too low to be an argument in itself for performing MRI-enterography in this group of patients.

COMMENTS

Background

Magnetic resonance imaging (MRI) is increasingly used in the assessment of small bowel Crohn's disease (CD). Unlike conventional radiology, MRI enables visualization of disease extension beyond the intestinal wall, i.e. abscesses and fistulas. However, some extra-intestinal findings are unexpected and without relation to CD (incidental findings).

Research frontiers

Only a few studies have described the clinical impact of incidental findings in abdominal MRI. Lesions may represent important diseases and benefit patients, but may also cause unnecessary morbidity because of the diagnostic work-up of benign lesions.

Innovations and breakthroughs

In 2 recent studies using abdominal MRI techniques, extra-intestinal lesions of major clinical importance were common. However, these studies did not include the results of subsequent diagnostic work-up to reveal the benefit from detection of these findings. In the present study, incidental findings were common in patients having MRI for evaluation of small bowel CD. Additional examinations revealed important disease in a minority of patients. However, a substantial number of patients experienced unnecessary morbidity arising from the additional examinations of benign or normal conditions.

Applications

Several modalities for diagnosing small bowel CD are available. The present study emphasized that the detection rate of important incidental lesions was too low to be an argument in itself for performing MRI-enterography in this group of patients.

Peer review

Jensen *et al* gave a nice and clear description of the research background, materials, methods, results and conclusions. Significant points have been presented and compared with data from prior research. Used methods are advanced, and detailed descriptions are provided allowing other investigators to reproduce or validate them. The statistical methods are appropriate. From the presented results, sufficient data can be drawn. In discussion, valuable conclusions are provided. References are appropriate and relevant. Tables and figures reflect the major findings of the study.

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BRIEF ARTICLE

Association of autoimmune type atrophic corpus gastritis with *Helicobacter pylori* infection

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Abstract

AIM: To study the association between *Helicobacter pylori* (*H. pylori*) infection and autoimmune type atrophic gastritis.

METHODS: Twenty-three patients with different grades of atrophic gastritis were analysed using enzyme immunoassay-based serology, immunoblot-based serology, and histology to reveal a past or a present *H. pylori* infection. In addition, serum markers for gastric atrophy (pepsinogen Ⅰ, pepsinogen Ⅰ/Ⅱ and gastrin) and autoimmunity [parietal cell antibodies (PCA), and intrinsic factor (IF), antibodies] were determined.

RESULTS: Of the 14 patients with severe gastric atrophy, as demonstrated by histology and serum markers, and no evidence for an ongoing *H. pylori* infection, eight showed *H. pylori* antibodies by immunoblotting. All eight had elevated PCA and 4/8 also had IF antibodies. Of the six immunoblot-negative patients with severe corpus atrophy, PCA and IF antibodies were detected in four. Among the patients with low to moderate grade atrophic gastritis (all except one with an ongoing *H. pylori* infection), serum markers for gastric atrophy and autoimmunity were seldom detected. However, one *H. pylori* negative patient with mild atrophic gastritis had PCA and IF antibodies suggestive of a pre-atrophic autoimmune gastritis.

CONCLUSION: Signs of *H. pylori* infection in autoimmune gastritis, and positive autoimmune serum markers in *H. pylori* gastritis suggest an etiological role for *H. pylori* in autoimmune gastritis.

Key words: *Helicobacter pylori*; Autoimmune gastritis; Gastric atrophy; Vitamin B12 deficiency

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INTRODUCTION

Autoimmune type corpus gastritis, formerly named type

A gastritis, is severe atrophy of gastric corpus associated with hypochlorhydria[1]. Even without total gastric atrophy, many of these patients have an inability to absorb vitamin B12 from food[2]. Generally, 15%-20% of vitamin B12 malabsorption in elderly patients is due to pernicious anaemia, as defined as deficiency of intrinsic factor (IF)[3]. Over 90% of patients with pernicious anaemia have parietal cell antibodies (PCA) and 50%-70% have elevated IF antibodies[1]. The autoantigen for PCA is H+/K+-adenosine triphosphatase, the proton pump[4].

In patients with *Helicobacter pylori* (*H. pylori*) infection, superficial gastritis proceeds to atrophic gastritis in about half of the patients[5]. Although this type of atrophic gastritis, which is associated with intestinal metaplasia, mainly involves the antrum, it can proceed to the corpus or affect the mucosa focally, *viz*. multifocal atrophic gastritis. Advanced atrophy develops over many years and *H. pylori* disappears from the gastric mucosa. In some patients, the antral intestinal metaplasia disappears and PCA appears; thus, the disease resembles classic autoimmune gastritis[6]. Gastric H+/K+-ATPase is also the major autoantigen in chronic *H. pylori* induced atrophic gastritis in corpus mucosa[7].

In *H. pylori* induced atrophic gastritis, the activated CD4+ Th1 cells infiltrating the gastric mucosa cross-recognize the epitopes of the gastric parietal cell proton pump and various *H. pylori* proteins[8,9]. It is not known if *H. pylori* is the initiating factor in activating Th1 cells, which leads to inflammation and apoptosis, or is only a coincidental bystander[10]. If the classic autoimmune type gastric atrophy is an end-stage of *H. pylori* induced gastric autoimmunity with atrophic gastritis, the prevalence of pernicious anaemia should decrease with declining prevalence of *H. pylori*. It is not known if vitamin B12 malabsorption in the late stages of gastric atrophy could be restored or prevented if *H. pylori* were eradicated earlier[11-14].

In the present study we investigated the signs of a previous *H. pylori* infection in patients with different grades of atrophic gastritis to assess the proportion of gastric atrophy not associated with *H. pylori* infection.

MATERIALS AND METHODS

All patients with an earlier gastroscopy reprint available and who had undergone a gastroscopy for clinical indications at Herttoniemi Hospital during 2004 and 2005[15] were included in the present study if their follow-up histology indicated they had atrophic gastritis. Twenty-three of the 38 patients with different grades of atrophic gastritis had a blood sample available and were included in the study. The median age was 65 years and 18 were females.

The Ethics Committee of the Hospital District of Helsinki and Uusimaa approved the study and all the participants gave their written informed consent.

Histology

Two biopsies from each of the antrum and the corpus were taken during gastroscopy and stained with haematoxylin-eosin, Alcian blue (pH 2.5)-periodic acid Schiff, and modified Giemsa stains. All the samples were examined by one pathologist who was unaware of the identity of the samples. The samples were assessed according to the updated Sydney system[16].

Serum tests

H. pylori antibodies were detected by an enzyme immunoassay (EIA) and by immunoblotting. Serum samples were taken after gastroscopy and stored (-20℃) until analyzed for IgG antibodies to *H. pylori* using a locally validated in-house EIA with high sensitivity and specificity[17]. Immunoblotting was performed by MP Diagnostics Helico blot 2.1 (MP Biomedicals, Singapore). The interpretation criteria for an *H. pylori* seropositive sample, according to the manufacturer, were: (1) fulfilling the criteria for CagA positivity; (2) the presence of any bands at 89 kDa, 37 kDa, or 35 kDa; or (3) the presence of both the bands at 30 kDa and 19.5 kDa. The criteria for CagA positivity were the presence of 116 kDa CagA band (a) in combination with current infection marker CIM; (b) in combination of the 30 kDa (UreA) and 19.5 kDa bands; or (c) in combination of at least one of the following bands 89 kDa (VacA), 37 kDa, or 35 kDa.

PCA were measured by Varelisa (Pharmacia Diagnostics, Freiburg, Germany) using H+/K+-ATPase as an antigen. According to the manufacturer's instructions, values > 15 U/mL were interpreted as positive but equivocal values (10-15 U/mL) were interpreted negative as well as values < 10 U/mL.

Serum IF antibodies of the blocking type were measured routinely with the haemoglobin charcoal adsorption assay. The cut off value used was 2 U/L.

Serum pepsinogen Ⅰ and Ⅱ and gastrin-17 levels were investigated with Gastropanel (Biohit PLC Diagnostics, Helsinki, Finland). The reference ranges were 30-120 μg/L for pepsinogen Ⅰ, 3-10 μg/L for pepsinogen Ⅱ, 3-20 for pepsinogen Ⅰ/Ⅱ, and 2-10 pmol/L for gastrin-17.

Statistical analysis

The differences between the groups were tested using two-tailed Fisher's exact test and the data were analysed using GraphPad software (QuickCalcs online calculators for scientists www.graphpad.com). *P* values < 0.5 were considered significant.

RESULTS

Of the 23 patients included in the study, 14 had severe gastric atrophy according both to histology and the serum markers, and the remaining nine patients had mild to moderate atrophic gastritis. The patients with severe atrophy were slightly younger (median age 64 years) than the other patients (median age 70 years). None of the patients with severe atrophy had either *H. pylori* in histology or elevated *H. pylori* antibodies in the EIA

Table 1 Histological and serum findings in patients with mild to moderate (n = 9) and severe (n = 14) atrophic changes in the corpus

Findings	Number of patients with atrophic corpus gastritis						*P*-value
	Grade 1 or 2			Grade 3			
	IB+ (n = 8)	IB- (n = 1)	Total	IB+ (n = 8)	IB- (n = 6)	Total	
Chronic corpus gastritis	7	1	8	8	6	14	NS
Chronic antral gastritis	7	1	8	3	3	6	0.04
Antral intestinal metaplasia	3	0	3	0	0	0	0.05
H. pylori in histology	4	0	4	0	0	0	0.01
Elevated EIA *H. pylori* IgG[1]	7	0	7	0	0	0	0.0001
Vitamin B12 therapy	1	0	1	4	6	10	0.009
Elevated PCA[2]	2	1	3	8	4	12	0.02
Elevated IF antibodies[3]	0	1	1	4	4	8	0.04
Low pepsinogen I[4]	0	0	0	8	6	14	0.0001
Elevated pepsinogen II[5]	5	0	5	4	1	5	NS
Low pepsinogen I / II[6]	1	0	1	8	6	14	0.0001
Elevated gastrin-17[7]	2	1	3	8	5	13	0.005

[1]In-house EIA positive ⩾ 700; [2]Parietal cell antibodies PCA elevated > 15; [3]Intrinsic factor IF elevated > 2; [4]Pepsinogen I low < 30; [5]Pepsinogen II elevated > 10; [6]Pepsinogen I / II low < 3; [7]Gastrin 17 elevated > 10. EIA: Enzyme immunoassay; *H. pylori*: *Helicobacter pylori*; PCA: Parietal cell antibodies; IF: Intrinsic factor; NS: Not significant; IB+: Immunoblot positive; IB-: Immunoblot negative. *P*-value: Total (grade 1 or 2) *vs* total (grade 3).

(Table 1); one patient had had a successful *H. pylori* eradication therapy 7 years earlier. Of the nine patients with mild to moderate atrophic gastritis, two had had a successful eradication therapy (4 mo and 6 mo earlier, respectively) and four had an ongoing infection shown in histology; seven had elevated *H. pylori* antibodies in the EIA (Table 1). All patients, except one with moderate atrophic gastritis, had chronic gastritis in the corpus, whereas the antrum was significantly less often affected in patients with severe atrophy compared to those with mild and moderate atrophic changes (P = 0.04, Fisher's exact test, Table 1). Antral intestinal metaplasia was not found in any of the patients with severe atrophy.

Serum markers for gastric autoimmunity were only rarely detected in patients with mild to moderate atrophic gastritis (PCA in three patients and IF antibodies in one patient, Table 1). In contrast, all 14 patients with severe atrophy had either elevated PCA or IF antibodies, six patients having both antibodies elevated. Furthermore, the levels of elevated PCA and IF antibodies were higher in patients with severe atrophy (eight of 12 patients with elevated PCA had a PCA titre over 100, and the mean IF antibody titre in eight patients with an elevated value was 8.7) compared to patients with only mild to moderate atrophic changes (only one of the three patients with elevated PCA had a PCA titre over 100 and the IF antibody titre in the only patient with an elevated value was 2.1).

H. pylori antibodies could be demonstrated by immunoblotting in 8/9 patients with mild to moderate atrophic gastritis and in 8/14 patients with severe gastric atrophy (Table 1). Patients with severe atrophy and a positive immunoblot result did not significantly differ from those with severe atrophy and negative immunoblot results as far as age, sex, histological findings, and serum results were concerned (Table 1). Although six patients with severe atrophy showed negative immunoblot results (according to the criteria of the manufacturer) four of them had a positive CagA band in the immunoblot; thus, only two patients showed no evidence of previous *H. pylori* infection. In addition, one patient with mild atrophic gastritis had no evidence (not even a CagA band) for ongoing or previous *H. pylori* infection (Table 1). This particular patient showed clearly elevated PCA (> 100 U/mL) and slightly elevated IF antibodies (2.1 U/L).

DISCUSSION

In our study, of the 14 patients having autoimmune type atrophic gastritis (severe gastric atrophy with elevated PCA and/or IF antibodies) only two had no signs of previous *H. pylori* infection. In addition, all except one of the patients with mild to moderate atrophic corpus gastritis had an ongoing *H. pylori* infection or signs of previous infection. The *H. pylori* negative patient with minor atrophic changes in the gastric corpus had elevated PCA and IF antibodies; whether this particular patient goes on to develop severe gastric atrophy of autoimmune type remains to be shown. To the best of our knowledge, she is the first patient described in the literature as having preatrophic autoimmune gastritis with elevated serum markers of pernicious anaemia and no signs of *H. pylori* infection, despite being investigated with both invasive and non-invasive methods: antrum and corpus histology in two gastroscopies with a 5.4 years interval and negative EIA serology and immunoblotting, including CagA.

In severe gastric atrophy, the exclusion of previous *H. pylori* infection is controversial, as the sensitivity of histology is low[18], and many of the EIA based serological tests are poorly validated[19]. In *H. pylori* gastritis, the antibodies in EIA serology decline below the cut-off values along with advanced atrophy[20], as

well as after eradication therapy[21]; thus, the previous *H. pylori* infection cannot be deduced by negative EIA serology. Immunoblotting with CagA antibodies can give positive results for years after the disappearance of *H. pylori*[22,23], but all *H. pylori* strains are not CagA positive. Discrepancies in CagA seropositivity yielded by immunoblotting in patients with severe gastric atrophy[24,25] may derive from the different sensitivities of the immunoblotting methods used[26].

Studies of patients with preatrophic autoimmune type of corpus gastritis are rare. In a population-based study, all 12 patients with autoimmune type atrophic gastritis (diffuse lymphocytic infiltration of the entire lamina propria in the corpus mucosa) without severe gastric atrophy showed *H. pylori* in histology or serology[27]. In the same study, of the 28 individuals with severe autoimmune type gastric atrophy six were *H. pylori* positive in histology and another 13 were positive in serology (altogether 68% positive for *H. pylori*). Considering the moderately high prevalence (2.8% in the Kalixanda study[27]) of the autoimmune type of gastric atrophy in general, the description in the literature of patients with *H. pylori* negative autoimmune type gastritis in preatrophic stage is rare.

Uibo described a 17-year-old female with no signs of gastritis and *H. pylori* in histology developing atrophic gastritis during a 12-year follow-up[28]. However, the exclusion of *H. pylori* infection in this case was based only on histology, and the childhood infection rate in this population cohort was nearly 100%. Kuipers described two patients who were negative for *H. pylori* and without gastritis at first visit, who then developed atrophic gastritis (one developed also intestinal metaplasia and pernicious anaemia) during more than 10 years of follow-up[29]. However, although in this study the *H. pylori* infection was assessed with serology and histology at the first visit, in cases of discrepant results, histology was considered predominant over serology unless atrophic mucosa was observed. Whether these two patients had positive serology at the first visit was not mentioned. In the study of Segni *et al*[30] of children with juvenile autoimmune thyroid disease, of the 18 children with elevated PCA who underwent gastroscopy, two children with hypergastrinaemia had *H. pylori* negative preatrophic gastritis, as shown by histology and EIA serology. Immunoblotting was not studied and follow-up has not been published. In the study of adult patients with Sjögren's syndrome, there was no difference in the prevalence of *H. pylori* infection, antigastric antibodies, or gastric histology between patients and controls, but after successful eradication therapy for *H. pylori*, the lymphocytic infiltration and atrophy in patients with Sjögren's syndrome, contrary to the controls, did not improve[31]. In addition, patients with Sjögren's syndrome who were positive for antigastric antibodies all had *H. pylori* infection and they more often had atrophic gastritis than the controls. In conclusion, from the previous studies, patients with autoimmune type atrophic gastritis without *H. pylori* infection might rarely exist, but at the moment a study showing preatrophic gastritis proceeding to total gastric atrophy without *H. pylori* infection is lacking. This is in accordance with our results; as the patient having preatrophic gastritis without signs of *H. pylori* infection did not proceed to total gastric atrophy during 5 years of follow-up.

Several studies suggest that autoimmune atrophic corpus gastritis is associated with *H. pylori* infection in the majority of cases. In one study, two-thirds of patients with atrophic corpus gastritis had evidence of *H. pylori* infection, when assessed with histology and serology[32]. In another study, 62% of the patients with pernicious anaemia and severe atrophic corpus gastritis had positive *H. pylori* serology[33]. In one further study, patients with atrophic corpus gastritis were negative for *H. pylori* in histology and in EIA-serology, but positive when studied by immunoblotting[25]. In another study of atrophic corpus gastritis, among 111 patients with negative *H. pylori* EIA serology, 95.5% were positive in immunoblotting[34]. In a study of 10 patients with severe atrophic corpus gastritis, all were *H. pylori* negative in histology and in EIA-serology, and only one was positive in immunoblotting[24]. However, in this particular study, the immunoblotting method used to measure CagA antibodies was less sensitive than EIA-serology in detecting an ongoing *H. pylori* infection. In the present study, all except three patients had a positive CagA band on the immunoblot, including all EIA-serology positive patients. We have studied the sensitivity and specificity of this particular immunoblotting method previously, with good results[23]. However, not even the immunoblotting method used in our present study can rule out a previous *H. pylori* infection with 100% certainty, as all *H. pylori* strains are not CagA positive. On the other hand, the common occurrence of *H. pylori* antibodies in patients with autoimmune type of atrophic gastritis could be a random effect, as the *H. pylori* infection rate has been nearly 100% in populations now presenting as the peak age group of autoimmune gastritis. This could also be one explanation why *H. pylori* prevalence studied by immunoblotting in patients with serological markers of autoimmune type gastritis (PCA and IF antibodies) was no different from patients with no such markers in our study.

Thus, it still remains to be shown if *H. pylori* infection is crucial for the development of autoimmune type atrophic gastritis. However, bacterial infections might be important in autoimmune processes, as recently suggested by Torchinsky *et al*[35]. In this *in vitro* study, phagocytosis of immune cells infected with bacteria and undergoing apoptosis promoted Th17 cell differentiation, the cell type having a potential role in autoimmunity. Thus, it is tempting to speculate that cells in the gastric mucosa infected with *H. pylori* could trigger an autoimmune response.

In conclusion, atrophic corpus gastritis, including

autoimmune type severe atrophy with vitamin B12 malabsorption, is associated with a longstanding *H. pylori* infection in most cases. There is an urgent need for population-based studies to assess the effect of *H. pylori* eradication on the development of vitamin B12 malabsorption.

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COMMENTS

Background

Autoimmune type atrophic gastritis is a severe gastric atrophy associated with pernicious anaemia with lifelong substitution therapy with vitamin B12. Longstanding *Helicobacter pylori* (*H. pylori*) infection proceeds in about 50% of patients to atrophic gastritis. *H. pylori* infection is much more prevalent than autoimmune type gastritis, and the association of these two conditions is possible without a causal relationship.

Research frontiers

Previous studies have shown that *H. pylori* shares several epitopes with the proton pump, and the β-subunit of this pump is the causative antigen in autoimmune gastritis. In animal models, the passive transfer of these antibodies does not cause disease, but CD4+ T-cells are responsible for the gastritis. Recently, it has been shown that bacterial infection can modify the immune response in the direction seen in autoimmune diseases, i.e. Th17 cell differentiation, thus linking infection and autoimmunity.

Innovations and breakthroughs

It is difficult to differentiate severe end stage *H. pylori* atrophic gastritis and autoimmune type gastric atrophy, because the autoimmune serum markers appear in *H. pylori* gastritis with increasing grade of atrophy, as shown in previous studies and confirmed in our study. The preatrophic stage of autoimmune type gastritis without *H. pylori* infection is an unknown entity. Several patients with autoimmune type gastric atrophy have signs of a previous *H. pylori* infection when studied with sensitive methods and remain positive for years, as shown in this study.

Applications

If *H. pylori* initiates the apoptosis that leads to gastric atrophy and vitamin B12 deficiency, eradication of the bacteria before the development of severe atrophic changes should abolish the development of pernicious anaemia and the need of lifelong vitamin B12 substitution therapy.

Peer review

This is a very interesting paper and asks quite an important question as to whether there is an association between *H. pylori* infection and autoimmune type atrophic gastritis. This work could be accepted after revision.

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BRIEF ARTICLE

On-treatment predictions of success in peg-interferon/ribavirin treatment using a novel formula

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Abstract

AIM: To predict treatment success using only simple clinical data from peg-interferon plus ribavirin therapy for chronic hepatitis C.

METHODS: We analyzed the clinical data of 176 patients with chronic hepatitis and hepatitis C virus genotype 1 who received 48 wk standard therapy, derived a predictive formula to assess a sustained virological response of the individual patient using a logistic regression model and confirmed the validity of this formula. The formula was constructed using data from the first 100 patients enrolled and validated using data from the remaining 76 patients.

RESULTS: Sustained virological response was obtained in 83 (47.2%) of the patients and we derived formulae to predict sustained virological response at pretreatment and weeks 4, 12 and 24. The likelihood of sustained virological response could be predicted effectively by the formulae at weeks 4, 12 and 24 (the area under the curve of the receiver operating characteristic: 0.821, 0.802, and 0.891, respectively), but not at baseline (0.570). The formula at week 48 was also constructed and validation by test data achieved good prediction with 0.871 of the area under the curve of the receiver operating characteristic. Prediction by this formula was always superior to that by viral kinetics.

CONCLUSION: These results suggested that our formula combined with viral kinetics provides a clear direction of therapy for each patient and enables the best tailored treatment.

Key words: Logistic regression analysis; Predictive formula; Prolongation of the therapy; Response-guided therapy; Viral kinetics

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INTRODUCTION

Persistent hepatitis C virus (HCV) infection is the major chronic liver disease in Japan, and pegylated interferon-α (PEG-IFN) plus ribavirin (RBV) therapy is the current mainstay of treatment. The goal of treatment is a sustained virological response (SVR), which is defined as undetectable serum HCV RNA, according to a polymerase

chain reaction-based assay, 6 mo after the cessation of therapy[1]. Patients who achieve SVR gain the benefits of regression of fibrosis, decreased incidence of hepatocellular carcinoma, and reduced morbidity and mortality. The genotype of HCV affects treatment efficacy and HCV RNA levels (viral load) also may have an effect. Only a small percentage of patients infected with HCV genotype 1b and high viral load achieved SVR with conventional IFN therapy for 6 mo[2]. Patients infected with HCV genotype 1 should receive 48 wk of PEG-IFN plus RBV, while 24 wk of treatment is recommended for patients with HCV genotype 2[3,4]. When PEG-IFN plus RBV is administered in this manner, around 50% of patients infected with HCV genotype 1 achieve SVR[5], which is a great improvement over the SVR associated with 24 wk conventional IFN-α therapy.

The likelihood of treatment success may also be predicted by viral kinetics on therapy as well as circulating core antigen and immune parameters[6,7]. In particular, recent studies have shown that SVR can be predicted by a rapid virological response (RVR), which is defined as an undetectable level of HCV RNA at 4 wk of treatment[8], and an early virological response (EVR), which is defined as either an undetectable level of HCV RNA or a drop in HCV RNA levels of at least 2 $\log_{10}$IU/mL after 12 wk of treatment[9].

Nevertheless, the current dosing regimens for PEG-IFN plus RBV could potentially under-treat some patients[10] and additional measurements of viral response are needed to facilitate individualization of therapy. Among predictive factors already reported[11-15], many are not readily available from daily clinical assessment, because they require genomic analyses and/or advanced experimental methods. There is increasing evidence to support extending the duration of treatment beyond 48 wk for patients with an HCV genotype 1 infection who display a slow virological response, which is defined by HCV RNA levels > 50 IU/mL at week 12 but undetectable at week 24. Several trials suggested that 72 wk of treatment with PEG-IFN plus RBV results in a better SVR rate than the same treatment for 48 wk[16-18]. However, it is difficult to accurately determine whether the individual should have their therapy extended at week 48, because the predictive value of a slow virological response may be insufficient alone. It would be very valuable to have a more accurate predictive marker of SVR at week 48, derived from clinically available measurements.

In this study, to try to make better prediction of patients who would or would not respond to 48 wk of PEG-IFN plus RBV therapy, we analyzed the clinical data of patients with chronic hepatitis C who received 48 wk of therapy, derived a predictive formula to assess the likelihood of an SVR for each individual patient using a logistic regression model and confirmed the validity of this formula.

MATERIALS AND METHODS

The study was approved by the Ethics Committee of the Keio University, School of Medicine, and was performed in accordance with the internationally accepted ethical standards for human experimentation. The study was conducted by the Keio Association for the Study of Liver Diseases (KASLD). All patients received explanations of the purpose and protocol of the study and written informed consent was obtained from each patient.

Patients

One hundred and seventy-six patients with chronic hepatitis C infected with HCV genotype 1b were enrolled prospectively and received PEG-IFN plus RBV therapy from December 2004 to May 2007. All patients had HCV RNA levels ⩾ 100 KIU/mL, measured by a quantitative polymerase chain reaction (PCR) assay (COBAS HCV Amplicor MONITOR™, sensitivity 500 IU/mL; Roche Diagnostic Systems, Inc., Tokyo, Japan). Pregnant women and women of childbearing potential, nursing mothers, male patients whose partner could have become pregnant, and those with anemia (hemoglobin concentration of 10 g/dL or less), leucopenia (1500 cells/μL or less), thrombocytopenia (80 000 cells/μL or less), severe dysfunction of organs other than the liver (these exclusion criteria are included in the instruction of the drug and provided by the manufacturer), infection with hepatitis B virus or human immunodeficiency virus, autoimmune hepatitis, primary biliary cirrhosis, and liver dysfunction caused by other etiologies were excluded. Some patients did not undergo a liver biopsy because not all of the centers could perform biopsies. All patients were treated for 48 wk and were followed for 24 wk after cessation of treatment. The formula was derived using data from the first 100 patients enrolled as selection data and validated using data from the additional 76 patients as test data. In this way it was possible to analyze the predictive accuracy and validity of the constructed formula.

Treatment and data collection

PEG-IFN-α2b (Schering-Plough K.K., Osaka, Japan) was administered weekly in doses adjusted for body weight according to the manufacturer's recommendations in Japan (45 kg or less, 60 μg; 46-60 kg, 80 μg; 61-75 kg, 100 μg; 76-90 kg, 120 μg; 91 kg or more, 150 μg). Similarly, RBV (Schering-Plough K.K.) was given in daily doses adjusted to body weight according to manufacturer's instructions (60 kg or less, 600 mg/d; 61-80 kg, 800 mg/d; 81 kg or more, 1000 mg/d). Serum levels of HCV RNA were quantified and, when the level was below 500 IU/mL, HCV RNA was measured with the COBAS HCV Amplification and Detection version 2.0, sensitivity 50 IU/mL, Roche Diagnostic Systems). Blood cell counts and chemistry were analyzed at the beginning of treatment and every 4 wk thereafter. A questionnaire was used to review demographic data (gender, age, weight, height), previous treatment, histologic activity grade, and fibrosis stage, dose of PEG-IFN, dose of RBV, presence of diabetes, HCV RNA levels, SVR, white blood cell

counts (WBC), neutrophil counts (NC), red cell counts (RBC), hemoglobin levels (Hb), platelet counts (PLT), serum aspartate aminotransferase levels (AST), and serum alanine aminotransferase levels (ALT).

Statistical analysis

The Mann-Whitney *U*-test was used to analyze continuous variables. Chi-squared and Fisher's exact tests were used for analysis of categorical data. One of our goals was to predict SVR using only simple clinical data, so a database was created containing the following basic information: for all patients, baseline age, sex, body weight (kg), height (cm), dose of PEG-IFN (μg/kg), dose of RBV (mg/kg), HCV RNA levels (KIU/mL), SVR (+/-), WBC (/μL), NC (/μL), RBC (/μL), Hb (g/dL), PLT (/μL), AST (IU/L), and ALT (IU/L). Statistical analyses were performed using the Statistical Package of Services Solutions (SPSS; SPSS Inc., Chicago, IL, USA) software, version 11.0. First, factors that differed significantly between SVR and non-SVR groups were identified at every time point by univariate analyses. The independent discriminative value of markers for predicting SVR was then assessed by logistic regression analysis. The third step was to construct a formula that combined independent factors. The best index for discrimination was the logistic regression function that combined the most discriminatory independent factors. The predictive formula was logically constructed by following basal formula:

$$1/p = 1 + \exp[-(\beta_0 + \beta_1 X_1 + \beta_2 X_2 + \ldots\ldots + \beta_r X_r)]$$

Diagnostic values of indices and isolated factors were assessed by sensitivity, specificity, positive and negative predictive values (PPV and NPV), and receiver operating characteristic (ROC) curves.

RESULTS

Patient profile

Of the 176 patients, 101 (59.8%) were men and the median age was 56 years (18-77), which is greater than has been reported from Western countries. The median values of body weight and BMI were 61 kg (41.2-90.5) and 22.8 (15.7-32.0), respectively, which are lower than has been reported from Western countries. These conditions are characteristic of recent trends in Japanese patients; older and less obese patients. Ninety-four patients (66.2%) were treatment naïve and the median value of HCV RNA was 2165 KIU/mL (130 to > 5000). The pretreatment median values were as follows; RBC 464 cells/μL, Hb 14.4 g/dL, PLT 165 × 10^3 cells/μL, WBC 4775 cells/μL, NC 2549 cells/μL, AST 51 IU/L, and ALT 63 IU/L.

Response rate and factors associated with svR

SVR was obtained in 83 (47.2%) patients and in 54 (54%) of the first 100 patients (selection data) enrolled in this study (Table 1). Of the 83, 43 were male; 60.6% of the male patients achieved SVR and there was a statistically significant gender difference (P = 0.020). The median age was significantly lower in the SVR group. There was no difference in body weight and BMI between the SVR group and non-SVR group for the patients used for selection data. The pretreatment HCV RNA level did not differ significantly between the SVR and non-SVR groups, while pretreatment RBC, Hb, PLT and ALT levels differed between the groups. The average cumulative dose of RBV administered up to the time point indicated was always much greater in the SVR group than in

Table 1 Basic demographic, virological, and clinical features of the 100 patients whose data were used as selection data

	SVR	non-SVR	*P* value
Number (%)	54 (54.0)	46	
Gender (%)			
Male	43 (60.6)	28	0.038
Female	9 (34.6)	18	
Age			
Median	53	57	0.0098
Range	18-72	37-77	
Weight (kg)			
Median	65.1	60	0.138
Range	42.5-90.5	43.9-86.0	
BMI (kg/m^2)			
Median	23.5	22.9	0.834
Range	17.5-31.8	18.2-31.2	
Previous + treatment	22	19	1.000
HCV RNA (KIU/mL)			
Median	1889	2263	0.554
Range	140 to < 5000	150 to < 5000	
RBC (× 10^{-4}/mL)			
Median	469	452	0.0041
Range	319-621	354-552	
Hb (g/dL)			
Median	15.0	14.1	0.0059
Range	10.9-17.7	11.3-17.2	
PLT (× 10^{-3}/mL)			
Median	172	159	0.039
Range	84-292	62-270	
WBC (/mL)			
Median	5000	4850	0.256
Range	3270-8900	2300-9200	
NC (/mL)			
Median	2550	2549	0.978
Range	1066-4231	1184-5626	
AST (IU/L)			
Median	54	50	0.898
Range	22-156	24-241	
ALT (IU/L)			
Median	76	55	0.027
Range	36-311	15-278	
PEG-IFN dose (48 wk) (μg/kg per day)			
Median	1.43	1.32	0.0043
Range	0.68-1.82	0.52-1.82	
RBV dose (48 wk) (mg/kg per day)			
Median	10.83	8.39	0.0002
Range	3.42-14.55	2.75-12.64	

SVR: Sustained virological response; BMI: Body mass index; RBC: Red blood cell count; Hb: Hemoglobin; PLT: Platelet count; WBC: White blood count; NC: Neutrophil count; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; PEG-IFN dose (48 wk): Total dose of PEG-IFN at 48 wk (μg/kg per wk); RBV dose (48 wk): Total dose of RBV at 48 wk (mg/kg per day).

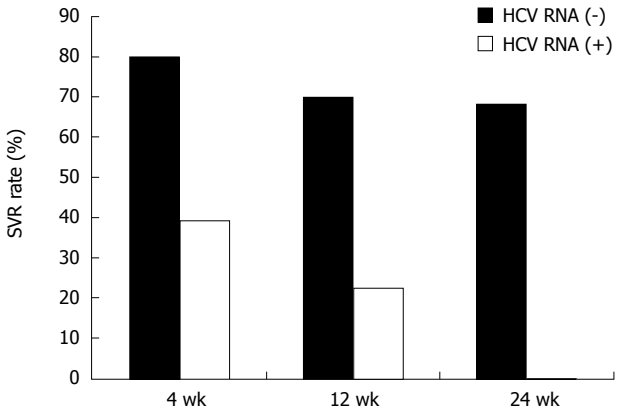

Figure 1 Sustained virological response rates (%) of the patients during peg-interferon plus ribavirin therapy for chronic hepatitis C at week 4 (4 wk), week 12 (12 wk) and week 24 (24 wk). HCV RNA (-) means patients whose serum HCV RNA became undetectable at the time point indicated. HCV RNA (+) means patients whose serum HCV RNA was not cleared at the indicated time point.

the non-SVR group. The average cumulative dosage of PEG-IFN differed between the groups at week 48.

Among the first 100 patients, serum HCV RNA decreased 1 log or more at week 4 in 67 (67.0%) and became undetectable in 31 (RVR, 31.0%). SVR was attained in 25 of the 31 RVR patients (80.6%). Likewise, a complete EVR was attained in 66 patients, among whom 46 (69.7%) finally achieved SVR. SVR was achieved in 52 of 76 patients (68.4%) whose serum HCV RNA had disappeared by week 24. SVR also was achieved in patients whose serum HCV RNA had not disappeared until week 12. SVR was attained in 39.3%, 22.6% and 0% of patients who failed to achieve RVR, complete EVR and HCV RNA negativity at week 24, respectively (Figure 1). From these data, PPV and NPV determined by viral kinetics at week 4 were 80.6% and 60.7%, respectively. PPV at weeks 12 and 24 were 68.7% and 68.4% respectively, and NPV were 77.4% and 100%, respectively.

Multivariate analysis for contributing factors to achieve SVR

Multivariate analysis was performed to determine the factors contributing to SVR. Analysis was made pretreatment and at weeks 4, 12 and 24. Factors available from pretreatment until at the time point were all included, and those calculated as $P < 0.1$ by univariate analysis at each time point were analyzed using the logistic regression method. A statistical difference was found in gender, age, RBC, Hb, PLT and log (ALT 0 wk: ALT levels at week 0) at pretreatment by univariate analysis. The independent factor contributing to SVR was RBC ($P = 0.024$) at pretreatment. Among significant factors found by univariate analysis at week 4, log (ALT 0 wk) ($P = 0.015$), RVR (4 wk) ($P = 0.0049$), and log (AST 4 wk) ($P = 0.042$) were independent factors by multivariate analysis. Similarly, log (ALT 0 wk) ($P = 0.0076$), EVR ($P = 0.0083$), WBC (4 wk) ($P = 0.035$), and average cumulative RBV dose ($P = 0.045$) were significant factors at week 12. Independent

Table 2 Logistic regression analysis of independent predictive factors for sustained virological response

Variables	Odds ratio	95% CI	*P* value
At pretreatment			
RBC ($\times 10^4$) (0 wk)	1.011	1.002-1.021	0.024
PLT ($\times 10^3$) (0 wk)	1.085	0.986-1.193	0.095
log (ALT 0 wk)	3.509	0.727-16.934	0.118
At week 4			
Age	0.941	0.885-1.000	0.051
log (ALT 0 wk)	27.090	1.891-388.001	0.015
RVR +/-	6.543	1.766-24.243	0.0049
log (AST 0 wk)	0.036	0.001-0.886	0.042
At week 12			
log (ALT 0 wk)	39.331	2.648-584.144	0.0076
RVR +/-	3.015	0.694-13.100	0.141
EVR +/-	8.340	1.728-40.265	0.0083
WBC (4 wk)	1.001	1.000-1.002	0.035
log (AST 12 wk)	0.049	0.002-1.037	0.053
RBV dose (12 wk)	1.519	1.010-2.284	0.045
At week 24			
log (ALT 0 wk)	68.688	3.669 to < 999.999	0.0047
RVR +/-	3.329	0.819-13.529	0.093
EVR +/-	31.775	2.840-355.460	0.0050
WBC (4 wk)	1.001	1.000-1.002	0.044
log (AST 12 wk)	0.036	0.001-0.918	0.044
RBV dose (12 wk)	1.607	1.021-2.528	0.040

contributing factors at week 24 were log (ALT 0 wk) ($P = 0.0047$), HCV RNA (-/+) (24 wk) ($P = 0.005$), WBC (4 wk) ($P = 0.044$), log (AST 12 wk) ($P = 0.044$) and average cumulative RBV dose (12 wk) ($P = 0.040$) (Table 2). It was intriguing in addition to RVR and EVR that baseline ALT level (log) always affected SVR prediction from pretreatment until week 24.

Predictive formulae of svr by logistic regression analysis

According to the method of logistic regression analysis, we derived four formulae to predict SVR of patients receiving 48-wk PEG-IFN plus RBV treatment in our cohort from significant factors selected by multivariate analysis at pretreatment and week 4, week 12 and week 24 as shown in Table 2. These formulae are as follows:

Pretreatment: $1/p = 1 + \exp\{-[-8.8065 - 0.0114 \times \text{RBC} (\times 10^4)\ 0\text{ wk} + 0.0812 \times \text{PLT} (\times 10^4)\ 0\text{ wk} + 1.2552 \times \log(\text{ALT } 0\text{ wk})]\}$

At week 4: $1/p = 1 + \exp[-(-1.8839 - 0.00607 \times \text{Age} + 3.2992 \times \log(\text{ALT } 0\text{ wk}) + 1.8784 \times \text{RVR} - 3.3364 \times \log(\text{AST } 4\text{ wk})]$

At week 12: $1/p = 1 + \exp\{-[-11.5278 + 3.672 \times \log(\text{ALT } 0\text{ wk}) + 1.1036 \times \text{RVR} + 2.1211 \times \text{EVR} + 0.000837 \times \text{WBC } 4\text{ wk} - 3.0134 \times \log(\text{AST } 12\text{ wk}) + 0.418 \times \text{RBV dose } 12\text{ wk}]\}$

At week 24: $1/p = 1 + \exp\{-[-14.5754 + 4.2296 \times \log(\text{ALT } 0\text{ wk}) + 1.2028 \times \text{RVR} + 3.4587 \times \text{HCV RNA } 24\text{ wk} + 0.0009 \times \text{WBC } 4\text{ wk} - 3.3224 \times \log(\text{AST } 12\text{ wk}) + 0.4741 \times \text{RBV dose } 12\text{ wk}]\}$

HCV RNA (-): 1, (+): 0.

ROC curve analysis was conducted to evaluate the accuracy of each prediction using both selection data and test data. The area under the curve of the ROCs

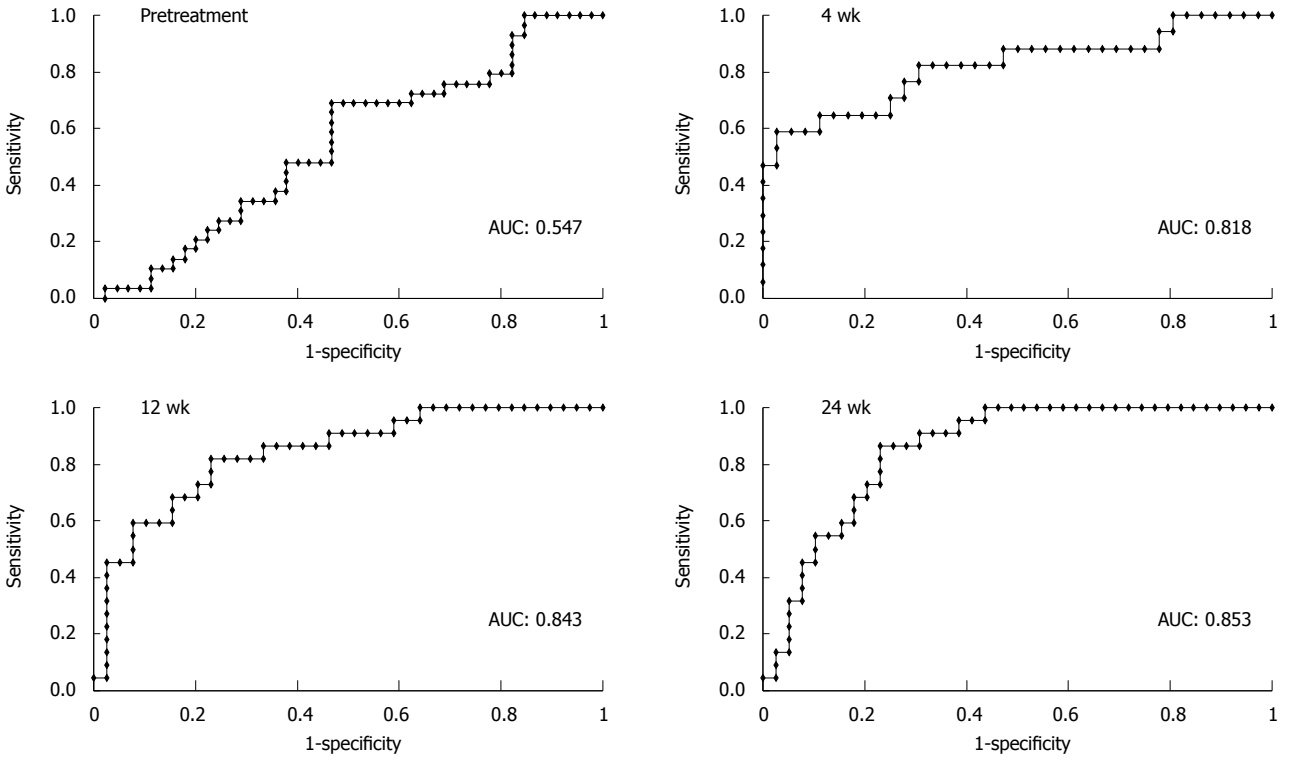

Figure 2 Receiver operating characteristic (ROC) curves and the area under curve (AUC) of the predictive values made by the formulae during peg-interferon plus ribavirin therapy for chronic hepatitis C at week 4, 8, 12 and 24.

(AUCs) of multiple logistic regression analyses using selection data (n = 100) at pretreatment and at weeks 4, 12 and 24 were 0.710, 0.828, 0.889, and 0.933, respectively. The predictive value at pretreatment was insufficient, but those after weeks 4, 12 and 24 were satisfactory.

The validity of the predictive formulae was evaluated further using test data (n = 76). The AUCs of logistic regression analyses using test data were 0.547, 0.818, 0.843, and 0.853 at pretreatment and weeks 4, 12 and 24, respectively (Figure 2). Unlike prediction by viral kinetics, our formula is always applicable to all patients and the final predictive value is fairly high according to the ROC analysis, as described above. The median calculated values for patients who ultimately attained SVR were 0.545, 0.661, 0.589 and 0.656, at pretreatment and weeks 4, 12 and 24, respectively, and these values were always significantly higher than those of non-SVR cases (Table 3). The statistical difference of predictive values between SVR and non-SVR patients increased with the duration of therapy.

Table 3 The median predictive values for sustained virological response (SVR) rates calculated using our formulae at pretreatment and weeks 4, 12 and 24

Median (range)	Patients who attained SVR	Patients with non-SVR	*P* value
Pretreatment	0.545 (0.286-0.926)	0.507 (0.108-0.945)	0.514
At week 4	0.661 (0.139-0.998)	0.251 (0.056-0.688)	0.000
At week 12	0.589 (0.079-0.994)	0.146 (0.001-0.952)	0.000
At week 24	0.656 (0.038-0.996)	0.026 (0.000-0.949)	0.000

The values were compared between patients who attained SVR and those who did not.

Prediction at week 48

It has been suggested that the prolongation of treatment is effective for patients who do not achieve HCV RNA negativity at week 12. There are also patients who achieve RVR and/or EVR but whose serum HCV RNA reappears after cessation of the treatment. We evaluated whether we can select such patients for whom treatment should be lengthened to 72 wk, using the same procedure as for on-treatment prediction of SVR with 48 wk of treatment. The predictive formula at week 48 was constructed from the data of the 100 patients used for selection data. This formula included the parameter whether HCV RNA disappeared during therapy. When HCV RNA disappeared at week 4, 8, 12 and 24, each value, such as 4, 8, 12 and 24, was inserted into the formula. For non-SVR cases, 100 was inserted into the formula. The formula was determined as follows:

$1/p = 1 + \exp\{-[-4.9107 - 0.0079 \times \text{Time of HCV RNA negative (wk)} + 0.1477 \times \text{PLT 0 wk} + 3.4941 \times \log(\text{ALT 0 wk}) - 1.7018 \times \log(\text{AST 12 wk})]\}$

The ROC curve obtained from the test data of 76 patients is shown in Figure 3. The AUC derived from the test data (n = 76) by logistic regression analysis was 0.871, suggesting that patients who can stop treatment at week 48 are predicted accurately by this formula. The median calculated value of patients who attained SVR was 0.775 (0.237-0.999) and that of patients who did not achieve

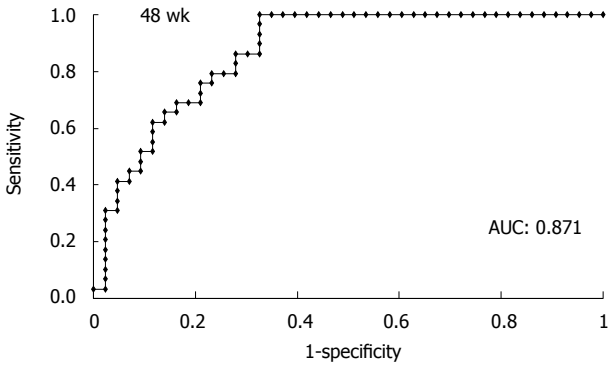

Figure 3 Receiver operating characteristic (ROC) curves of the predictive values calculated by the formula from data up to 48 wk of peg-interferon plus ribavirin therapy for chronic hepatitis C.

SVR was 0.004 (0-0.966, $P < 0.00001$). PPVs and NPVs calculated by the formula with various cut-off points are shown in Table 4. When we set the cut-off point at 0.2, NPV was 100% and 28 patients were included in this category.

There were 10 patients who relapsed after attaining complete EVR in 76 patients (4 cases achieved negativity of HCV RNA at week 8 and others achieved at week 12), and two of them could be pointed out as non-SVR by our calculation if the cut-off point was set at 0.5 (the value of one predicted person was 0.129 and another was 0.421).

DISCUSSION

We propose here a method using formulae for the prediction of SVR in patients with chronic hepatitis C treated for 48 wk with PEG-IFN plus RBV. The predictive potential was very high when judged by AUC analysis, which was more than 0.8 from week 4. In particular, the validity at week 24 was more than 0.85 of AUC. The simplest method of prediction of SVR may be viral kinetics and a response-based on-treatment prediction, such as RVR and EVR, which are the outcomes of totally integrated viral and host factors. The PPV of the formulae were better at weeks 12 and 24 than the prediction with viral kinetics, and the NPV of the formulae were better at weeks 4 and 12. Evaluation of the formulae using data from the test patients revealed a very high AUC value of more than 0.85. These results suggest that formulae based on simple clinical data are superior to prediction by viral kinetics. These formulae, however, cannot be permanent, and may vary among different groups of patients, so should be re-evaluated or re-constructed, even for our series when the number of patients has increased. The most important outcome of this study is that we can predict SVR of our patients accurately with 48-wk PEG-IFN plus RBV therapy using only "simple" clinical data. Individual tailoring of treatment duration may be an option in the future to reduce relapse rates in HCV type 1-infected patients. The concept that extension of treatment duration can reduce relapse rates should be adopted only for a limited proportion of type 1-infected patients because the possibility of SVR may be very low in those whose serum HCV RNA remains positive at week 24. The patients who will benefit most from prolongation of therapy include those whose serum HCV RNA is positive at week 12 but negative at week 24, including even patients with RVR and EVR. The formulae we suggest might be helpful for such patients who are expected to achieve SVR but did not do so. For those individuals, our method based on logistic regression analysis will show a clear direction of therapy in each case and enable the best tailored treatment. Further prospective studies should be performed to determine whether this approach really increases the SVR rate by selection of patients and extension of treatment duration up to week 72.

Table 4 Positive and negative predictive values calculated by the formula constructed with data at week 48

Cut-off point	PPV (%)	NPV (%)	*P* value
0.2	65.9	100	< 0.00001
0.3	66.7	96.7	< 0.00001
0.4	66.7	86.1	< 0.00001
0.5	68.8	82.5	0.000013
0.6	71.4	79.5	0.000024
0.7	77.3	76.0	0.000048
0.8	80.0	70.2	0.00074

The calculated value was applied to the cut-off points and the numbers of patients with sustained virological response were evaluated.

RVR may be valuable for determining treatment duration but is not sufficient for predicting the response to treatment[8]. When SVR is predicted by RVR, the confirmed SVR rate within whole patients may be low. In the study of Yu *et al*[19], SVR was achieved in 42 of 100 patients, and all the patients who attained RVR achieved SVR. However, SVR was also attained in patients who did not attain RVR, and another 30 SVR patients who were not included in the RVR group. If 100% of patients with RVR attain SVR, the final prediction of SVR at this point (week 4) is 53.2%. In the study of Jensen *et al*[20], 95 of 374 (25.4%) genotype 1 patients attained RVR. The SVR rate of these 95 patients was 82% compared to 20.8% among the 374 treated subjects. The PPV of EVR for SVR is estimated to be less robust, less than 70%, and the validity may decrease more when we predict SVR by RVR only. We could predict SVR patients with 57.9% if the cut-off point was set at 0.5, and with 64.7% if that was set at 0.6 at week 4. The potent SVR of patients who did not achieve RVR could be predicted by our formula and the combination with viral kinetics may further improve predictive value.

Ferenci *et al*[21] investigated response-guided treatment based on RVR and 78.8% of HCV genotype 1 patients with RVR attained SVR. Around 20% of patients with RVR failed to achieve SVR in their study. The SVR rate of patients with a complete EVR is around 80% and that

of patients without an EVR is around 15%. Therefore, there are several unfortunate patients with RVR and EVR but in whom serum HCV RNA reappears after the cessation of treatment. On the other hand, it may be difficult to attain SVR for patients whose serum HCV RNA does not disappear until after week 24 (late responders), even if they are treated for more than 48 wk with PEG-IFN plus RBV. Recent studies suggested that the extension of treatment to 72 wk would help to achieve SVR in such "unfortunate" patients, who should have responded well to the 48 wk therapy. It is not realistic that all patients who attain RVR and EVR should receive 72 wk therapy to ensure SVR. Our method of deriving a formula, predicting success or failure of response to 48 wk treatment, may serve as a good compass to identify patients who require extended treatment to achieve SVR. Of course, further prospective study is necessary and there has been no evidence that prolongation of therapy really decreases relapse rate. However, using the formula of week 48, we could predict patients who will benefit from an additional 24 wk of treatment and achieve an SVR. In fact, we could recognise 2 of 10 EVR patients as non-SVR by our formula and they would be rescued if they receive additional 24 wk therapy.

Perhaps the rule of stopping the treatment of patients with a decrease of less than 2 $\log_{10}$ in HCV RNA level within the initial 12 wk of therapy should be reconsidered because the high NPV of this rule (98%-100%) could be confirmed only for the 48 wk treatment group and not for the 72 wk group. As seen in our series, Japanese patients with chronic hepatitis C are older and have thinner physiques than those in Western countries. Because the time of infection was approximately 60 years ago[22], and much earlier than elsewhere in the world, patients of an older age require treatment with PEG-IFN plus RBV[23]. However, these patients easily become anemic, probably because of their older age combined with their physical characteristics[24], and the adherence to RBV, which may be critical for attaining SVR, is usually low. An RBV dosage of 1000-1200 mg/d is administered rarely in Japanese studies. The higher dose of PEG-IFN and RBV in 48 wk therapy suggested by Fried *et al*[25], who studied patients with a mean age of approximately 47, is almost impossible in Japan. When older patients opt for PEG-IFN plus RBV therapy, it is a "one-chance-treatment" and they always endure patiently the side effects of therapy but rarely agree to re-treatment after the cessation of therapy. Therefore, the on-treatment prediction of SVR is very important for older patients and, if the probability of success is reasonable, they would choose prolongation of the therapy. In this case, our formula is a very useful tool to decide whether the patient should receive additional therapy at week 48.

Many factors affecting the SVR rate have been reported, including viral- and host-related factors. Among the viral factors, amino acid substitutions in the interferon sensitivity determining region (ISDR) located in HCV nonstructural region 5A[11] and the core region (71st and 90th codons)[12] are well established. We also reported amino acid substitutions in the RNA-dependent RNA polymerase (NS5B) region from our cohort study[26,27]. On the other hand, host factors, such as pretreatment intrahepatic CD8+ cell count[28], the T-helper type 1 and 2 (Th1/Th2) ratio[13,29,30] and T-helper activity[31], have also been demonstrated. Other factors, such as metabolic and diabetic factors, have been implicated in the efficacy of IFN therapy[32]. RBV plasma concentration at week 4[14] and a new index, named accordion index[33], have also been proposed. These significant viral and host factors, except for the metabolic factors, are difficult to examine and daily clinical assessment is not practical. Shirakawa *et al*[15] published an excellent report recently on the classification of patients according to their responsiveness to PEG-IFN plus RBV therapy. They predicted SVR successfully with pretreatment data, but the prediction included particular determinations, such as ISDR sequences and Th1/Th2 ratios, which are not easily available in clinics and are uneconomical. In contrast to their report, the formulae proposed in this study involve factors included among readily available data, and moreover, the validity was very high, especially at weeks 24 and 48.

In conclusion, our predictive formula, which is easily constructed in every institution with simple clinical data, would offer better prediction of SVR and non-SVR than the prediction by viral kinetics. Further study including extended protocol (72 wk treatment) and analysis with other measurement of HCV RNA, such as real-time PCR, should be evaluated in the future.

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COMMENTS

Background

The likelihood of treatment success of 48 wk peg-interferon (PEG-IFN) plus ribavirin (RBV) therapy for chronic hepatitis C may be predicted by viral kinetics on therapy. In particular, recent studies have shown that sustained virological response (SVR) can be predicted by a rapid virological response (RVR), and

an early virological response (EVR). Nevertheless, the current dosing regimens could potentially under-treat some patients and additional measurement of viral response is needed to facilitate individualization of therapy. Among predictive factors already reported, many are not readily available from daily clinical assessment, because they require genomic analyses and/or advanced experimental methods.

Research frontiers

It is difficult to accurately determine whether the individual should have their therapy extended at week 48, because the predictive value of a slow virological response may be insufficient alone. It would be very valuable to have a more accurate predictive marker of SVR at week 48, derived from clinically available measurements. According to the method of logistic regression analysis, the authors of this study derived formulae to predict SVR of patients receiving 48 wk PEG-IFN plus RBV treatment in their cohort from significant factors selected by multivariate analysis at pretreatment and weeks 4, week 12 and week 24.

Innovations and breakthroughs

The most important outcome of this study is that it is possible to predict SVR accurately with 48 wk PEG-IFN plus RBV therapy by formulae using only "simple" clinical data.

Applications

This study may enable the best tailored treatment especially for patients with a high expectation of sustained virological response with 48 wk peg-interferon and ribavirin therapy for chronic hepatitis C but whose responses relapse. Prospective studies informed by this method will be of considerable value.

Terminology

RVR is defined as an undetectable level of HCV RNA at 4 wk of treatment, and EVR is defined as either an undetectable level of HCV RNA or a drop in HCV RNA levels of at least 2 $\log_{10}$IU/mL after 12 wk of treatment. Recent studies reported that these on-treatment viral kinetics are useful for prediction of SVR.

Peer review

Conclusively, establishing a new predictive formula to assess the likelihood of a SVR of the individual patient chronically infected with HCV is very important.

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BRIEF ARTICLE

Reoperation for early postoperative complications after gastric cancer surgery in a Chinese hospital

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Abstract

AIM: To investigate the occurrence of postoperative complications of gastric cancer surgery, and analyze the potential causes of reoperation for early postoperative complications.

METHODS: A total of 1639 patients who underwent radical or palliative gastrectomies for gastric cancer were included in the study. The study endpoint was the analysis of postoperative complications in inpatients.

RESULTS: About 31% of patients had early post-operative complications, and complications of infection occurred most frequently. Intra-abdominal hemorrhage and anastomotic leak were the main causes of reoperation, which accounted for about 2.2%. Mortality was 11.1% in the reoperation group, but was only 0.8% in other patients. The duration of postoperative stay in hospital was significantly longer and the total expenditure was markedly higher in the patients who underwent reoperation ($P < 0.001$). There was no significant association of any available factors in this study with the high rate of reoperation.

CONCLUSION: Reoperation significantly increases the mortality rate and raises the burden of the surgical unit. More prospective studies are required to explore the potential risk factors.

Key words: Reoperation; Gastric cancer; Surgery; Postoperative complications

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Sah BK, Chen MM, Yan M, Zhu ZG. Reoperation for early postoperative complications after gastric cancer surgery in a Chinese hospital. *World J Gastroenterol* 2010; 16(1): 98-103 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i1/98.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i1.98

INTRODUCTION

Though the occurrence of postoperative complications and mortality rate after surgery for gastric cancer have significantly decreased over the past years, they are still considered high[1,2]. Radical gastrectomy with D2 lymph node dissection is widely accepted, but the extent of lymph node dissection is controversial among different centers[3-10]. It is well accepted that the extent of surgery (particularly aggressive dissection of the lymph nodes) does not extend the overall survival, and postoperative complications were significantly related to the extent of surgery, particularly the extent of lymph node dissection. This was proved by Japanese surgeons who conducted several clinical randomized controlled trials (RCTs)[3,9-11]. Sasako *et al*[9] conducted a RCT in 24 hospitals in Japan to compare D2 lymphadenectomy alone with D2 lymphadenectomy plus para-aortic nodal dissection

(PAND) in patients undergoing gastrectomy for curable gastric cancer. They concluded that compared with D2 lymphadenectomy alone, D2 lymphadenectomy plus PAND does not improve the survival in curable gastric cancer; extended D2 lymphadenectomy plus PAND should not be performed to treat curable stage T2b, T3, or T4 gastric cancer; and that D2 gastrectomy is associated with the low mortality and reasonable survival of the patients.

Many researchers at leading centers for gastric cancer, including those in Korea and China, have indicated that combined resection of other organs is not of long-term benefit and it significantly increased the prevalence of postoperative complications and mortality[3,4,11-13]. The occurrence rates of postoperative complications in the spleen-preservation group and splenectomy group were 11.6% and 29.3%, respectively. There was a higher frequency of pleural effusion, intra-abdominal abscess, and pancreatitis in splenectomized patients. A higher recurrence was observed in the splenectomy group (40.4%) compared with the spleen-preservation group (25.1%). The mean survival time was 72.0 mo in the spleen-preservation group compared with 56.7 mo in the splenectomy group[13]. Investigation of early postoperative complications would therefore be beneficial to optimize the extent of gastric cancer surgery.

Reoperation after routine surgery, particularly after gastric cancer surgery, increases the overall burden for both the the surgical ward and the patients. We therefore investigated the factors causing reoperation and their effects on the recovery of patients who undergo surgery for gastric cancer.

MATERIALS AND METHODS

A total of 1639 patients who underwent radical or palliative gastrectomies for gastric cancer in five consecutive years were included in the study (Table 1). Data were collected directly by comprehensive review of the original records of all patients. Sixty-seven patients with missing data and 13 patients who underwent emergency surgery were excluded from the analysis. Exclusion criteria were disease other than gastric cancer, and any type of palliative surgery (including exploratory laparotomy and gastrojejunal anastomosis) other than gastrectomy. The median age of the patients was 59 years (range, 17-93 years). The ratio of male and female patients was approximately 7:3.

Patients with early and resectable advanced gastric cancer underwent radical surgery (gastrectomies with D2 lymphadenectomy). Patients with late-stage gastric cancer underwent palliative gastrectomy. Most patients were diagnosed to be in stage Ⅲ. The tumor invaded the serosa or adjacent structures in 38.1% of patients which was classified as pT3, and in 11.4% of patients classified as pT4 (Table 1). With respect to combined organ resection, 19 splenectomies, 14 partial pancreatectomies with splenectomy, 14 partial colectomies, two partial colectomies with splenectomy, seven partial hepatectomies (lobectomy),

Table 1 Demographic data of the patients

Items	Percentage (%)
Age group (yr)	
≤ 60	54.1
61-70	25.2
≥ 71	20.7
Sex	
Male	68.9
Female	31.1
Diagnosis	
Primary gastric cancer	97.4
Gastric stump cancer[1]	2.6
Site of tumor	
Proximal	17.6
Body	13.5
Distal	43.3
Large or multiple	25.6
No. of procedures	
Partial gastrectomy	76.0
Total gastrectomy	24.0
Type of resection	
Radical gastrectomy	91.6
Palliative gastrectomy	8.4
Combined resection	
Yes	4.4
No	95.6
Type of anastomosis	
Billroth Ⅰ	47.6
Billroth Ⅱ	13.9
Billroth reverse[2]	13.4
Roux-en-Y	21.6
Roux-en-Y (P-shape)	3.0
Others	0.5
Unknown	0.3
TNM stage	
ⅠA	12.1
ⅠB	8.5
Ⅱ	18.1
ⅢA	20.8
ⅢB	16.2
Ⅳ	24.3

[1]Including recurrent gastric cancer; [2]Oesophago-gastric anastomosis.

one total hysterectomy and one partial pancreatectomy were carried out in 58 patients (Table 1). About 25% of the patients underwent total gastrectomy. Billroth I, typical Roux-en-Y, and Billroth reverse (esophagogastric anastomosis) were the preferred methods for anastomosis after distal gastrectomy, total gastrectomy and proximal partial gastrectomy. Above 85% patients underwent surgery by senior surgeons with experience of 20-30 years. The minimal working experience of the surgeons was > 15 years. No surgical fellow or surgeons-in-training was allowed to perform the surgery independently. All the patients were managed by senior attendants under direct supervision of the surgeons.

The endpoint was analysis of postoperative complications and postoperative mortality in inpatients. Complications were recorded according to the definitions stated in the Physiological and Operative Severity Score for the Enumeration of Mortality and Morbidity (POSSUM)[14]. As there are many complications that are not covered by its definitions, an undefined complication was therefore

recorded as "innominate" and the details were provided in separate tables. Severities of all complications were stratified according to Rui Jin Hospital Classification of Complications[15].

We also audited the overall expenditure in US dollars ($) of patients during their stay in hospital and compared it between the reoperation group and non-reoperation group.

Statistical analysis

The statistical analysis was done using the Statistical Package for Social Science (SPSS) version 13.0 for Windows (SPSS, Incorporated, Chicago, IL, USA). Non-parametric methods were used to test the data without normal distribution. $P < 0.05$ was considered significant.

RESULTS

About 31% of patients had different types of complications according to POSSUM criteria. The prevalence of individual complications was not equal to the total number of complications. Multiple complications were possible in a single patient (Table 2). Postoperative infection was the most common complication. The occurrence of anastomotic leak was about 2%, and postoperative mortality was only 1%.

There were numerous innominate complications (Table 3), most of which were accompanied by complications described in POSSUM. Most patients had pleural effusion or/and seroperitoneum, most of which were accompanied by low fever but pathological diagnosis of infection could not be confirmed. A substantial number of patients had persistent fever or recurring fever of unknown origin. About 5% of patients had persistent nausea or vomiting caused by gastroplegia or enteroplegia, anastomosis edema, or ileus. Some patients were clinically suspected to have a minor leak but there was no sufficient objective evidence to support this finding. Although these patients were managed by conservative treatment (mainly NPO, intravenous antibiotics, and total parenteral nutrition), they still increased the burden on the surgical ward. Complications were rare such as pancreatic fistula, chyle leak, and bleeding at the anastomosis site.

Innominate complications were recorded empirically and merged to calculate different levels of complication type according to the Rui Jin Hospital Classification of Complications[15]. Most complications were minor (11.0%) or moderate (15.2%), only 8.3 % of patients had severe complications.

Patients were categorized into three levels according to the length of postoperative stay in hospital. About 75% of patients were discharged in good condition in less than 15 d after uneventful recovery and removal of sutures, 19.7% of patients discharged within 16-30 d and only 6% of patients stayed in hospital for more than a month.

There was a significant difference in the occurrence rate of overall complications between partial and total gastrectomy with radical lymphadenectomy, but no significant difference between partial and total gastrectomy with palliative lymphadenectomy was observed (Table 4).

Table 2 Details of complications

Complications	Frequency	Percentage (%)
Hemorrhage		
Wound	1	0.1
Deep	16	1.0
Wound dehiscence		
Superficial	9	0.5
Deep	5	0.3
Anastomotic leak	38	2.3
Infection		
Wound	15	0.9
Deep	80	4.9
Pyrexia of unknown origin	253	15.4
Septicemia	7	0.4
Chest	172	10.5
Urinary tract infection	20	1.2
System failure		
Renal	28	1.7
Respiratory	22	1.3
Cardiac	13	0.8
Hypotension	16	1.0
Deep venous thrombosis	4	0.2
Death	17	1.0
Overall	506	30.9

Table 3 Innominate complications

Complications	Frequency	Percentage (%)
Pleural effusion	213	13
Continuous or relapsing pyrexia of unknown origin	170	10.4
Seroperitoneum	118	7.2
Gastro or enteroplegia, anastomosis edema, ileus	75	4.6
Suspicious or sub-clinical anastomotic leak	57	3.5
Pancreatitis	22	1.3
Central vein catheter infection	14	0.9
Anastomosis site or upper GI bleeding	10	0.6
Chyle leak	8	0.5
Pancreatic fistula	6	0.4

The occurrence of complications of infection (including deep infection, pulmonary infection), system failure, and mortality was significantly higher in total gastrectomy with radical lymphadenectomy. After stratification of patients into partial and total gastrectomy groups, we noted no significant difference in complication occurrence between radical lymphadenectomy and palliative lymphadenectomy (Table 5).

Thirty-six patients underwent reoperation for different causes, with. intra-abdominal hemorrhage and anastomotic leak as the main causes (Table 6). There was no significant difference in physiological score (PS; $P = 0.382$) and operative severity score (OSS; $P = 0.849$) between patients in the reoperation group and the non-reoperation group. Median values of PS and OSS in the reoperation group were 14.5 (range, 12-25) and 18 (range, 16-24) respectively, whereas they were 15 (range, 12-38) and 18 (range, 11-28) in the non-reoperation group. Mortality was significantly higher in patients who underwent reoperation ($P < 0.001$), being 11.1% in the reoperation group but only 0.8% in

Table 4 Difference of complication rate between partial and total gastrectomy

LN dissection	Radical			Palliative		
Gastrectomy	Partial	Total	Sig	Partial	Total	Sig
Overall	308 (26.7)	142 (40.9)	< 0.001	32 (34.8)	24 (52.2)	NS
Reoperation	23 (2.0)	12 (3.5)	NS	1 (1.1)	0	NS
Hemorrhage						
Wound	1 (0.1)	0	NS	0	0	
Deep	11 (1.0)	4 (1.2)	NS	1 (1.1)	0	NS
Wound dehiscence						
Superficial	7 (0.6)	1 (0.3)	NS	1 (1.1)	0	NS
Deep	4 (0.3)	1 (0.3)	NS	0	0	
Leak	22 (1.9)	12 (3.5)	NS	1 (1.1)	3 (6.5)	NS
Infection						
Wound	8 (0.7)	6 (1.7)	NS	1 (1.1)	0	NS
Deep	42 (3.6)	30 (8.6)	< 0.001	5 (5.4)	3 (6.5)	NS
PUO	165 (14.3)	65 (18.7)	0.044	13 (14.1)	10 (21.7)	NS
Septicemia	1 (0.1)	6 (1.7)	< 0.001	0	0	
Chest	86 (7.5)	61 (17.6)	< 0.001	14 (15.2)	11 (23.9)	NS
UTI	13 (1.1)	6 (1.7)	NS	1 (1.1)	0	NS
System failure						
Renal	15 (1.3)	12 (3.5)	0.008	1 (1.1)	0	NS
Respiratory	12 (1.0)	7 (2.0)	NS	3 (3.3)	0	NS
Cardiac	5 (0.4)	6 (1.7)	0.034	2 (2.2)	0	NS
Hypotension	8 (0.7)	6 (1.7)	NS	2 (2.2)	0	NS
DVT	1 (0.1)	3 (0.9)	NS	1 (1.1)	0	NS
Death	6 (0.5)	8 (2.3)	0.007	2 (2.2)	1 (2.2)	NS

Sig: Significance; LN: Lymph node; PUO: Pyrexia of unknown origin; UTI: Urinary tract infection; DVT: Deep vein thrombosis.

Table 5 Difference of complications rate between radical and palliative LN dissection

LN dissection	Partial			Total		
Gastrectomy	Radical	Palliative	Sig	Radical	Palliative	Sig
Overall	308 (26.7)	32 (34.8)	NS	142 (40.9)	24 (52.2)	NS
Reoperation	23 (2.0)	1 (1.1)	NS	12 (3.5)	0	NS
Hemorrhage						
Wound	1 (0.1)	0	NS	0	0	NS
Deep	11 (1.0)	1 (1.1)	NS	4 (1.2)	0	NS
Wound dehiscence						
Superficial	7 (0.6)	1 (1.1)	NS	1 (0.3)	0	NS
Deep	4 (0.3)	0	NS	1 (0.3)	0	NS
Leak	22 (1.9)	1 (1.1)	NS	12 (3.5)	3 (6.5)	NS
Infection						
Wound	8 (0.7)	1 (1.1)	NS	6 (1.7)	0	NS
Deep	42 (3.6)	5 (5.4)	NS	30 (8.6)	3 (6.5)	NS
PUO	165 (14.3)	13 (14.1)	NS	65 (18.7)	10 (21.7)	NS
Septicemia	1 (0.1)	0	NS	6 (1.7)	0	NS
Chest	86 (7.5)	14 (15.2)	0.008	61 (17.6)	11 (23.9)	NS
UTI	13 (1.1)	1 (1.1)	NS	6 (1.7)	0	NS
System failure						
Renal	15 (1.3)	1 (1.1)	NS	12 (3.5)	0	NS
Respiratory	12 (1.0)	3 (3.3)	NS	7 (2.0)	0	NS
Cardiac	5 (0.4)	2 (2.2)	NS	6 (1.7)	0	NS
Hypotension	8 (0.7)	2 (2.2)	NS	6 (1.7)		NS
DVT	1 (0.1)	1 (1.1)	NS	3 (0.9)	0	NS
Death	6 (0.5)	2 (2.2)	NS	8 (2.3)	1 (2.2)	NS

other patients (Table 7). In the reoperation group, the mortality rate of patients with radical lymphadenectomy was higher than that of patients who underwent palliative lymphadenectomy. Mortality rate was higher in patients who underwent total gastrectomy than in those who

Table 6 Causes of reoperation

Causes	Surgical management	Frequency
Intra-abdominal hemorrhage[1]	Simple hemostasis	16
Anastomotic leak	Repair and placement of drainage	10
Deep wound dehiscence	Closure of abdominal wall	4
Abdominal infection	Debridement and placement of drainage	3
Ileus	Adhesiolysis of small intestine	2
Anastomotic obstruction	Reconstruction	1

[1]Including 2 cases of anastomosis site bleeding.

Table 7 Potential causes of death

Complications	Reoperation *n* (%)	
	Yes (*n* = 36)	No (*n* = 1603)
Death	4 (11.11)	13 (0.81)
Extent of surgery		
LN dissection		
Radical	4 (11.11)	10 (0.62)
Palliative	0	3 (0.18)
Gastrectomy		
Partial	1 (0.03)	7 (0.44)
Total	3 (8.33)	6 (0.37)
Complications		
Intra abdominal hemorrhage	1 (0.03)	0
Anastomotic leak	2 (5.55)	0
Infection		
Deep	1 (0.03)	2 (0.12)
Pyrexia of unknown origin	0	3 (0.18)
Septicemia	0	1 (0.06)
Chest	3 (8.33)	7 (0.44)
Urinary tract	2 (5.55)	0
System failure		
Renal	3 (8.33)	8 (0.49)
Respiratory	4 (11.11)	10 (0.62)
Cardiac	2 (5.55)	9 (0.56)
Hypotension	1 (0.03)	9 (0.56)
Deep venous thrombosis	0	3 (0.18)
Pancreatitis	0	1 (0.06)
Anastomosis site bleeding	0	1 (0.06)

underwent partial gastrectomy (Table 7).

Except for four patients with wound dehiscence who were discharged within one month, the other 32 patients were treated in hospital for more than one month. The length of postoperative stay was significantly longer in patients who underwent reoperation ($P < 0.001$). The mean duration of postoperative stay was 44.6 d (standard deviation, SD = 29.41 d) in patients with reoperation, but was only 14.6 d (SD = 8.09 d) in other patients.

Reoperation caused a significant economic burden for patients. There was a significant difference in the total expenditure between groups of patients with or without reoperation ($P < 0.001$). The median expenditure in patients with reoperation was 7946.36 $ (SD = 8930.38 $) but it was only 3238.32 $ (SD = 4404.63 $) in other patients.

Univariate analysis of the data revealed no significant association of any available factors in this study with the higher rate of reoperation, including age, hypertension,

anemia, hypoalbuminemia, hyperglycemia, type of gastrectomy, combined organ resection, type of anastomosis, surgeon's experience (number of operations performed), tumor stage.

DISCUSSION

In the surgical approach for early and selective advanced gastric cancer, gastrectomy with D2 lymphadenectomy is justified[6,16-19]. The procedure of surgery, particularly the extent of lymphadenectomy for gastric cancer, varies among individual centers. The occurrence of postoperative complications was higher in inexperienced hands, and there was a considerable difference in early surgical outcomes among centers[3,20]. Postoperative complications were inversely correlated with the number of patients undergoing treatment in a surgical unit[21]; similar results were published for patients undergoing surgery for gastric cancer[15]. Overall survival rate was higher at specialized centers. It was therefore stressed in many articles that gastric cancer surgery was safe at specialized centers[3,6,22,23].

The postoperative complications at our institution were in the acceptable range because most patients had a smooth recovery and postoperative mortality was not high. Overall surgical outcome was acceptable because of the occurrence rate of complications was below the moderate level. Postoperative infection was the commonest complication. There are several complications (e.g. gastroplegia or enteroplegia, suspicious anastomotic leak, pleural effusion) which are not covered by POSSUM. These complications cannot be ignored because they have a big impact on the overall burden (patient-related and economic) of our hospital. A substantial number of patients had persistent fever without a clear diagnosis; appropriate investigation was necessary to find the cause. Further investigation was required to classify or define the diagnosis of sub-clinical anastomotic leak. The Ruijin Hospital Classification of Complication, stratifies complications to different levels according to the severity of the disease, and is a validated classification[2,15]. We suggest that other hospitals use this classification for assessment of surgical outcome.

Reoperation was in the acceptable range as compared with a recent report from the Korean Institute, and the mortality caused by reoperation was low[24]. Most reoperations were carried out for intra-abdominal hemorrhage, which may be related to the experience of surgeons and necessitates additional efforts to examine the easily missed bleeding sites (particularly anastomosis sites). The four cases of rupture of the abdominal wall may be attributed to the poor surgical technique because these patients had their linea alba closed by an interrupted silk suture. We did not observe this complication in patients with linea alba closed by a continuous absorbable suture. Anastomotic leak was followed by intra-abdominal infection which often caused peripancreatic abscess, and eventually pancreatic fistulas in some cases. Improvement of surgical technique is therefore crucial to lower the occurrence of intra-abdominal hemorrhage and anastomotic leak.

In conclusion, although the overall occurrence of postoperative complications was high after gastric cancer surgery, the occurrence rate of severe complications and mortality were low. Reoperation after gastric cancer surgery significantly increases the mortality and overall burden of the surgical unit. As the gastric cancer surgery is considered as a routine surgery, it is important to control the postoperative complications. Univariate analysis of the data revealed no significant association of any available factors in this study with the high rate of reoperation; however, more prospective studies are required to explore the potential risk factors for the higher rate of reoperation after gastric cancer surgery.

COMMENTS

Background

Though the occurrence of postoperative complications and mortality after surgery for gastric cancer have significantly decreased over the past years, they are still considered high. It was well accepted that the extent of surgery does not extend the overall survival and that postoperative complications were significantly related to the extent of surgery. Therefore, surgical extent should be seriously considered and postoperative complications should not be ignored.

Innovations and breakthroughs

The postoperative complication is highly variable among different centers. However, surprisingly there are very few reports on this issue, especially from Chinese surgical centers. This study was conducted at a leading center for gastric cancer surgery in China, and analyzed a large cohort of patients for a long period. It provides the details on the occurrence of postoperative complications and analyzed its impact on patients and surgical ward. The finding of this study certainly provides very useful reference to the surgeons working in this field.

Applications

The better understanding about the occurrence of different types of the postoperative complications and its underlying causes may help surgeons reduce the postoperative complications and upgrade the quality of surgical treatment.

Terminology

"POSSUM" is an internationally accepted scoring system which is applied for the evaluation of surgical treatment. "Rui Jin Hospital Classification of the complications" is a novel system which stratifies all the complications in three different levels and provides objective idea about the severity of complications.

Peer review

The article has some very good information and is worthy of publication.

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BRIEF ARTICLE

Electro-acupuncture to prevent prolonged postoperative ileus: A randomized clinical trial

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Abstract

AIM: To examine whether acupuncture can prevent prolonged postoperative ileus (PPOI) after intraperitoneal surgery for colon cancer.

METHODS: Ninety patients were recruited from the Fudan University Cancer Hospital, Shanghai, China. After surgery, patients were randomized to receive acupuncture (once daily, starting on postoperative day 1, for up to six consecutive days) or usual care. PPOI was defined as an inability to pass flatus or have a bowel movement by 96 h after surgery. The main outcomes were time to first flatus, time to first bowel movement, and electrogastroenterography. Secondary outcomes were quality of life (QOL) measures, including pain, nausea, insomnia, abdominal distension/fullness, and sense of well-being.

RESULTS: No significant differences in PPOI on day 4 (P = 0.71) or QOL measures were found between the groups. There were also no group differences when the data were analyzed by examining those whose PPOI had resolved by day 5 (P = 0.69) or day 6 (P = 0.88). No adverse events related to acupuncture were reported.

CONCLUSION: Acupuncture did not prevent PPOI and

was not useful for treating PPOI once it had developed in this population.

Key words: Acupuncture; Gastrointestinal motility; Gastrointestinal disorders; Gastrointestinal neoplasms

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INTRODUCTION

Bowel dysfunction following abdominal surgery is common and usually temporary, lasting no more than 3 d; however, if patients are unable to tolerate an oral diet, pass flatus, or have a bowel movement by postoperative day 4, they are considered to have prolonged postoperative ileus (PPOI). PPOI is uncomfortable for patients and potentially dangerous. The collection of gas and secretions related to PPOI causes pain and discomfort with bloating, distention, and often emesis[1]. Delayed gastric emptying also increases the risk of aspiration in patients during the early postoperative period. Patients with PPOI cannot be discharged from the hospital until the ileus has resolved. Few published studies have estimated the cost of PPOI, but expenses related to longer hospital stays, nursing care, laboratory/diagnostic testing, and interventional treatments are likely to be considerable.

Current treatment for postoperative ileus in China is primarily supportive and includes nasogastric suction, intravenous fluids, parenteral nutrition, and gradual ambulation with simple exercises. However, a variety of preventive interventions[2-6] for PPOI, such as preoperative carbohydrate loading, wrapping patients in warm blankets in the operating room, chewing gum, and rocking in a rocking chair postoperatively to stimulate gastrointestinal function, have been mentioned in the literature. Further research is needed, however, to evaluate the efficacy of these approaches. Two studies published by Sculati and colleagues have concluded that a preoperative bran-enriched diet (lasting 8-10 d) may help prevent PPOI[7,8], but this is often not practical for patients undergoing gastrointestinal surgery.

Although prevention and treatment of PPOI with various pharmacologic agents has been explored for several years, success has been limited[9-17]. Alternatives to systemic opioid analgesia, such as thoracic epidural analgesia[18] and non-opioid analgesics such as ketorolac tromethamine[10,11], have been shown to shorten the duration of PPOI when compared with opioids, but non-opioid analgesia does not adequately control pain in all patients[10,11]. The combined use of a local anesthetic for chemical sympathectomy and sparing amounts of narcotic for improved pain control has been proposed, but there is no clear guidance as to which combination best promotes bowel motility while maintaining adequate pain control[17].

A 2008 Cochrane review of the use of prokinetic agents in PPOI has concluded that there was no evidence to support the use of erythromycin, and insufficient evidence for cisapride, cholecystokinin-like drugs such as cerulein, and dopamine antagonists such as metoclopramide, propranolol and vasopressin[19]. Neostigmine rapidly decompresses the colon and has shown some potential in PPOI; however, side effects such as bradycardia, bronchospasm, and increased risk of anastomotic dehiscence are of major concern[17,20]. Lubiprostone, a bicyclic fatty acid that acts as a chloride channel opener and thereby increases intestinal water secretion, has been shown to be effective in constipation[21] and is currently being investigated in PPOI[16].

Narcotic receptor antagonists represent another major class of drugs studied in the treatment of PPOI. Naloxone, for example, is limited by its central nervous system effects and potential to reverse analgesia[17]. Methylnaltrexone, a quaternary derivative of naltrexone that does not cross the blood-brain barrier, has shown some efficacy in opioid-induced constipation[22,23], but preliminary results from two trials in PPOI showed no benefit over placebo[24]. In May 2008, alvimopan, a selective mu-receptor antagonist, was the first drug to receive United States Food and Drug Administration (FDA) approval specifically for the treatment of PPOI after showing benefit in several phase Ⅲ trials[25,26]. However, there are concerns about the cost-benefit ratio of this drug, given that it shows only a modest reduction in hospital stay (7-15 h) and costs nearly $1000/treatment cycle[27,28].

Some research has suggested that traditional Chinese herbal medicines can also help bowel motility[29,30]. For example, *saussura cappa* and the formula *Liu Jun Zi Tang* have been associated with improved stomach and intestinal emptying time and increased plasma motilin levels[29,31]. Although herbal medicine shows some benefit for gastrointestinal motility, abdominal surgery patients generally cannot have anything by mouth during the perioperative period.

Acupuncture has been used in China for thousands of years to treat a variety of gastrointestinal problems[32]. The advantages of acupuncture are that it is a cost-effective, minimally invasive procedure with a very low incidence of side effects. Although prior studies have investigated the effects of acupuncture on gastrointestinal motility in humans[33], few randomized clinical trials have been published. Controlled animal studies supported

by plausible physiological and laboratory evidence have, however, shown that acupuncture has positive effects on gastric and intestinal motility[34-38]. Although the exact mechanisms are not fully understood, one hypothesis is that acupuncture may help regulate the gastrointestinal tract *via* the autonomic nervous system. Several animal studies have revealed that the effect of acupuncture on gastrointestinal function is mediated through sympathetic and parasympathetic efferent pathways[39,40].

To the best of our knowledge, only one study[41] has evaluated the efficacy of acupuncture in preventing PPOI after abdominal surgery. However, in that trial, the incidence of PPOI assessed at postoperative day 4 after ileostomy/colostomy closure was too small to show significance between the treatment and control groups. Therefore, in the current prospective, randomized study, we investigated whether acupuncture could prevent PPOI after invasive colon cancer surgery. Bowel motility was determined by time to first flatus, time to first bowel movement, and by electrogastroenterography (EGEG), a device that detects electrical signals from the abdomen. Secondary objectives were to compare postsurgical quality of life (QOL) between the treatment and control groups in terms of pain, nausea, insomnia, abdominal distension/fullness, and sense of well-being.

MATERIALS AND METHODS

Patient eligibility

Patients were recruited from the Fudan University Cancer Hospital between July 2004 and October 2006 and were enrolled in the trial after providing written informed consent. Regardless of sex or ethnicity, all patients 18-75 years old, who had colon cancer with a Duke A to D stage diagnosis (as long as metastatic disease did not affect bowel function) and were scheduled to undergo intraperitoneal surgery, were identified at the time of preoperative evaluation and screened for eligibility. Eligible patients had to meet the following criteria: physical status classification of category Ⅲ or better according to criteria established by the American Society of Anesthesiologists[42]; planned use of epidural infusion for post-surgical pain management; no upper or lower extremity deformity or local skin infections that could interfere with accurate acupuncture point location; no active systemic infection; no chronic functional constipation as defined by Rome Ⅰ criteria prior to the cancer diagnosis[43]; and no history of cerebrovascular accident or spinal cord injury. Patients were also excluded if they had chronic pain currently treated with any form of major opioid or with weak opioids at morphine equivalent doses > 30 mg/24 h; had a cardiac pacemaker; were mentally incapacitated or had a significant emotional or psychiatric disorder that precluded study participation; were pregnant; were using laxatives or other medicines known to affect bowel function, such as herbal preparations, high-dose vitamins, or iron sulfates; had known bleeding abnormalities or were on heparin or warfarin; had any parasurgical complications needing intensive care; or were currently using acupuncture.

Procedures

The study was designed collaboratively and conceived by faculty from Fudan University Cancer Hospital and M.D. Anderson Cancer Center, The University of Texas. An experienced statistician was involved in all stages of study development and analysis. The protocol was approved by both Institutional Review Boards. Two nurses from Fudan Cancer Hospital spent 3 mo at M.D. Anderson Cancer Center undergoing research nurse training, two physicians underwent 2 mo of faculty research training, and the acupuncturist from Fudan University Cancer Hospital spent 1 mo at M.D. Anderson Cancer Center. The acupuncturist was trained specifically in aspects of quality control and fidelity to study-related acupuncture procedures. During the course of the trial, faculty and staff from M.D. Anderson Cancer Center also visited Fudan Cancer Hospital four times to review the trial. Video conferences were conducted twice each month.

At the Fudan University Cancer Hospital, patients are generally admitted for preoperative evaluation 3-5 d prior to surgery. All patients were recruited during this time. The first 30 patients were randomized using simple randomization; however, group differences were noted for route of anesthesia administration. Therefore, from the 31st patient forward, in order to ensure group balance for anesthesia administration, patients were randomized into either treatment or control groups by a form of adaptive randomization, minimization[44]. Before a participant was assigned to a treatment group, the number of already randomized participants with similar covariate characteristics was totaled. The totals were computed based on marginal sums so that each covariate was considered separately. The treatment assignment for a participant was then based on which treatment group assignment would produce the best overall balance with respect to the covariate characteristics. The patient characteristic used for group assignment was the mode of anesthesia (iv, epidural, or iv plus epidural).

After surgery, if the patient continued to meet all eligibility criteria, she/he was randomized with equal probability into either the treatment or control group. Data were collected and recorded twice a day for 6 d or until the first bowel movement. Patients had to have a bowel movement prior to discharge; therefore, participation ended at the time of discharge for anyone leaving prior to the sixth postoperative day, regardless of whether they had received acupuncture treatment.

Acupuncture treatment

The acupuncture treatments were performed in the patient's room by a hospital accredited physician who has over 30 years of acupuncture experience and is routinely involved in postoperative care to patients. Patients in the treatment group received acupuncture

once a day, starting on postoperative day 1, for six consecutive days or until the first bowel movement, whichever came first. After the skin was prepped with 70% alcohol, the needles were inserted and remained in place for approximately 20 min with each treatment. The treatment frequency was agreed upon by a small panel of experienced acupuncturists. Patients in the control group received standard postoperative care with no acupuncture.

During the acupuncture treatment, each patient lay in a supine position in his or her hospital bed. A tight abdominal dressing and binder was placed after surgery and not removed for 2-3 d, therefore, only points located on the extremities were selected. Electrical stimulation was applied concomitantly and continuously to two pairs of points [SJ-6 (positive) and GB-34 (negative)] bilaterally by placing lead wires on the needles connected to an electro-acupuncture stimulator (Model 980; Shanghai Medical Equipment Co. Ltd., Shanghai, China). This unit applied consistent stimulation throughout the treatment at a frequency of 2 Hz.

The acupuncture needles used (Huatuo, Suzhou, China) conformed to the requirements of the ISO 9002, EN46002 and CE certification, United States FDA International Good Manufacturing Practices, and the World Health Organization's standards for quality and safety. These stainless steel needles are 1.5 *cun* in length by 32-gauge diameter and are provided in individual sterile packages.

Standardized techniques for point location were used and were based on anatomical landmarks as well as proportional measurements of the patient's body. For example, finger breadth refers to the patient's middle finger, and the proportional unit of measure was the "*cun*", defined as the distance between the two medial ends of the creases of the interphalangeal joints when the patient's middle finger is flexed[32]. The following bilateral acupuncture points were selected specifically for the purpose of improving bowel motility. (1) SJ-6: located 3 *cun* proximal to the dorsal crease of the wrist, on the line connecting *Yangchi* (SJ-4) and the tip of olecranon, between the radius and ulna, on the radial side of the extensor digitorum communis muscle. The Chinese name for this point is *Zhigou*. According to the underlying theory of Traditional Chinese Medicine (TCM), this is a major point for stimulating the intestines[45] and is often paired with GB-34 to treat constipation. (2) GB-34: located in the depression anterior and inferior to the head of the fibula. The Chinese name for this point is *Yanglingquan*. This point is often paired with SJ-6 to treat constipation due to *qi* stagnation or heat[45]. (3) ST-36: on the lateral surface of the leg, 3 *cun* distal to the lower border of the patella, one finger's breadth from the tibia tuberosity between the tibia digitorum tibialis anterior muscle and the tendon of longus pedis. The Chinese name for this point is *Zusanli*. It lies just over the deep peroneal nerve and is commonly used by acupuncturists to harmonize (i.e. regulate) the gastrointestinal tract[45]. (4) ST-37: located 3 *cun* below *Zusanli* (St-36) and one finger's breadth (middle finger) from the anterior border of the tibia. The Chinese name for this point is *Shangjuxu*. Based on TCM theory, this point also regulates the intestines[45].

Outcome measures

The main outcome measure of bowel motility was assessed by asking patients to record the exact date and time that they first passed flatus and the exact date and time of their first bowel movement after surgery. Time 0 was the time anesthesia ended according to the anesthetic record. The total numbers of hours between time 0 and the passage of flatus and between time 0 and the first bowel movement were then calculated. PPOI was defined as having no bowel movement for more than 96 h (4 d) after surgery.

Secondary measures included EGEG and QOL assessments. Electrical signals from the stomach and intestines associated with gastrointestinal motility were monitored *via* EGEG (Huake Electronic Technical Research Institute, Beijing, China). The surgeon placed the leads for EGEG monitoring at the time of wound closure and prior to placement of the abdominal dressing and binder. According to standard postoperative care at this hospital, the abdominal dressing and binder were not removed until postoperative day 2 or 3; therefore, assessment of bowel sounds was not performed. EGEG monitoring occurred twice per day. After attachment of the electrodes to the leads, the patient rested for 1 min, and then two consecutive 3-min recordings were obtained. Both frequency (per minute) and amplitude were measured using two channels.

Data related to postoperative QOL were obtained from the nursing and physician progress notes and the patient's self-evaluation using the Quality of Life Status (QOLS) assessment tool, which is based on the Edmonton Symptom Assessment System (ESAS)[46-48]. The QOLS used in this study was a slightly modified version of the ESAS and consisted of five items (pain, nausea, insomnia, abdominal distention, and general sense of well-being), which were rated using a 0-10 numeric rating scale.

Statistical analysis

Our primary analysis was to determine the proportion of patients with ileus in each group at day 4 and to determine if the two groups differed in the proportions of ileus observed, using a binomial test. We powered our study to be able to estimate each proportion to within at most 16% by including 40 patients in each group. For example, if 50% of patients in one group (20 out of 40) developed ileus, the 95% confidence interval of this estimate would be 34%-66%. In addition, if we found that 40% of patients (16 out of 40) in one group developed ileus and 11% or fewer ($\leqslant$ 4) in the second group developed ileus, this difference would be considered statistically significant with 80% power and a two-sided significance level of 5%.

The time of occurrence of bowel motility indicators (i.e. first passage of flatus and first bowel movement)

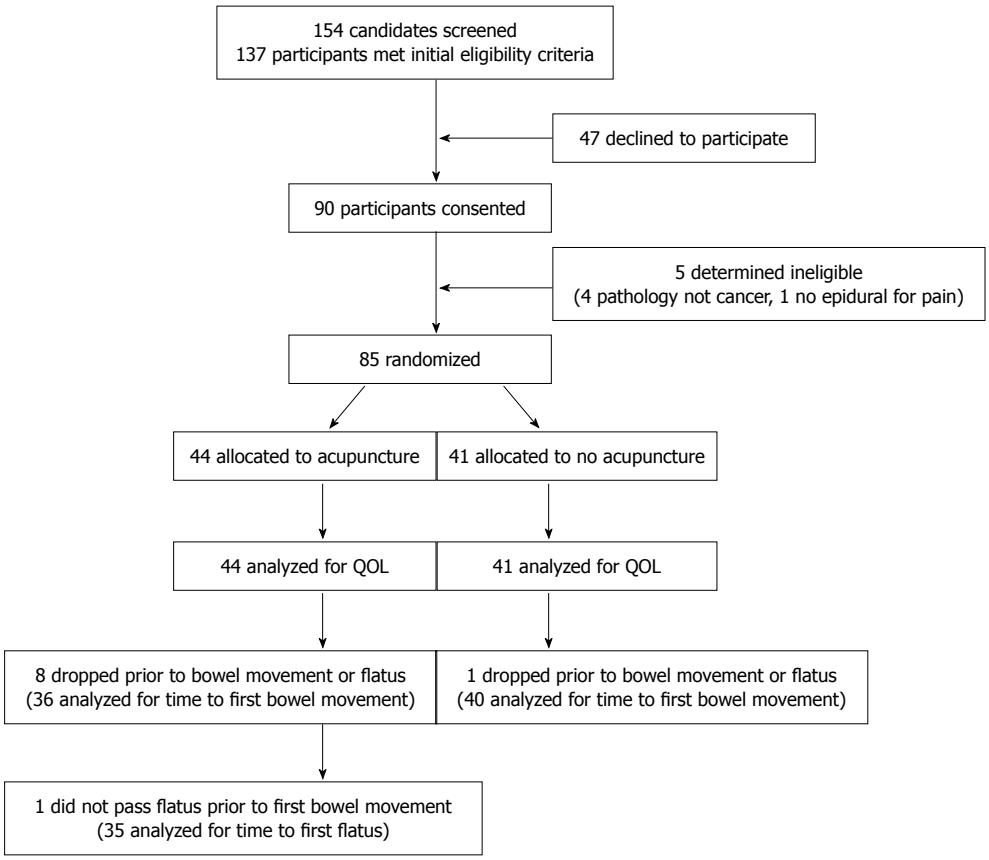

Figure 1 Flow of participants through the study.

was subtracted from the time anesthesia ended, and compared between groups using the Wilcoxon two-samples test. Unpaired *t* tests were used to compare EGEG indicators and QOL outcomes between groups.

RESULTS

Of the 154 subjects screened, 137 met all the initial eligibility criteria. Forty-seven patients who were eligible declined to participate. Therefore, 65.7% of eligible patients (90 patients) agreed to participate. The overall mean age of the participants was 53.7 years (range, 29-73 years), and there were no group differences in mean age (acupuncture, 54.3 years; control, 53.1 years). There were 38 (45%) women and 47 (55%) men, with an even balance between groups (43% and 46% women in the acupuncture and control groups, respectively). All patients received intraperitoneal surgery, and a representative sample of the population that we wanted to assess was obtained as follows: 47 (52.2%) proctectomy, 21 (23.3%) right hemicolectomy, 11 (12.2%) sigmoidectomy, eight (8.9%) left hemicolectomy and three (3.3%) transverse colectomy. Of 90 patients, 70 (77.78%) received epidural anesthesia (37 acupuncture; 33 controls), 18 (20%) received both epid ural and general anesthesia (7 acupuncture, 11 in controls), and two (2.2%) patients in the acupuncture treatment group received general anesthesia only. There were no differences between groups with regard to diagnosis, operation methods, operation time, blood loss, and postoperative complications.

No participants were lost to follow-up. Five participants were dropped from the study after consent but prior to randomization either because a subsequent pathology report indicated that they did not have a cancer diagnosis, or because they did not receive epidural infusion for post-surgical pain management. Eighty-five patients were randomized (acupuncture = 44; no acupuncture = 41). Although some postoperative QOL data were obtained on all patients, eight in the acupuncture group and one in the no acupuncture group were dropped prior to first bowel movement or first passage of flatus, and before PPOI could be assessed on postoperative day 4, because the inconvenience of study participation (acupuncture-4 dropped on postoperative day 1, 2 on day 2, 1 on day 3, and 1 on day 4; no acupuncture-1 dropped on postoperative day 2). Figure 1 shows the flow of participants through enrollment, randomization, follow-up, and analysis.

Table 1 provides a summary of the study results. There were no significant differences between the groups in terms of bowel motility indicators, EGEG, or post-surgical QOL, thus, the null hypothesis was not rejected.

There were also no group differences when the data were analyzed on the basis of the subset of 46 patients who developed PPOI by day 4 (21 of 36 acupuncture patients *vs* 25 of 40 control patients, $P = 0.71$), and

Table 1 Summary of study results

Outcome	Acupuncture			Control			*P* value
	n	mean ± SD	Median (range)	*n*	mean ± SD	Median (range)	
Hours to first flatus	35	68.26 ± 23.38	72.25 (26.75-124.63)	40	65.24 ± 17.5	64.88 (30.25-105.17)	0.36
Hours to first bowel movement	36	119.04 ± 47.97	108.67 (34.00-241.17)	40	119.38 ± 60.21	104.25 (37.00-359.00)	0.77
EGEG at morning (Freq/min)	44	9.66 ± 1.34	9.32 (7.34-12.87)	41	9.59 ± 1.63	9.65 (6.80-14.98)	0.83
EGEG at afternoon (Freq/min)	44	9.76 ± 1.51	9.6 (6.11-15.93)	41	9.81 ± 1.46	9.94 (6.74-14.31)	0.65
EGEG at morning Amplitude (uv)	44	245 ± 61.1	243 (119-391)	41	239 ± 48.3	237 (131-343)	0.75
EGEG at afternoon Amplitude (uv)	44	250 ± 59.8	250 (131-380)	41	239 ± 51.7	222 (144-345)	0.39
Pain[1]	44	2.51 ± 1.74	2.22 (0-6)	41	2.37 ± 1.52	2.09 (0-5.73)	0.82
Nausea[1]	44	0.91 ± 1.67	0 (0-8)	41	0.45 ± 0.99	0 (0-5.25)	0.35
Insomnia[1]	44	5.11 ± 1.9	5 (1.83-9.00)	41	5.18 ± 2.06	5.17 (0.67-9.33)	0.76
Abdominal distention[1]	44	0.98 ± 1.35	0.21 (0-4.75)	41	0.76 ± 1.3	0.13 (0-6.50)	0.46
General well-being[1]	44	4.11 ± 1.57	4 (1.67-8.00)	41	3.73 ± 1.3	3.67 (1.17-6.29)	0.34

[1]Based on a 0-10 numeric rating scale.

whether their PPOI had resolved by day 5 (8 of 21 acupuncture patients and 11 of 25 controls, $P = 0.69$) or day 6 (13 of 21 acupuncture *vs* 16 of 25 controls, $P = 0.88$). The remaining 17 patients experienced a bowel movement by day 7. There were no adverse events greater than grade I (according to the Common Terminology Criteria for Adverse Events v3.0 (CTCAE)[49] related to the acupuncture treatment reported during the study.

DISCUSSION

PPOI developed in 46 patients, and there was no significant difference in the number of patients with PPOI in the acupuncture group ($n = 21$) *vs* the control group ($n = 25$). Similarly, there were no group differences in bowel activity as assessed by EGEG or in self-reported pain, nausea, insomnia, abdominal distention, or general well-being. In this patient population, PPOI occurred in up to 50% of participants in both groups, which suggested a lack of efficacy of acupuncture to prevent PPOI. Furthermore, analyses of the subset of patients who developed PPOI on day 4 and the resolution of PPOI on days 5 or 6 also revealed no group differences. Although this was a secondary analysis based on a small number of patients, it suggested that acupuncture was not effective in treating PPOI once it developed in this population.

Several important facts, however, were learned that will help with the development of future trials. First, standard postoperative care is different in China than in the United States. At Fudan University Cancer Hospital, patients undergoing this type of surgery often do not ambulate until after postoperative day 3, and in many cases, patients may not ambulate until postoperative day 5 or 6. In addition, patients have a nasogastric tube in place for several days and, thus, progression in diet is much slower than in the United States. Finally, a tight abdominal dressing and binder is placed after surgery and not removed for 2-3 d. Each of these factors could play an important role in gastrointestinal motility postoperatively and were not analyzed separately in the current study. As this was a randomized clinical trial, however, these factors were likely balanced between both groups. No patients in either group were given enteral or nasogastric feeding before passage of first flatus, and all patients in both groups were given similar iv fluids that included fat emulsion, amino acids, glucose and equilibrium liquids.

One limitation of the current study is that the use of epidural anesthesia in the majority of patients may have diminished the possible effects of acupuncture because of the blockade of afferent and efferent pathways, as acupuncture may act on gastrointestinal motility through neural mechanisms. Moreover, opioid use was not tracked and analyzed formally; however, through the process of randomization and based on a brief review of patient records showing consistency in pre- and postoperative medication, the authors believe this was likely to have been similar in both groups. Nevertheless, it is important to note that the acupuncture treatment in this study was designed to stimulate gastrointestinal motility. The efficacy of acupuncture for pain control is well established[50], and future trials should explore whether or not acupuncture designed to reduce pain can also reduce the amount of opioids used, and thus lessen the occurrence of PPOI. Although acupuncture alone may not be sufficient to reduce the risk of developing postoperative ileus, adding acupuncture to a regimen that includes other preventive measures such as less opioid use, increased ambulation, and progressive diet could have a synergistic effect and help prevent this debilitating complication following abdominal surgery. Future acupuncture trials should, therefore, be designed to include evaluation of these factors, as well as different point combinations, treatment schedules, and type of needle stimulation (i.e. manual *vs* electrical).

To the best of our knowledge, only one previous randomized trial has examined the use of acupuncture to prevent PPOI[41]. That study was conducted in the United States among a population of cancer patients who underwent ileostomy/colostomy reversal, a procedure which is not commonly performed in China. Unfortunately, the incidence of PPOI in that study was too small to determine any statistically significant

differences between the groups. It is important to note that no adverse events related to acupuncture were reported during either the previous or current trial.

Providing acupuncture treatment to post-surgical patients at the bedside was found to be feasible, and the logistics of so doing were determined in the current study. Future research should evaluate the efficacy of acupuncture in a different population and for other postoperative conditions, such as anxiety, pain, nausea, vomiting, and wound healing.

In conclusion, this study confirmed findings from previous research[42] that acupuncture can be safely administered in a postoperative setting; however, it was not found to be effective in preventing PPOI in this population. Future studies examining the use of acupuncture for PPOI should include assessment of activity, diet, and postoperative pain control, as well as different point combinations, treatment schedules, and type of needle stimulation. Finally, this was a prevention study, and the efficacy of acupuncture in treating PPOI once it has developed has not been evaluated in a prospective randomized trial. Since prior animal and human data[34-38] have shown that acupuncture can regulate gastrointestinal function, further investigation is warranted.

COMMENTS

Background

Postoperative ileus is a common problem in patients who have major abdominal surgery. The duration is usually short, but prolonged postoperative ileus (PPOI) may lead to increased hospital stay and costs. Acupuncture is often used to treat gastrointestinal disorders in China, but it is still not known whether it is effective for preventing or treating PPOI.

Research frontiers

Information from this study may help surgeons choose appropriate therapy for PPOI after abdominal surgery.

Innovations and breakthroughs

This study was conducted as part of a unique collaboration between researchers in the United States and China. Only one previous randomized trial, conducted in the United States, has examined the use of acupuncture to prevent PPOI in cancer patients. Standard postoperative care is very different in the United States than in China, and some of these treatment differences could play an important role in postoperative gastrointestinal motility.

Applications

Future studies examining the use of acupuncture to prevent or treat PPOI should include assessment of activity, diet, and postoperative medication for pain control.

Terminology

For the purposes of this study, PPOI was defined as an inability to pass flatus or have a bowel movement by 96 h after surgery.

Peer review

In this study, the authors investigated whether acupuncture can prevent PPOI after intraperitoneal surgery for colon cancer. Their results show that acupuncture cannot prevent PPI in this population. Subset analyses also suggest that acupuncture is not useful for treating PPOI. This was a prospective, randomized study, with novelty and innovation. It will be helpful for the surgeons to choose appropriate therapy for PPOI after abdominal surgery. Presentation and readability of the manuscript is good for publication.

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BRIEF ARTICLE

Silencing Fas-associated phosphatase 1 expression enhances efficiency of chemotherapy for colon carcinoma with oxaliplatin

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Abstract

AIM: To investigate whether silencing Fas-associated phosphatase 1 (FAP-1) expression enhances the efficiency of chemotherapy for colon carcinoma with oxaliplatin.

METHODS: Expression of FAP-1 in mRNA and protein was detected by reverse transcription polymerase chain reaction (RT-PCR) and flow cytometry. Small interfering RNA (siRNA) was designed according to the FAP-1 mRNA sequence. Cell proliferation was evaluated by methyl thiazolyl tetrazolium (MTT) assay. Anenxin V- and propidine iodine (PI) were assayed by flow cytometry for the detection of apoptosis.

RESULTS: The expression of FAP-1 was increased in SW480 cells after chemotherapy with oxaliplatin. Transfection of FAP-1 siRNA into SW480 cells silenced the expression of FAP-1 and consequently abolished the inhibitory function of Fas/FasL-mediated apoptosis pathway, thus increasing the efficacy of chemotherapy for colon carcinoma with oxaliplatin.

CONCLUSION: RNA interference combined with conventional chemotherapy is more effective against colon cancer.

Key words: Colon carcinoma; Fas-associated phosphatase 1; RNA interference; Oxaliplatin; Chemotherapy

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INTRODUCTION

The incidence of colon carcinoma is increasing worldwide[1]. Although great achievements have been made in surgery, chemotherapy and even some novel molecule-targeted drugs, such as bevacizumab (Avastin) used in treatment of colon carcinoma, their efficacy is still limited[2]. Since tumorigenesis is a multiple step event involving multiple genes, a single treatment modality just targets a part of the pathogenesis of colon carcinoma. The mechanism underlying the limited efficacy of the above treatment modalities still needs to be further explored in order to prolong the survival time of such patients.

Fas/FasL system is the essential pathway of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells to induce cell apoptosis[3]. Fas, an apoptotic message receptor, is belonged to the tumor necrosis factor family and expressed in many normal tissues and malignancies. Its expression pattern is mainly located in activated T lymphocytes and NK cells. By binding to Fas antibody or FasL, Fas-induced apoptotic pathway can be activated, initiating tumor cell apoptosis[4-6]. However, it has been reported that Fas receptors are highly expressed in colon carcinoma cells at both mRNA and protein levels, and FasL levels are high in blood and tissues of colon carcinoma patients[7,8], suggesting that colon carcinoma cells can escape from the immune clearance, resist to the cytotoxic activity of host immunocytes, and are, thus, insensitive to FasR- mediated apoptosis. Although the mechanism underlying the failure of immune system to protect humans against colon malignancies remains unclear, it has been recently shown that Fas-associated phosphatase 1 (FAP-1) may play an important role in the pathogenesis of colon malignancies[9]. FAP-1, a tyrosine phosphatase, inhibits FasR-mediated apoptosis. By interacting with the cytoplasmic death domain of Fas receptors, FAP-1 acts as a negative switch in the Fas pathway[10]. Transfection of FAP-1 into Fas-sensitive cells can block FasL-induced apoptosis[11]. Fas and FAP-1 are expressed in colon cancer tissue and the expression of FAP-1 is associated with resistance against Fas-mediated apoptosis and interrupting the correspondence between FAP-1 and Fas can reverse the anti-Fas inducing apoptosis function of FAP-1[12]. Down-regulating the expression of FAP-1 by interleukin 2 can promote the sensitivity of colon cancer cells to Fas-induced apoptosis[13] and interrupting FAP-1 also increases chemosensitivity to certain kinds of cancer[14], suggesting that FAP-1 can be used as a target for treatment of malignancies. These findings lead to a question of whether interrupting FAP-1 sensitizes chemotherapy for colon carcinoma.

It has been recently shown that RNA interference (RNAi) plays an important role in the treatment of malignancies, virus infection, and other diseases[15-19]. Small interfering RNA (siRNA) is small in size, and can easily infiltrate cell membranes and other structures. Its efficiency and specificity are higher than those of anti-sense oligonucleotide[20-23]. RNAi may be used as a novel gene therapeutic procedure combining with chemotherapy for colon carcinoma.

In this study, the expression of FAP-1 was up-regulated after treatment with oxaliplatin. Silencing FAP-1 by siRNA effectively reversed the apoptotic resistance and increased the efficacy of chemotherapy for colon carcinoma with oxaliplatin.

MATERIALS AND METHODS

Colon cancer cell line and culture

Human colon adenocarcinoma cell line SW480, obtained from Chinese Type Culture Collection Committee Cell Bank (Shanghai, China), was maintained in RPMI-1640 medium (GIBCO, Grand Island, NY, USA), supplemented with 10% heat-inactivated fetal calf serum (FCS) at 37℃ in a humidified atmosphere containing 5% CO_2.

FAP-1 siRNA design

Three different sequences of FAP-1 specific siRNA, including positive control glyceraldehyde phosphate dehydrogenase (GAPDH) siRNA and negative control siRNA, were designed and synthesized by Genepharma (Shanghai, China). The sequence of each siRNA is shown in Table 1.

siRNA transfection

siRNA was transfected with a siPORT™ *NeoFX*™ transfection agent (AMBION) following its manufacturer's instructions.

Detection of FAP-1 by reverse transcription polymerase chain reaction (RT-PCR)

RNA was harvested from colon cancer cells by extracting Trizol (Invitrogen) following its manufacture's instructions. cDNA was synthesized from 2 μg of total RNA in a 20 μL reaction system containing 0.5 μL of PrimeScript™ RTase, 4 μL of 5 × PrimeScript™ buffer, 0.5 μL of RNase inhibitor, 1 μL of Oligo dT, 2 μL of dNTP, 11 μL of RNase free H_2O. The mixture was incubated for 60 min at 42℃ and then for an additional 30 min at 53℃. The unhybridized RNA was digested with 10 units of RNase H at 37℃ for 10 min.

PCR was performed on cDNA using the sense and anti-sense primers to amplify FAP-1 and a house keeping gene, GAPDH. All primers were designed according to the published sequences: FAP-1: (sense) 5'-AGGTCTGCAGAGAAGCAAGAATAC-3' and (anti-sense) 5'-GAATACGAGTGTCAGACATGG-3'; GAPDH: (sense) 5'-AACGGATTTGGTCGTATTG-3' and (anti-sense) 5'-GGAAGATGGTGATGGGATT-3'.

The PCR conditions were as follows: denaturation at 95℃ for 5 min, followed by 30 cycles at 94℃ for 30 s, at 50℃ for 45 s, at 72℃ for 1 min, and a final extension at 72℃ for 7 min for FAP-1, and denaturation at 95℃ for 5 min, followed by 30 cycles at 94℃ for 30 s, at 58℃ for 45 s, at 72℃ for 1 min, and a final extension at 72℃ for 7 min for GAPDH. Primers were used at a final concentration of 0.1 μmol/L each, dNTPs at 50 μmol/L,

Table 1 siRNA sequence

siRNA	From 5' to 3'
FAP-1 siRNA 1709	Sense: CGAAGGAAAGUAAACAUAATT
	Anti-sense: UUAUGUUUACUUUCCUUCGGT
FAP-1 siRNA 6267	Sense: CAGGUACAUUAAAGAUGAATT
	Anti-sense: UUCAUCUUUAAUGUACCUGGA
FAP-1 siRNA 3264	Sense: GGGAGAUCACCUUAGUGAATT
	Anti-sense: UUCACUAAGGUGAUCUCCCTT
GAPDH positive control	Sense: GUAUGACAACAGCCUCAAGTT
	Anti-sense: CUUGAGGCUGUUGUCAUACTT
Negative control	Sense: UUCUCCGAACGUGUCACGUTT
	Anti-sense: ACGUGACACGUUCGGAGAATT

siRNA: Small interfering RNA; FAP-1: Fas-associated phosphatase 1; GAPDH: Glyceraldehyde phosphate dehydrogenase.

$MgCl_2$ at 1.5 mmol/L, Taq DNA polymerase at 1.0 μg per 50 μL reaction mixture. The 607 bp and 208 bp PCR products were the predicted FAP-1 and GAPDH, respectively, separated by electrophoresis on 2% agarose gel and stained with colloidal gold. The target bands were analyzed by densitometry. FAP-1 cDNA was semi-quantitated by densitometric comparison with GAPDH from the same sample.

Immunofluorescence analysis for FAP-1

Approximately 10^6 cells were incubated with 10 g/mL rabbit anti-human FAP-1 polyclone antibody (Santa Cruz) for 30 min at 4℃ and washed with PBS containing 2% FCS. PE-conjugated secondary goat anti-rabbit antibody (Boster, Wuhan, China) was added to the cells for 30 min at 4℃. The cells were washed again with PBS containing 2% FCS and then the intensity of fluorescence was analyzed. Isotype-matched control antibody was used to determine the nonspecific binding. A total of 10000 cells were examined for each determination. Data were expressed as relative fluorescence intensity (RFI = mean fluorescence intensity of cells stained with anti-FAP-1 pAb/mean fluorescence intensity of cells stained with control pAb).

Cell proliferation assay

Cell proliferation was evaluated by methyl thiazolyl tetrazolium (MTT) assay. SW480 cells were seeded into a 96-well plate at the concentration of 3000 cells per well. Oxaliplatin (Henrui Co, LTD, Jiangsu Province, China) was administrated at a concentration of 5 μg/mL 24 h after the cells were plated. The proliferation status of SW480 cells was observed at 24, 48, 72, and 96 h, respectively, after treatment with oxaliplatin. Each group was quadruplicates and its mean OD value was used to represent the proliferation status of the group. MTT (Merck) was dissolved in RPMI 1640 and prepared at 1 mg/L for use. The medium was removed, the cells were washed three times with PBS, and 100 μL MTT solution was added into each well and incubated in dark at 37℃. Then, the MTT solution was removed and 100 μL DMSO (Sigma) was added into each well to dissolve the remaining formazan by gently shaking the plate for 15 min. Finally, a 495 absorption value of each well was obtained with a spectrophotometer (Labsystems Dragon).

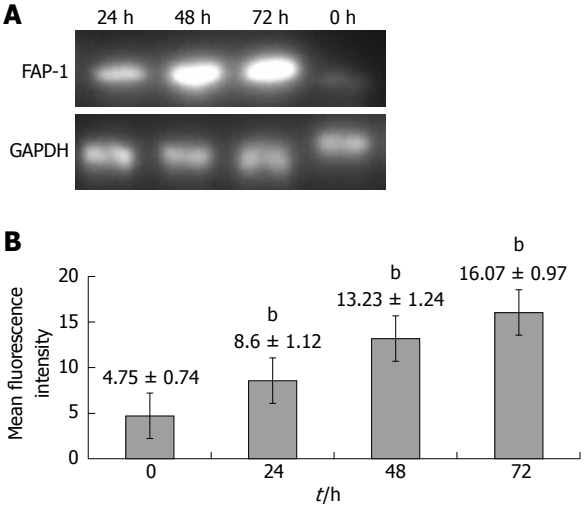

Figure 1 Expression of Fas-associated phosphatase 1 (FAP-1) mRNA (A) and protein (B) after oxaliplation administration. Oxaliplatin promotes FAP-1 expression of SW480 cells at 0, 24, 48 and 72 h after chemotherapy. $^bP < 0.01$ *vs* 0 h group.

Detection of apoptosis by flow cytometry

Anenxin V-FITC kit (Bender Medsystems) and propidine iodine (PI) were used to calculate the cells undergoing apoptosis with a flow cytometer (Facscalibur, Becton Dickinson). Anenxin V (+) PI (-) represents apoptotic cells, whereas Anenxin V (+) PI (+) represents dead cells. The procedure was carried out according to the manufacturer's instructions for the Anenxin V-FITC kit.

Statistical analysis

All experiments were performed in triplicate. The results were expressed as mean ± SE. Statistical analysis was performed by one-way analysis of variance (ANOVA) and comparisons among groups were performed by Bonferroni's multiple-comparison *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Oxaliplatin promoted FAP-1 expression in SW480 cells

To investigate whether FAP-1 is resistant to chemotherapy for colon carcinoma with oxaliplatin, RT-PCR and flow cytometry were carried out to detect the FAP-1 expression in SW480 colon carcinoma cells at 0, 24, 48 and 72 h after chemotherapy for colon carcinoma with oxaliplatin. The FAP-1 expression was increased at both mRNA (Figure 1A) and protein levels ($P < 0.01$), and reached its peak at 48 h (Figure 1B).

siRNA silenced FAP-1 expression

siRNA was transfected into SW480 cells with a transfection agent, siPORT(AMBION). Three sequences of FAP-1 siRNA (1709, 6267 and 3264) were designed.

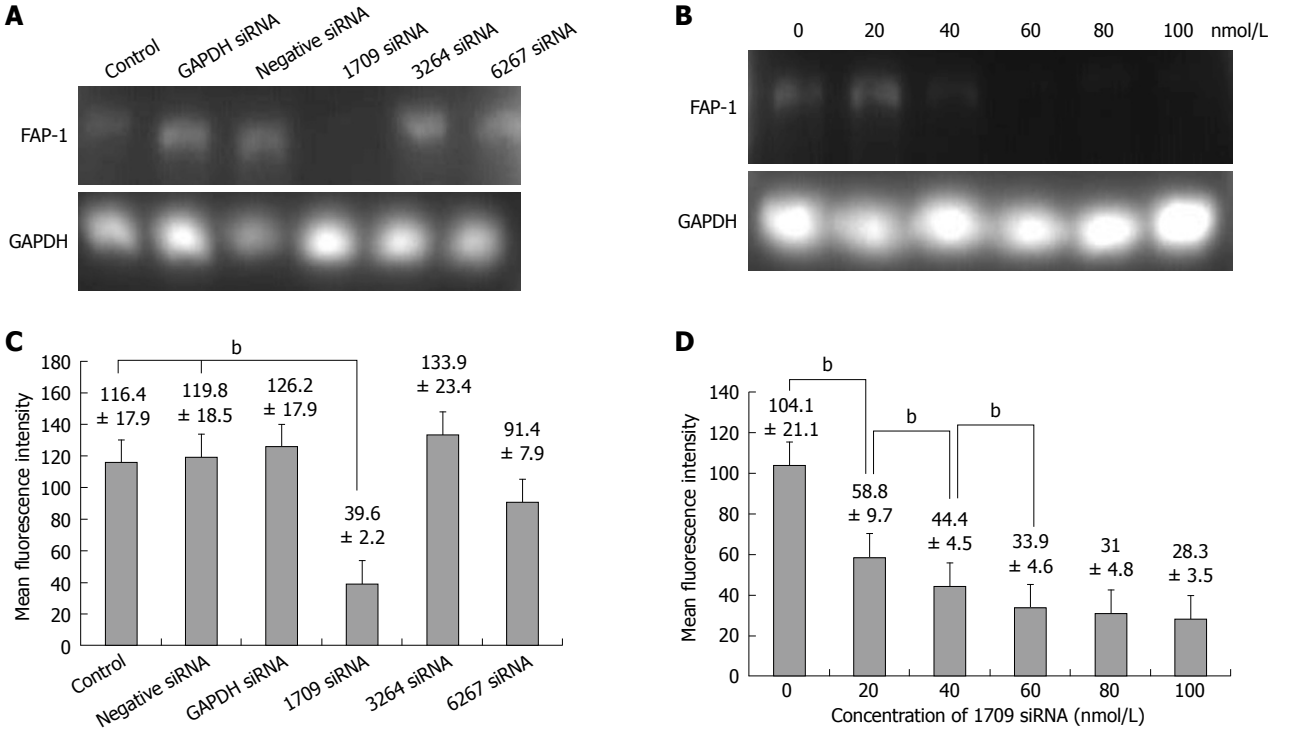

Figure 2 Small interfering RNA (siRNA) silencing FAP-1 expression in siRNA 1709 group (A and C) and at the concentration of 60 nmol/L (B and D). $^{b}P < 0.01$ *vs* 0 h group.

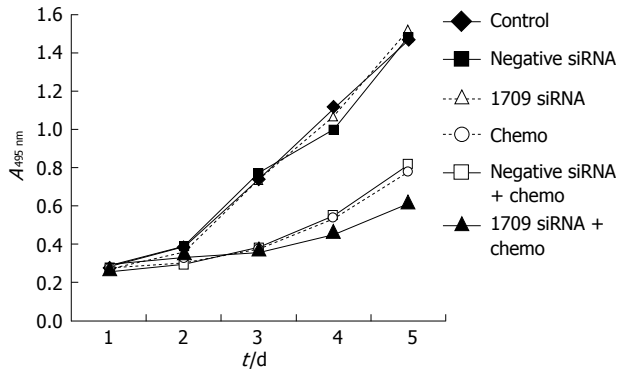

Figure 3 FAP-1 siRNA increasing the inhibitory effect of oxaliplatin on proliferation of SW480 cells.

Forty-eight hours after transfection of siRNAs into SW480 cells, the FAP-1 expression at mRNA and protein levels was detected by RT-PCR and flow cytometry. The FAP-1 protein was expressed in siRNA 1709 group (Figure 2A and C) and at the concentration of 60 nmol/L (Figure 2B and D), respectively ($P < 0.01$).

FAP-1 siRNA combined with oxaliplatin inhibited proliferation of SW480 cells

To investigate whether FAP-1 siRNA enhances the sensitivity of SW480 cells to oxaliplatin, cell proliferation was assayed in 6 groups including negative control group, negative siRNA group, siRNA 1709 group, oxaliplatin group, oxaliplatin+negative siRNA group, and oxaliplatin + siRNA 1709 group. Transfection was performed when the cells were seeded. After 24 h, the culture medium was removed and washed three times with PBS and oxaliplatin dissolved in the culture medium (5 μg/mL) was added. The culture medium was replaced daily to keep the consistent concentration of oxaliplatin. Cell growth was observed daily for five days. Transfection of negative siRNA and siRNA into SW480 cells did not inhibit cell proliferation. Transfection of oxaliplatin combined with transfection of negative siRNA reduced cell proliferation. The greatest proliferation inhibition was found after treatment with oxaliplatin combined with transfection of siRNA 1709 (Figure 3).

FAP-1 siRNA combined with oxaliplatin increased apoptosis of SW480 cells

To investigate whether transfection of FAP-1 siRNA into SW480 cells combined with oxaliplatin increases apoptosis of colon carcinoma cells, the apoptotic rate of colon carcinoma cells was detected by flow cytometery and Anenxin V and PI immunofluorescence. Transfection was performed when the cells were seeded. After 24 h, the culture medium was removed and washed three times with PBS and oxaliplatin dissolved in culture medium at the concentration 5 μg/mL was added and the cells were harvested. The apoptotic rate of the negative control was 9.56% ± 2.38%, and similar in both siRNA transfection groups ($P = 0.416$). The apoptotic rate of oxaliplatin combined with siRNA 1709 transfection group was 26.7% ± 3.67%, which was higher than that of the oxaliplatin treatment group ($P < 0.01$, Figure 4), suggesting that oxaliplatin promotes FAP-1 expression in SW480 cells.

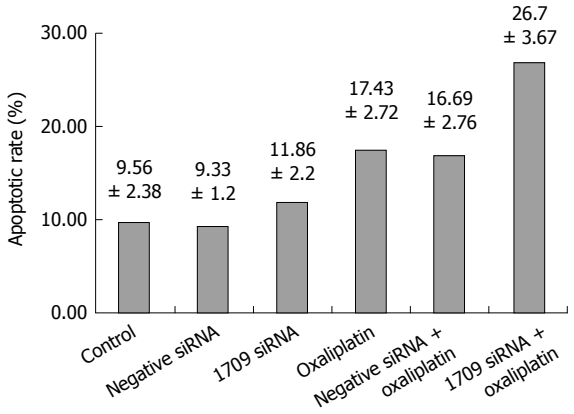

Figure 4 FAP-1 siRNA enhancing the apoptosis inducing effect of oxaliplatin.

DISCUSSION

Colon cancer represents a major public health problem, resulting in more than 1 million new cases diagnosed each year and approximately a half million deaths worldwide. Colectomy is the only procedure that may cure colon carcinoma, but the 5-year survival rate mainly depends on the stage of tumor at the time of diagnosis. The majority of patients with colon carcinoma are at an advanced stage beyond surgical treatment when they visit a doctor[1]. For patients who cannot be cured by surgery, chemotherapy is another important and complementary treatment[24]. Among the chemotherapeutic drugs, oxaliplatin is commonly used in treatment of colon carcinoma. However, its efficacy, especially in patients at advanced stage, is still limited[2].

The main mechanism of action of oxaliplatin is mediated through the formation of DNA adducts[25-27]. When the platinum compound enters the cells, one chloride ligand is dissociated to form a reactive monoaquamonochloro complex, which reacts rapidly with the guanines on DNA to form monoadducts. The subsequent dissociation of the second chloro ligand allows conversion of the transiently formed monoadducts to a variety of stable diadducts[28,29]. The majority are intrastrand diadducts binding to a guanine residue[30,31]. Since intrastrand adducts are the most abundant adducts and capable of blocking both DNA replication and transcription, they are considered the major cytotoxic lesions. As a final result, oxaliplatin induces primary and secondary DNA lesions leading to apoptosis of human cancer cells[32].

It has been shown that DNA lesion repair mechanism, over-expression of copper transporters, and enhanced drug detoxification result in an increased chemo-resistance to oxaliplatin[33,34]. However, the mechanism may be more complicated. Some researchers hold that the major process leading to chemotherapy resistance is the ability of cancer cells to evade cell death signals[35]. In our study, the expression of FAP-1, a negative switch in Fas-mediated apoptosis, was elevated in SW480 colon cancer cells after treatment with oxaliplatin. We quantified the transcription level only by densitometry rather than by RT-qPCR. The role of MMP7 (matrix metallopeptidase 7) and its cross-talk with the FAS/FASL system during the acquisition of chemo-resistance to oxaliplatin have been reported[36]. Raymond D[37] also showed that oxaliplatin can activate the Notch-1 signaling pathway in colon cancer cells and enhance its chemo-resistance to SW480 colon cancer cells, indicating that the functional disorder of the Fas apoptosis pathway mediated by FAP-1 elevation may protect SW480 cells against apoptosis and is involved in chemo-resistance effect.

In our study, since FAP-1 was elevated after treatment with oxaliplatin and might account for chemo-resistance, the FAP-1 expression was inhibited by RNA interference to make clear whether it sensitizes chemotherapy. The apoptotic rate of oxaliplatin combined with siRNA transfection was higher than that of oxaliplatin only. The greatest proliferation inhibition was found in the group of oxaliplatin combined with siRNA transfection, suggesting that the elevated FAP-1 expression is involved in the mechanism enabling SW480 cells to be insensitive to oxaliplatin treatment. Based on the fact that siRNA used to silence the expression of FAP-1 and treatment with oxaliplatin increased the apoptosis of SW480 cells and reduced their proliferation, we can develop a novel therapeutic measure to enhance the efficacy of chemotherapy. The similar phenomenon was also observed in other malignances. Etodolac, a selective cyclo-oxygenase-2, can enhance carboplatin-induced apoptosis of human tongue carcinoma cells by down-regulating FAP-1 expression[38] and sphingosine kinase isoforms can regulate oxaliplatin sensitivity to human colon cancer cells through ceramide accumulation and Akt activation[39]. Secretase inhibitors have been recently used to abrogate oxaliplatin-induced activation of the Notch-1 signaling pathway in colon cancer cells, which can enhance chemo-sensitivity[37]. In the present study, FAP-1 siRNA combined with oxaliplatin reduced the proliferation of colon carcinoma SW480 cells compared with oxaliplatin alone. No study is available so far on the Fas/FasL system and FAP-1 interacting to influence cell proliferation. We hold that the higher reduction of proliferation is due to the enhanced apoptotic rate of FAP-1 siRNA combined with oxaliplatin treatment, decreasing the number of cells.

Since the pathogenesis of colon carcinoma remains largely unclear, a variety of chemotherapies have been designed to inhibit tumor growth. So far, no single strategy can solve all the complicated problems in the treatment of colon carcinoma. Our study is an attempt to integrate gene therapy targeting FAP-1 and conventional chemotherapy for colon cancer.

In conclusion, oxaliplatin increases the expression of FAP-1. RNAi can knockdown FAP-1 and sensitize chemosensitivity, and RNA interference combined with conventional chemotherapy is more effective against colon cancer.

COMMENTS

Background

Colon cancer represents a major public health problem, resulting in more than one million new cases diagnosed each year and approximately a half million deaths worldwide. Chemotherapy is an important and complementary treatment modality for colon carcinoma. Among the chemotherapeutic drugs, oxaliplatin is a commonly used in treatment of colon carcinoma, but its efficacy, especially in patients at advanced stage, is still limited.

Research frontiers

The mechanism underlying oxaliplatin chemo-resistance is complicated. DNA lesion repair mechanism, over-expression of copper transporters, and enhanced drug detoxification cannot fully explain its mechanism. In this study, Fas-associated phosphatase-1 (FAP-1) was elevated in colon carcinoma cells after oxaliplatin treatment, implicating that the functional disorder of the Fas apoptosis pathway mediated by FAP-1 elevation may protect colon carcinoma cells against apoptosis and is involved in the chemo-resistance effect. Chemotherapy can be sensitized by inhibiting FAP-1 expression with RNA interference.

Innovations and breakthroughs

Since the pathogenesis of colon carcinoma remains largely unclear, a variety of chemotherapeutic treatment modalities available have been designed. So far, no single treatment modality can solve all the complicated problems. This study is an attempt to integrate gene therapy targeting FAP-1 and conventional chemotherapy for colon cancer.

Applications

Oxaliplatin can increase the expression of FAP-1. RNAi can knockdown FAP-1 and sensitize chemosensitivity. This in vitro study showed RNA interference combined with conventional chemotherapy is more effective against colon cancer.

Terminology

FAP-1 is a tyrosine phosphatase, which inhibits FasR-mediated apoptosis. By interacting with the cytoplasmic death domain of Fas receptors, FAP-1 acts as a negative switch in the Fas pathway. Transfection of FAP-1 into Fas-sensitive cells can block FasL-induced apoptosis.

Peer review

This study investigated the important factors that inhibit the apoptotic effect of oxaliplatin on colorectal cancer cells, which is of significance in the treatment of colon carcinoma.

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CASE REPORT

Malignant schwannoma of the pancreas involving transversal colon treated with *en-bloc* resection

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Stojanovic MP, Radojkovic M, Jeremic LM, Zlatic AV, Stanojevic GZ, Jovanovic MA, Kostov MS, Katic VP. Malignant schwannoma of the pancreas involving transversal colon treated with *en-bloc* resection. *World J Gastroenterol* 2010; 16(1): 119-122 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i1/119.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i1.119

Abstract

Pancreatic schwannoma is a very uncommon tumor of the pancreas, with only 27 cases reported. Most pancreatic schwannomas are benign, with only four malignant tumors reported. We describe a case of giant malignant schwannoma of the pancreatic body and tail, which involved the transverse colon. The tumor was treated successfully with *en bloc* distal splenopancreatectomy and colon resection. This is believed to be the first reported radical operation for malignant schwannoma of the pancreatic body, with infiltration of the transverse colon, with excellent long-term results. The patient is alive and well 28 mo after the operation. The authors conclude that pancreatic schwannomas should be considered in the differential diagnosis of cystic neoplasms of the pancreas, although the diagnosis can only be confirmed by microscopic examination. In the case of the benign tumors, local excision is adequate, but in the case of malignant schwannoma, oncological standards must be fulfilled.

INTRODUCTION

Mesenchymal tumors derived from Schwann cells that envelope the peripheral nerves (schwannoma/neurilemmoma) can be found throughout the body, with the most common localization in the extremities, trunk, head and neck, retroperitoneum, mediastinum and pelvis[1]. Visceral schwannomas, which arise from sympathetic and parasympathetic nerve fibers, are very rare[2]. Pancreatic schwannoma is notably uncommon, with only 27 cases reported in the English and Japanese literature to date[3-7]. Most pancreatic schwannomas are benign, with only four malignant tumors reported.

We describe a case of giant malignant schwannoma of the pancreatic body and tail, which involved the transverse colon. The tumor was treated successfully with *en bloc* distal splenopancreatectomy and colon resection.

CASE REPORT

A 24-year-old woman was hospitalized because of unclear

abdominal symptoms, dyspepsia, weight loss and palpable tumor in the left hypochondrium. She had no anamnesis of pancreatitis or trauma, and there was no sign of von Recklinghausen's disease. The laboratory data (complete blood count, hepatic and pancreatic function tests) were within normal limits. Tumor marker levels also were within normal limits [carcinoembryonic antigen (CEA), 2.3 U/mL and carbohydrate antigen 19-9 (CA 19-9), 16.8 U/mL].

Ultrasonography of the abdomen revealed a well-demarcated, large, predominantly hyperechogenic mass, with hypoechogenic components in the body and tail of the pancreas, and compression of the posterior gastric wall. Computed tomography (CT) showed a well-circumscribed round hypodense mass (32 HU), 18 cm in diameter, which occupied the body and tail of the pancreas and displaced the splenic vein. There was suspect infiltration of the stomach and transverse colon (Figure 1).

Percutaneous needle aspiration was performed and a very small amount of the fluid was aspirated. Biochemical analysis showed normal amylase (48 U/L), CEA (1.9 ng/mL) and CA 19-9 (13 U/L) levels. Cytology revealed rare large cells with high nuclear-cytoplasmic ratios, prominent nucleoli, and cytoplasmic vacuoles, which were suggestive of malignancy. The cell block contained fragments of connective tissue and stroma with some spindle cells and hemosiderin-laden histiocytes.

We decided on operative treatment under the tentative diagnosis of a cystic neoplasm of the pancreas. Laparotomy revealed an encapsulated solid tumor in the pancreatic body and tail, which involved the transverse colon. There was no macroscopic regional lymphadenopathy. The patient underwent *en bloc* resection that consisted of hemipancreatectomy, splenectomy, omentectomy and transverse colon resection. Systematic lymphadenectomy was performed with removal of the 7, 9, 10 and 11 groups of lymph nodes, according to the Japanese Gastric Cancer Association Classification. Overall number of lymph nodes was 12, with micrometastases founded in two. The postoperative course was uneventful and the patient was discharged on postoperative day 11. No chemo or radiotherapy was added. She is free of symptoms 28 mo after surgery.

Surgical biopsies were fixed in 10% formaldehyde overnight, embedded in paraffin wax, and cut at a thickness of 4 μm. The sections were stained with hematoxylin and eosin, Alcian blue/periodic acid-Schiff, van Gieson and immunohistochemical avidin biotin complex techniques, by using S-100 antibody for detection of schwannoma, and Ki 67 antibody to evaluate mitotic activity in tumor cells.

Microscopy showed that tumor cells infiltrated only the serosa of the transverse colon. The nuclei and cytoplasm of the spindle-shaped neoplastic cells were diffuse (Figure 2A) and strongly immunoreactive for S-100 protein (Figure 2B and C). Tumor cells showed increased proliferative activity, with numerous mitotic figures. In the hypercellular areas, we registered > 10 mitotic figures/

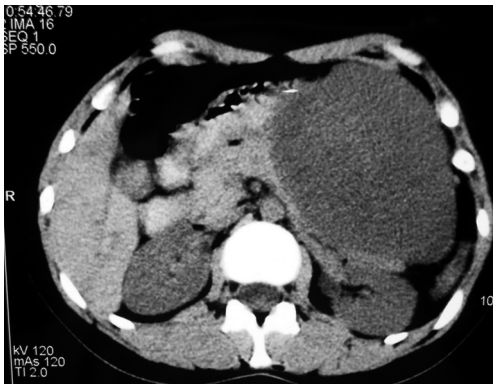

Figure 1 Computed tomography (CT) of pancreatic schwannoma with involvement of the transverse colon.

high-power field. Intense nuclear staining for Ki67 (*MIB1*) in the neoplastic cells was observed (Figure 2D). There was no cystic component or secondary elements of mature cartilage or bone.

DISCUSSSION

Mesenchymal tumors derived from Schwann cells that envelope peripheral nerves (schwannoma/neurilemmoma) are uncommon. They can be found throughout the body, with the most common localization in the extremities, trunk, head and neck, retroperitoneum, mediastinum and pelvis[1]. Visceral schwannomas, which arise from sympathetic and parasympathetic nerve fibers are very rare[2]. Pancreatic schwannoma is notably uncommon, with only 27 cases reported in the English and Japanese literature to date[3-7]. The patients ranged in age from 41 to 87 years (mean 61 years), with a nearly equal sex distribution. The tumor size ranged from 1.5 to 20 cm (mean: 6.5 cm), with the pancreatic head involved in 44%, and the body and tail in 56% of cases[1]. Schwannomas usually occur as solitary lesions, but are occasionally multiple when associated with von Recklinghausen's disease[8].

Although three cases of small solid pancreatic schwannomas have been reported[9-11], typical presentation of schwannomas is in the form of cystic, thin-walled, and hemorrhagic masses[3]. In our case, we found a large, solid schwannoma with a well-defined capsule, along with infiltration of the transverse colon.

Typical microscopic features of schwannoma are two microscopic components: a highly ordered cellular component (Antoni A areas), and a loose myxoid component with degenerative changes (Antoni B areas)[12]. Tumor cells are invariably immunoreactive for S100 protein, vimentin and CD56, and negative for cytokeratin AE1/3, CD34, CD117 (c-kit), desmin, and smooth muscle myosin[13].

A review of the literature has revealed only four cases of malignant schwannoma of the pancreas[2,12,14,15]. Malignant transformation of a benign schwannoma is extremely rare[8]. Also, malignant pancreatic schwannomas

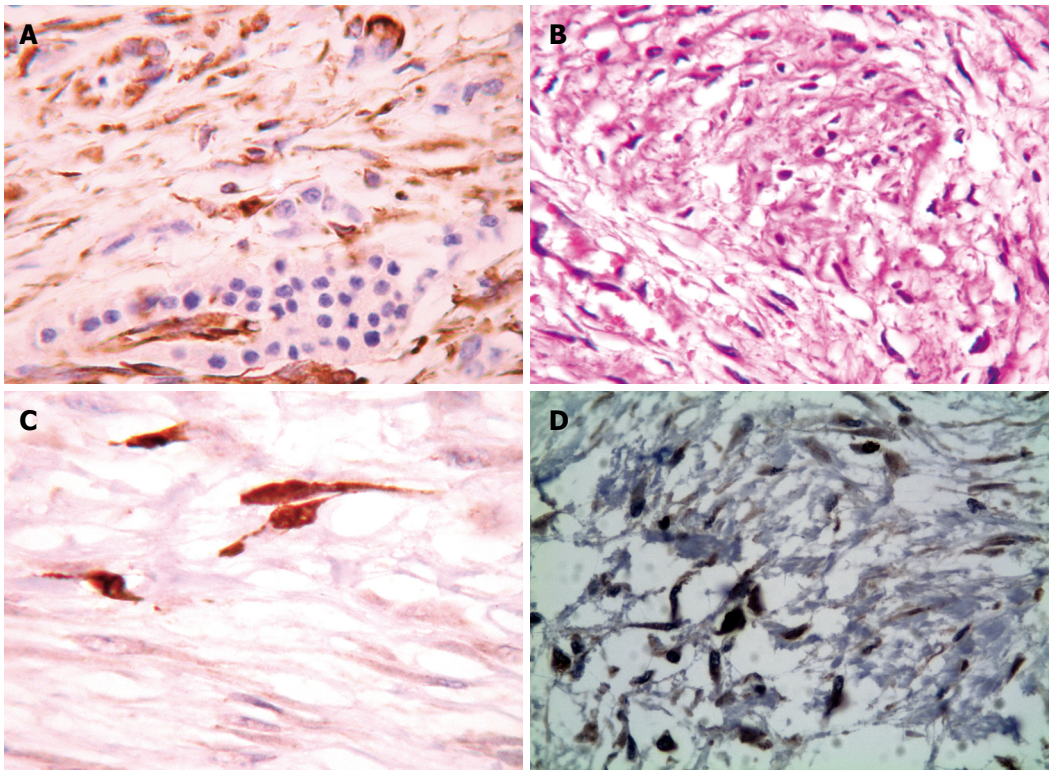

Figure 2 Microscopy of tumor cells infiltrating the serosa of the transverse colon. A: Schwannoma pattern of growth: Antoni B type. HE, × 200; B: Characteristic elongated cells with cytoplasmic processes in area with loose, myxoid background. Immunohistochemical ABC method, × 400; C: Pancreatic schwannoma with subtotal pancreatic tissue. Immunohistochemical ABC method, × 400; D: Intense nuclear staining for Ki67 (MIB1) in the neoplastic cells. Immunohistochemical ABC method, × 400.

that were associated with von Recklinghausen's disease have been reported, but none of the benign pancreatic schwannomas were associated with von Recklinghausen's disease[2,15].

Clinically, schwannoma is asymptomatic for a long time, or it is accompanied by nonspecific abdominal pain and discomfort. In the late clinical course, compression of the surrounding organs might be noticed.

To establish the diagnosis of pancreatic schwannoma, CT is the initial investigation of choice. CT findings usually show well-defined, round masses with multiple, low-attenuation, cystic necrotic areas. In tumors that are predominantly or exclusively composed of Antoni A areas (cellular component), CT shows inhomogeneous, hypodense, solid masses with contrast enhancement. When the tumor is predominantly composed of Antoni B areas (loose myxoid), CT shows homogeneous cystic masses without significant contrast enhancement[16]. On magnetic resonance imaging, schwannomas are present as masses of low signal intensity on T1-weighted images and of high signal intensity on T2-weighted images[17].

Deep tumors tend to grow larger, therefore, they are more likely to show secondary degenerative changes such as cyst formation, calcification, hemorrhage, and hyalinization, and are known as ancient schwannomas[1]. As a result of these changes and the content of predominantly loose tissue, pancreatic schwannoma is often misdiagnosed as a pseudocyst or other cystic neoplasm of the pancreas.

Definitive preoperative diagnosis is proven using fine-needle aspiration. However, this method correctly diagnoses only one in eight histologically proven schwannomas[18,19]. Definitive diagnosis requires histological examination and complex immunohistochemistry or ultrastructural examination[12]. For the definition of malignancy, which is often difficult in mesenchymal tumors, we use the following criteria: accentuated cell pleomorphism, high mitotic activity, rare stained necrosis, infiltration of the adjacent organs (colon), and locoregional lymph node micrometastases[2,12].

The malignant transformation of pancreatic schwannoma is uncommon, therefore, simple enucleation of benign schwannoma is usually sufficient if the pathology is confirmed before surgery[4].

In the case of the malignant schwannoma, oncological resection is indicated. A review of the literature has revealed one case of unresectable tumor of the pancreatic body, which was resolved by drainage[14]. In the case of pancreatic head localization, simple excision was performed in one case[2], and radical (Whipple) operation was performed in two other cases. However, in the present literature, only short-term follow-up has been reported (maximum, 9 mo), with no data about maximum survival[12,15].

As far as we are aware, this is the first report of radical surgery for malignant schwannoma of the pancreatic body, with infiltration of the transverse colon, with excellent long-term results. Our patient is alive and well 28 mo after the operation.

Radiotherapy has been shown to decrease tumor

growth and regression in neurogenic schwannoma, but there have been no previous reports of chemoradiation therapy[20]. However, the role of chemoradiation therapy in the management of pancreatic schwannoma has not been proven. Surgical excision with close follow-up and surveillance remain the mainstay of treatment[3].

In conclusion, pancreatic schwannoma is very rare, but an important pathological condition. It should be considered in the differential diagnosis of cystic neoplasms of the pancreas, although the diagnosis can only be confirmed by microscopic examination. In the case of benign tumors, local excision is adequate, but in the case of malignant schwannoma, oncological standards must be fulfilled.

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CASE REPORT

Successful endoscopic sclerotherapy for cholecystojejunostomy variceal bleeding in a patient with pancreatic head cancer

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Abstract

Variceal bleeding outside the esophagus and stomach is rare but important because of its difficult diagnosis and treatment. Bleeding from cholecystojejunostomy varices has been reported to be a late complication of palliative biliary surgery for chronic pancreatitis. Such ectopic variceal bleeding has never been reported after palliative surgery for pancreatic cancer, probably because of the limited lifespan of these patients. Herein, we report our successful experience using endoscopic cyanoacrylate sclerotherapy to treat bleeding from cholecystojejunostomy varices in a 57-year-old man with pancreatic head cancer. To our knowledge, this is the first case report in the literature of this rare complication.

Key words: Ectopic varix; Pancreatic cancer; Cholecystojejunostomy; Sclerotherapy

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Hsu YC, Yen HH, Chen YY, Soon MS. Successful endoscopic sclerotherapy for cholecystojejunostomy variceal bleeding in a patient with pancreatic head cancer. *World J Gastroenterol* 2010; 16(1): 123-125 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i1/123.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i1.123

INTRODUCTION

Variceal bleeding outside the esophagus and stomach is rare but important because of its difficult diagnosis and treatment. These ectopic varices are usually associated with cirrhosis and, less often, may result from portal vein thrombosis, chronic pancreatitis, mesenteric venous thrombosis, or adhesion caused by prior surgery[1]. Bleeding from cholecystojejunostomy varices has been reported to be a late complication of palliative biliary surgery for chronic pancreatitis[1-4]. To our knowledge, such bleeding has never been reported after surgery for pancreatic cancer, probably because of the limited lifespan of such patients[1]. Herein, we report our successful experience using endoscopic cyanoacrylate sclerotherapy to treat bleeding from cholecystojejunostomy varices in a 57-year-old man with pancreatic head cancer.

CASE REPORT

A 57-year-old man was admitted to our hospital because of tarry stool passage. His surgical history included a Billroth-Ⅱ operation for peptic ulcer 30 years ago and cholecystojejunostomy for biliary palliation due to pancreatic head cancer diagnosed 6 mo before this admission. The tumor progressed with portal vein invasion and multiple hepatic metastases during this period despite chemotherapy.

Hemoglobin count was 60 g/L on presentation and his coagulation profiles were within normal limits. An upper endoscopy disclosed no hemorrhagic lesion in

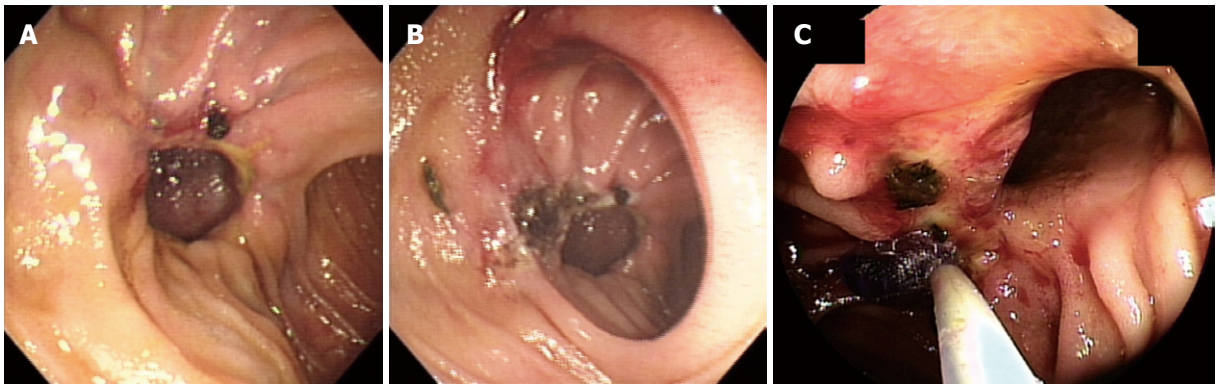

Figure 1 Endoscopic view. A: An anastomotic ulcer was observed in the cholecystojejunostomy; B: Anastomotic ulcer after argon plasma coagulation therapy; C: n-butyl-2-cyanoacrylate injection into the cholecystojejunostomy varices.

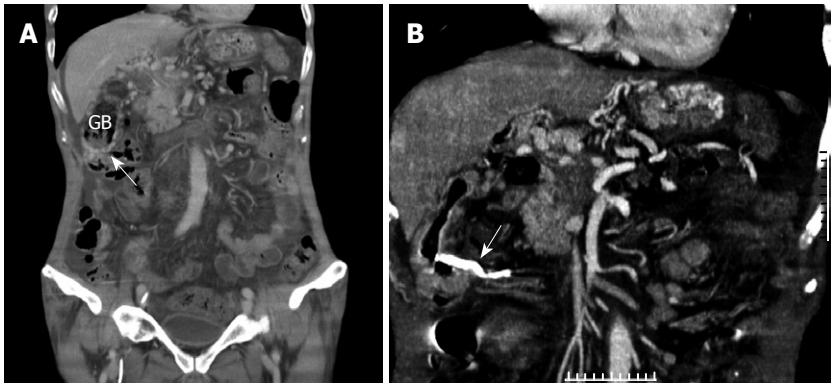

Figure 2 Abdominal computed tomography. A: Prominent vessels (arrow) formed around the gall bladder; B: Obliterated varicose vein (arrow).

the esophagus or stomach. The endoscope was then advanced to the cholecystojejunostomy area where shallow ulcers over the anastomosis were found (Figure 1A). The patient was administered intravenous proton pump inhibitor and blood component replacement therapy. The bleeding persisted and colonoscopy subsequently revealed a bleeding point above the terminal ileum. Therefore, a decision was made to perform local therapy for the anastomotic ulcer with argon plasma coagulation (Figure 1B).

The patient continued to bleed despite endoscopic therapy. abdominal computed tomography (CT) was performed, which revealed portal vein tumor invasion with collateral circulation formation. Prominent varices were found around the gall bladder and cholecystojejunostomy (Figure 2A). No tumor invasion to the bowel was noted. Retrospective review of the endoscopic images revealed that the folds were mildly engorged with mild blue color, which suggested underlying varices.

After discussion, the patient agreed to endoscopic therapy with n-butyl-2-cyanoacrylate for these varices on the 12th d of hospitalization. Endoscopic ultrasound with miniprobe was used to confirm the presence of varices beneath the anastomotic ulcer, and injection therapy with n-butyl-2-cyanoacrylate was carried out smoothly (Figure 1C). A follow-up CT revealed successful obliteration of the collateral circulation (Figure 2B). The patient died of his disease 4 mo later and had no recurrent gastrointestinal bleeding during the intervening period.

DISCUSSION

Esophageal or gastric variceal bleeding is a common cause of severe gastrointestinal bleeding. Ectopic variceal bleeding from the duodenum[5-7], jejunum[8], ileum[9], and colon[10,11] have been reported in the literature as a diagnostic and therapeutic challenge to clinicians. This ectopic variceal bleeding usually results from portal hypertension, portal vein thrombosis, mesenteric vein thrombosis, chronic pancreatitis, adhesion after surgery, or inflammatory bowel disease[1,12]. Our reported case, suffering from cholecystojejunostomy variceal bleeding, is even rarer and such bleeding has been reported to be associated only with chronic pancreatitis[1-4] in the literature. This is probably because these cancer patients have a limited lifespan, dying before such varices can develop[1].

The diagnosis of ectopic varices is usually made after endoscopic examination: mesenteric venography[1,12], abdominal ultrasound[13], enteroclysis[3], or CT[14]. In our case, the diagnosis was difficult to make during the initial endoscopic examination because the varicose vein was not prominent and was masked by an overlying anastomotic ulcer. A careful review of the CT and endoscopic images in this case suggested the presence of varices in the cholecystojejunostomy and led us to approach the case with different endoscopic measures. Consequently, we suggest that endoscopic ultrasound is mandatory, as in our case, to confirm the presence of varices prior to endoscopic therapy. Alternatively, needle aspiration or

injection of contrast may prove useful for diagnosis.

Unlike esophageal and gastric varices, the treatment options for ectopic varices have varied in the literature. Surgical resection[1] or radiological methods to decrease portal hypertension[15] have previously been reported. Both endoscopic band ligation[10] and sclerotherapy[15] have been successfully used to treat such ectopic varices. Endoscopic cyanoacrylate sclerotherapy was chosen in this patient with advanced cancer because it is minimally invasive and has previously been used to treat gastric varices endoscopically[16]. A follow-up CT demonstrated successful obliteration of the varices and thus, permanent hemostasis was achieved for our case.

In conclusion, we report a case of pancreatic cancer with bleeding from cholecystojejunal varices. The diagnosis was made by CT, endoscopy, and endoscopic ultrasound. Cyanoacrylate sclerotherapy was a successful method to achieve hemostasis in this case.

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CASE REPORT

Ileum perforation due to delayed operation in obturator hernia: A case report and review of literatures

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Abstract

A 83-year-old woman was admitted to our hospital because of intermittent abdominal colicky pain and vomiting for 26 h. The pain localized over the periumbilical area with radiation along the medial side of the thigh. Computed tomography scan with three-dimensional reconstruction revealed a loop of small bowel protruding into the left obturator canal. Incarcerated obturator hernia was diagnosed and emergency laparotomy was arranged immediately. Unfortunately, her family refused surgery because of her worsening condition. On the third evening after admission, the patient developed peritonitis and sepsis. Perforation of small bowel due to the incarceration was noted during laparotomy. Bowel resection and an end-ileostomy were performed. She recovered well despite of the complication of multiple organ dysfunction syndrome. Literature is reviewed, and the pathogenesis, clinical manifestation, imaging features and treatment are discussed.

Key words: Obturator hernia; Intestinal obstruction; Computed tomography

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INTRODUCTION

Obturator hernia is considered to be rare and accounts for 0.05%-0.4% of all hernias[1]. The first case of obturator hernia was published in 1724 by Arnaud De Ronsil. Throughout the centuries, obturator hernia has been considered as an uncommon but important cause of mechanical intestinal obstruction, which usually occurs in elderly, emaciated women. Although some characteristics relevant to obturator hernia have been introduced and various imaging modalities have been applied, it remains a diagnostic and therapeutic challenge for surgeons. Here we report a case with correct diagnosis but delayed operation that directly results in bowel perforation, septic shock and multiple organ dysfunction syndrome (MODS).

CASE REPORT

A 83-year-old woman was admitted to our hospital because of intermittent abdominal colicky pain and vomiting for 26 h. The pain localized over the periumbilical area with radiation along the medial side of the thigh. The patient had had several episodes of similar syndrome during the past 13 mo, but it relieved spontaneously or by treatment with intravenous fluids and nasogastric suction. A

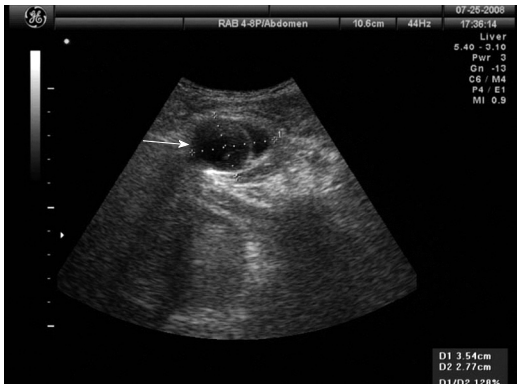

Figure 1 Ultrasonographic image of the left groin demonstrating a fluid-filled loop of bowel with the neck of the hernia uncertainly identified (arrow).

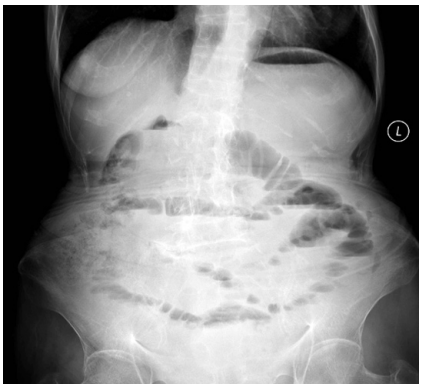

Figure 2 Plain abdominal film revealing multiple dilated loops of small bowel in an emaciated woman with scoliosis.

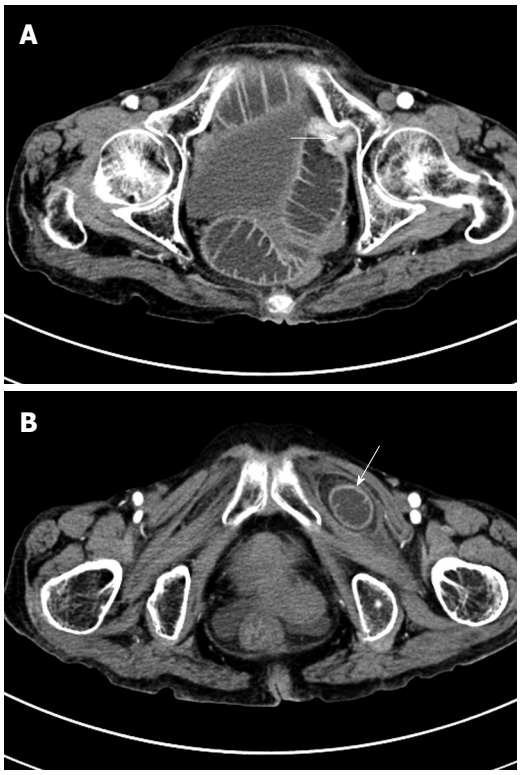

Figure 3 Abdominal axial CT image. A: Severe dilated small bowel and abrupt stenosis at the terminal ileum in the pelvic cavity (arrow); B: A low-density mass with clear border located between the pectineus and the left external obturator muscles (arrow).

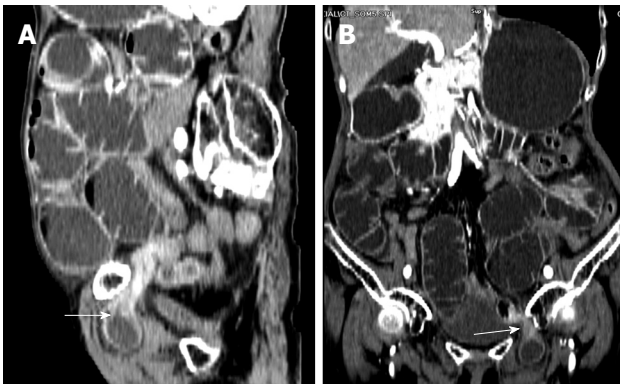

Figure 4 Three-dimensioned reconstructed CT (A: Sagittal section; B: Coronal section) finding extensive dilation of the small bowel loops and a loop of small bowel protruding into the left obturator canal with the transition zone from dilated to collapsed bowel (arrows).

diagnosis of intestinal obstruction was made 7 mo prior to current admission. But the cause of disease was unclear. She had a similar attack 5 mo earlier, was suspected of having incarcerated hernia by ultrasonography. A loop of small bowel at the left groin region was noted (Figure 1). But subsequent investigations and treatment were refused after the relief of pain. She also had a history of chronic constipation, ischemic heart disease and chronic bronchitis. She had no previous abdominal surgery. On her arrival, the body temperature was 36.8℃, blood pressure was 105/75 mmHg, heart rate was 92 beats per minute. Her body mass index was 18 (body weight 39 kg, height 147 cm). Physical examination revealed an emaciated lady with a soft but distended abdomen, visible bowel coils, mild tenderness over the lower abdomen, and hyperactive bowel sounds. No mass or rebound pain of the abdomen was noted. There was no palpable hernia in the groin. No abnormal signs were found on rectal and vaginal examinations. The Howship-Romberg sign, which characterized by pain or paresthesia in the hip with radiation down the medial thigh to the knee on the affected side, was positive. The white blood cell count was 11.2 × 10^9/L and other serum analyses were within normal limits. The initial plain abdominal radiography showed dilated loops of small bowel (Figure 2). Computed tomography (CT) scan demonstrated an abrupt stenosis at the terminal ileum in addition to the dilation of small bowel (Figure 3A). A low-density mass was also noted between the pectineus and the left external obturator muscles (Figure 3B). Three-dimensional reconstructed CT revealed a loop of small bowel protruding into the left obturator canal. The stenosis of the lumen and distended small bowel loops in the abdomen were well visualized (Figure 4). Incarcerated obturator hernia was diagnosed and emergency laparotomy was arranged immediately. Unfortunately, her family refused surgery because of her worsening condition, general weakness and the high risk involved. The patient was managed conservatively with

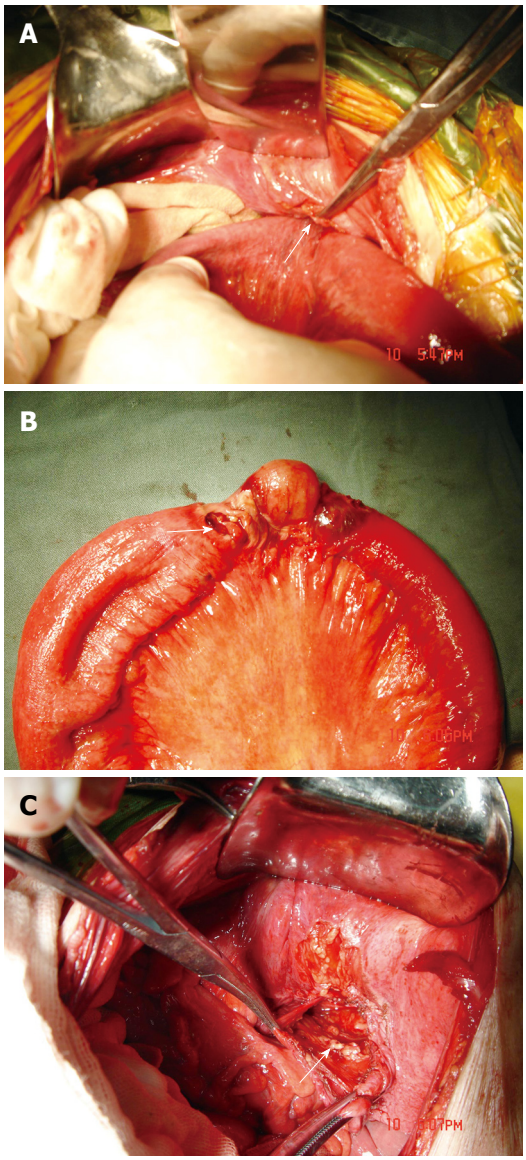

Figure 5 Intraoperative photography illustrating. A: Incarcerated small bowel at left obturator foramen (arrow); B: Perforation of ileum proximal to the site of incarceration (arrow); C: A defect in the left obturator canal (arrow).

intravenous fluids and nasogastric suction. On the third evening after admission, her abdominal pain suddenly became worse and constant. Subsequently, she developed restlessness and more unbearable generalized abdominal pain. On examination, the body temperature was 37.7℃, the blood pressure was 75/55 mmHg, and the heart rate was 128 beats per minute. There were obvious signs of peritonism and bowel sounds were absent. The diagnosis of perforation of small bowel and septic shock was made. The patient underwent emergency surgery after explanation to her guardian. Laparotomy revealed a loop of the terminal ileum about 80 cm from the ileocecal valve, herniating into the left obturator canal (Figure 5A). Perforation of small bowel was noted proximal to the port of incarceration (Figure 5B). The peritoneal contamination was severe. In view of the patient's poor conditions, we resected the perforated ileal segment and performed an end-ileostomy with closure of the distal loop. The defect at the hernia site was 1.5 cm in diameter and was closed with a few interrupted non-absorbable sutures (Figure 5C). The operating time was 75 min. Postoperatively, the patient was complicated with MODS and received subsequent treatment in intensive care unit. She recovered well and discharged 21 d after surgery. The second operation for re-established intestinal continuity was suggested 3 mo after operation. But the patient declined and now she is still uncertain about it. No recurrence of the hernia was noted during the 5 mo follow-up.

DISCUSSION

An obturator hernia is a herniated viscera through the peritoneal defect that is bounded superiorly by the pubic ramus and inferiorly by the free edge of the obturator membrane[2]. The incidence was approximately nine times higher in women than in men due to the wider pelvis and relatively greater diameter of the obturator foramen in females[3]. Recent published series show nearly exclusive incidence in women[4-7]. It occurs most commonly in emaciated elderly women between 70 and 90 years of age. The loss of protective preperitoneal fat from aging or malnutrition makes a larger space in the obturator canal and facilitates the formation of a hernia[8,9]. Thus, it is not surprising that most of the patients are markedly underweight. The majority of obturator hernias occur on the right side probably because the sigmoid colon may cover the left obturator foramen and prevents herniation[10]. About 6% of cases are bilateral and some may be associated with other types of hernia, such as the indirect inguinal hernia, the direct inguinal hernia, or the femoral hernia[11]. It is uncertain if concomitant conditions that lead to constant and increased intra-abdominal pressure such as constipation, ascites, chronic obstructive pulmonary disease, kyphoscoliosis, or multiparity are risk factors for obturator hernia. Our case presented most of these predisposing factors, including old age, emaciation, constipation and chronic bronchitis.

There are three anatomic stages in the development of obturator hernia[8]. Stage one: preperitoneal connective tissue and fat enter the pelvic orifice of the obturator canal. Stage two begins with a peritoneal dimple through the canal and progresses to the formation of a peritoneal sac. Stage three is characterized by clinically significant symptoms produced by the entrance of an organ, usually the ileum, sometimes the omentum or part of the bladder, into the sac. Rarely, it was reported that ovary or Meckel's diverticulum can also be incarcerated[12,13]. Our case appeared obviously to be in the third stage of development at the time of admission because of the presence of intestinal obstruction.

The most common presentation of obturator hernia is mechanical small bowel obstruction caused by incarceration of the bowel into the obtutator canal. The symptoms may be acute or intermittent if the hernia content reduces into the peritoneal cavity spontaneously.

In some cases, the initial symptoms are mild nausea, vomiting and anorexia, probably due to incomplete herniation or Richter's type. Obturator neuralgia is also an important complaint that extends from the inguinal crease to the anteromedial aspect of the thigh. The Howship-Romberg sign is reported to be present in nearly 50% of cases which refers to pain along the medial thigh and sometimes in the hip caused by compression of the obturator nerve by the hernia sac[10,12]. Flexion of the thigh usually relieves the pain. Extension, abduction, or medial rotation of the hip may exacerbate the pain. The clinical diagnosis is often difficult to make when this sign is absent. However, the Howship-Romberg sign was generally masked by the severe abdominal symptoms, and it was always neglected before operation. Another possible sign on presentation is the Hannington-Kiff sign[14]. It is characterized by an absent adductor reflex in the thigh, resulting from obturator nerve compression. The obturator canal is easily identified by digital vaginal examination at either 2 o'clock or 10 o'clock position. Palpation of a tender mass in the obturator region is of great value in obtaining the correct diagnosis. However, it is evident in only a few cases because the incarcerated mass is usually small and deeply situated.

Various imaging modalities have been applied to establish accurate preoperative diagnosis of this rare disease, including plain abdominal radiography, herniography, ultrasonography, CT, and gastrointestinal imaging with contrast medium. Plain radiography provides no specific findings apart from a dilated bowel loop, and can not reveal any significant information as to the cause of intestinal obstruction[15]. It is almost unuseful in diagnosing obturator hernia. Herniography can directly demonstrate the hernial sac, but has no place in the emergency diagnosis of obturator hernia and is used only in elective cases. Ultrasonography has been considered as a reliable modality with the presence of a hypoechoic tubular structure or a cystic lesion in the region of obturator canal. However, it is not easily identified due to the deep location within the pelvic musculature and smaller hernia sac[1]. In 1983, CT was first reported to be useful for detecting obturator hernia by Cubillo, and now is regarded as the standard diagnostic modality for obturator hernia with a documented accuracy of 80%[10,16]. CT images may demonstrate an air-filled or fluid-filled bowel loop in the region of the obturator foramen. In a study of a series of patients, Kammori *et al*[10] reported that 15 of 16 patients who were confirmed to have obturator hernia were diagnosed by CT alone. With the advent of multi-slice helical CT scan and the three-dimensional reconstruction technique, the accurate images are of great help in identifying obturator hernia and understanding the relationship between obturator canal and small bowel, as was seen in our study. In our case, the history taking, clinical manifestation and Howship-Romberg sign only gave the suspicion of obturator hernia. The definitive diagnosis was made by CT images.

There is a general agreement that obturator hernia must be treated surgically. A variety of operative approaches to obturator hernias have been described including retropubic approach, obturator approach, inguinal extraperitoneal approach, transperitoneal approach and combined approach with either a laparotomy or laparoscopy[3,17-19]. However, because of the rarity of this condition, there is no consensus on the most proper approach. In patients with an established preoperative diagnosis, an extraperitoneal approach is the best surgical procedure. However, a transperitoneal approach will be necessary in those patients with intestinal obstruction of uncertain cause. We prefer transperitoneal approach because it can obtain adequate exposure, avoid vessel damage, facilitate the reduction of the incarcerated bowel, identify and allow resection of the strangulated bowel when necessary, and easy repair of the defect as well. It should be emphasized that careful dissection of the hernia sac is essential to avoid injury of the obturator nerve or vessels, and the contralateral side must be routinely explored because of the chance of underlying hernia[18]. Recently, with the advantage of minimal invasion, laparoscopic technique has been applied in management of obturator hernia. This mini-invasive method may provide some benefits for these high-risk patients, such as less postoperative pain, fewer complications, earlier ambulatory and shorter hospital stay[17]. However, experience with laparoscopy is largely based on isolated case report. The laparoscopic operation for obturator hernia is infeasible in an emergency and is still limited to be performed widely due to the technical problems[20]. As in other hernias, after reduction of the contents, the defect of obturator canal should be repaired. Methods of repair vary from a simple suture or using autogenous adjacent tissue like broad ligament, ovary or uterus to permanent prosthetic mesh[21,22]. But the use of mesh is not advised in the presence of peritonitis or bowel resection because of the potential risk of infection.

Early surgical intervention is essential to the appropriate treatment of obturator hernia. However, surgeons are often reluctant to operate and family members always hesitate in proceeding with clinical management because of the age, the concomitant disease and the general condition of the patient. Conservative medical treatments were attempted in hopes that the obstruction would be reduced, which undoubtedly result in delayed surgical intervention and increased the morbidity and mortality rates. In our study, the duration from onset of symptoms to surgery was 3.7 d. Here we felt deeply regret that the delayed operation led to the gut resection with ileostomy and severe complications. Fortunately, the patient recovered well.

In conclusion, obturator hernia is relatively rare and is a significant cause of intestinal obstruction, particularly in emaciated elderly women without a history of abdominal surgery. It is important that physicians consider obturator hernia in mind when making a diagnosis in patients with small bowel obstruction. For the diagnosis of obturator hernia, the intermittent attacks of intestinal obstruction,

a positive Howship-Romberg sign, a palpable tender mass in the groin area by digital vaginal examination are helpful. Abdominal CT is useful for viewing a loop of small bowel herniated into the obturator canal. The point of early surgical intervention is highlighted because it is the only hope to lower the high morbidity and mortality associated with this condition.

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CASE REPORT

Biliary cystadenocarcinoma diagnosed with real-time contrast-enhanced ultrasonography: Report of a case with diagnostic features

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Abstract

Biliary cystadenocarcinoma is a very rare malignant cystic tumor of the liver, which is often misdiagnosed due to a poor recognition of it. We report a case of a 60-year-old man with biliary cystadenocarcinoma with his real time contrast enhanced ultrasound (CEUS) characteristics compared to those of computed tomography (CT) and magnetic resonance imaging (MRI), which were correlated with the surgical and pathologic findings. Cystic wall enhancement, internal septations and intra-cystic solid portions in the arterial phase were observed on CEUS after contrast agent injection. The enhancement was washed out progressively and depicted as hypo-enhancement in the portal and late phases. CT revealed a large irregular cystic lesion in the left liver lobe with no clear septations and solid components. MRI showed an irregular cystic occupying lesion with septations.

Key words: Biliary cystadenocarcinoma; Cystic neoplasm; Hepatic disease; Contrast medium; Ultrasonography

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INTRODUCTION

Biliary cystadenocarcinoma is a very rare malignant cystic tumor of the liver. It is often misdiagnosed because of an insufficient recognition of it[1,2] and is hard to differentiate it from benign cystic lesions, such as simple cysts, hydatid cysts and its benign counterpart, cystadenoma. Although these cystic lesions of the liver are more frequently discovered because of the advances in abdominal imaging over the past several years, they are often incorrectly diagnosed, resulting in inadequate therapy[1-5]. In recent years, real time contrast-enhanced ultrasonography (CEUS) has gained substantial attention in liver imaging, and its role in differentiating benign from malignant focal liver lesions has been well established. The enhancement characteristics of common benign and malignant focal liver lesions on CEUS have been well described and analyzed, some of which are considered criteria for the differential diagnosis of focal liver lesions[6-8]. However, to

the best of our knowledge, no reports are available on the enhancement characteristics of biliary cystadenocarcinoma on real time CEUS, or on comparison between CEUS, computed tomography (CT) and magnetic resonance imaging (MRI) findings. We report a case of a 60-year-old man with a surgically proven biliary cystadenocarcinoma with its CEUS, CT, MRI and histopathologic findings compared.

CASE REPORT

A 60-year-old man was admitted to a local hospital due to an intermittent abdominal pain for 9 d, which was especially severe in the epigastrium. Physical examination revealed a palpable upper abdominal mass with tenderness in the epigastrium. Except for a 5-year history of diabetes mellitus, laboratory test showed normal carcinoembryonic antigen (CEA), alpha-fetoprotein (AFP), hepatitis B surface antigen (HBsAg), and anti-hepatitis B surface antigens (anti-HBs), but an elevated serum CA19-9 level of 1090 U/mL.

CT scan performed in the local hospital revealed a large irregular cystic lesion in the left liver lobe with no clear septations and solid components (Figure 1). After admitted to our hospital, two-dimensional ultrasonography and CEUS were performed with Philips iU22 (Philips Medical Systems, Bothell, WA) using a 1.0-5.0 MHz probe C5-1 transducer with a pure wave crystal technology to obtain B-mode and color Doppler images. The acoustic power output was adjusted to a low mechanical index of approximately 0.04 for CEUS.

B-mode ultrasonography showed a 12.5 cm × 10.6 cm × 8.2 cm anechoic cystic mass with a well-defined thick wall, mural nodules, and multiple internal septa (Figure 2A), as well as multiple thick and coarse mural and septal calcifications or stones within the septated cysts (Figure 2B). Color Doppler image demonstrated affluent blood flow in the internal septa (Figure 2C). After a conventional sonographic examination to depict the size, location, echogenicity, and internal color Doppler flow signals of the mass, pulse inversion harmonic imaging mode was initiated to examine the real-time CEUS using a sulfur hexafluoride microbubble contrast agent (SonoVue; Bracco SpA, Milan, Italy). A contrast agent (2.4 mL) was administrated intravenously in a bolus fashion *via* an antecubital vein, followed by a flush of 5 mL normal saline solution. CEUS displayed hyper-enhancement of the cystic wall, internal septations and intra-cystic solid portions in the arterial phase at 18 s after contrast agent injection. The intensity reached its peak at 31 s and the enhancement was washed out progressively and depicted as hypo-enhancement in the portal and late phases (Figure 3). MRI showed an irregular cystic occupying lesion with separations (Figure 4).

Finally, hepatic lobectomy was performed during which a large cystic mass was found with mucinous fluid present in some portions of the lesion. The mass was finally diagnosed as a biliary cystadenocarcinoma based on the pathological findings which confirmed the thick and coarse calcifications and stones on ultrasonography (Figure 5).

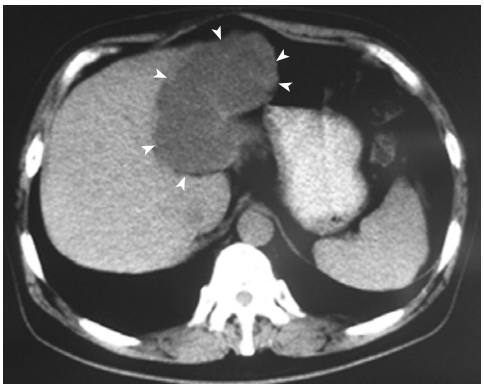

Figure 1 Biliary cystadenocarcinoma. Computed tomography shows a huge cystic tumor (arrowheads) in the left liver lobe, with no clear septations and solid components.

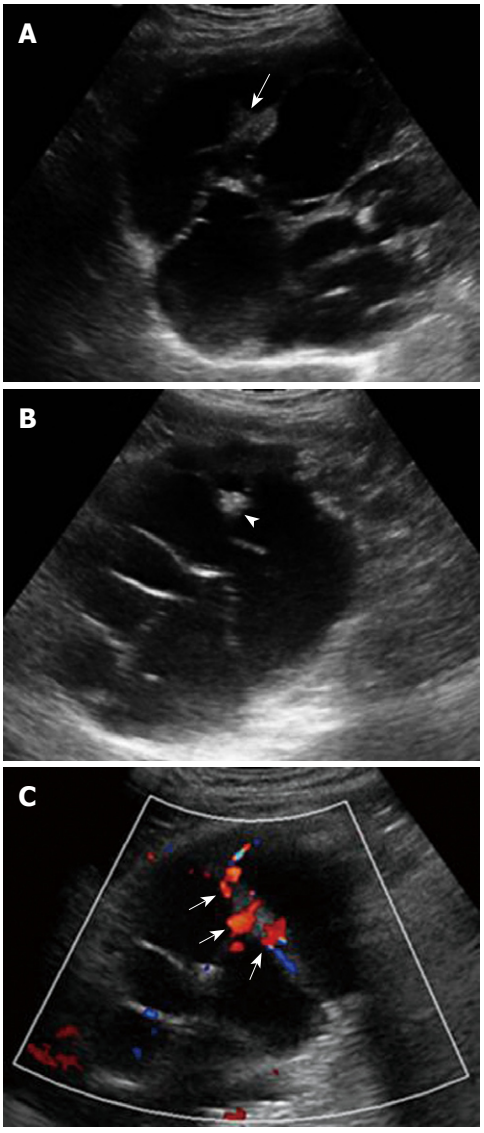

Figure 2 Biliary cystadenocarcinoma. A: Conventional sonography shows a multilocular cystic lesion in left lobe of the liver with nodular thickening of internal septa and mural nodules projecting into the cyst (arrow); B: Conventional sonography shows septal calcifications or stones (arrowhead); C: Color doppler flow imaging (CDFI) shows affluent vascularity in the internal septa (arrows).

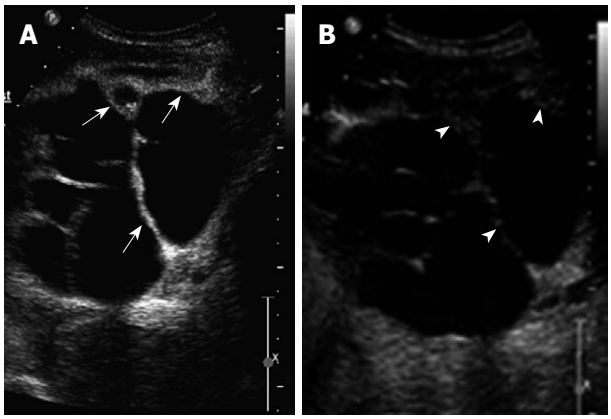

Figure 3 Biliary cystadenocarcinoma. A: Contrast enhanced ultrasound (CEUS) shows hyperenhancement of the cystic wall, internal septations and intracystic solid portions during the arterial phase (arrows); B: CEUS shows hypoenhancement of the cystic wass, internal septations and intracystic solid portions during the portal and late phases (arrowheads).

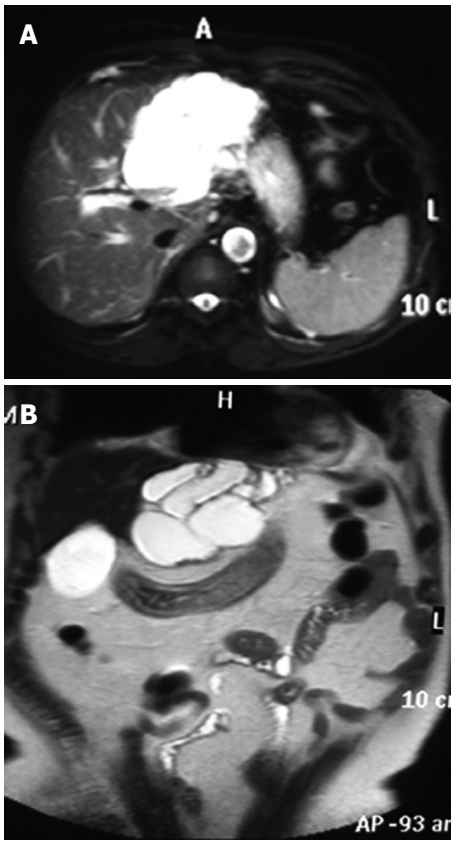

Figure 4 Biliary cystadenocarcinoma. A and B: Magnetic resonance imaging (MRI) scans showing a multilocular cyst in the left lobe of the liver.

DISCUSSION

Biliary cystadenocarcinoma is a very rare malignant cystic tumor of the liver with an incidence of 0.41%[1]. Since it was first reported in 1943[9], only a few cases of biliary cystadenocarcinoma have been reported in the literature[2,9]. Most biliary cystadenocarcinomas are primary malignant tumors originating from the intra-hepatic bile duct or from congenital intra-hepatic biliary malformation, or from benign cystadenoma[10-12], or from very slowly growing bile ducts[2].

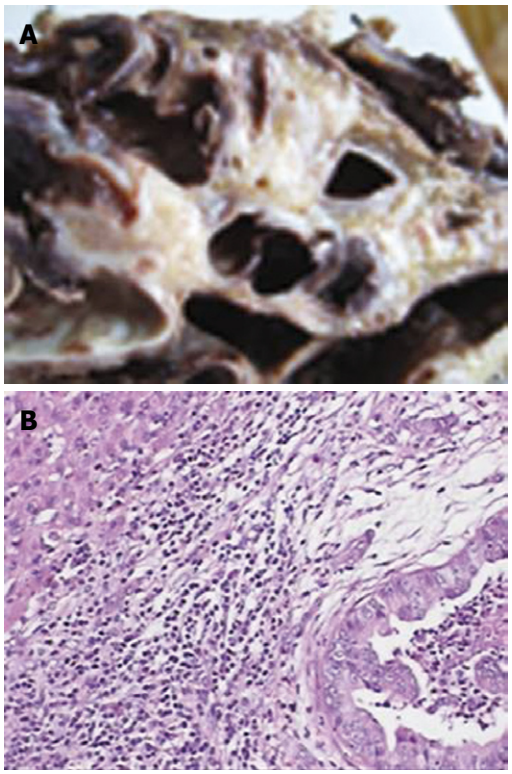

Figure 5 Biliary cystadenocarcinoma. A: Gross appearance of a cross-section of the formalin fixed specimen showing a multilocular mucinous cyst; B: Microscopically, tumor tissues showing moderately differentiated adenocarcinoma with a papillary growth pattern (HE, original magnification × 100).

Symptoms of biliary cystadenocarcinoma include abdominal pain, infection, dyspepsia, anorexia, nausea, vomiting, and occasionally, jaundice due to ductal compression. Most biliary cystadenocarcinoma patients are symptomatic with palpable upper abdominal masses[2]. Abdominal pain and a palpable upper abdominal mass were noted in our case with no jaundice. Because of insufficient knowledge about the biliary cystadenocarcinoma and its indistinctive clinical presentations, it is often misdiagnosed as a hepatic abscess, or a hydatid cyst, or a metastatic tumor with cystic degeneration, or even a simple cyst. It is most difficult to differentiate biliary cystadenocarcinoma from cystadenoma, due to their very similar clinical presentations and imaging features. Cystadenoma occurs predominately in women while 38%-44% of biliary cystadenocarcinomas occur in men[13-15]. Choi *et al*[16] reported that involvement of the left liver lobe is much more common. The majority of biliary cystadenocarcinomas are large in size, usually exceeding 10 cm in diameter, ranging 3.5-25 cm[16,17]. In our case, the lesion was found in the left liver lobe with a diameter of 12.5 cm.

Preoperative imaging studies are of key importance in differential diagnosis. The characteristic CT finding in these tumors is low-density intra-hepatic lesions with internal septa and mural nodules. Contrast enhancement can be seen along the internal septa and wall[2,3,5,17]. In our case, however, CT scan only revealed a large irregular

cystic lesion in the left liver lobe with no clear septations and solid components, while sonography showed internal septa and affluent blood signals. It was reported that CT scan fails to show any definite or probable septa while sonography reveals internal septa in the same cases. Because contrast enhanced CT was not performed for our case, CT enhancement characteristics of biliary cystadenocarcinoma could not be observed, suggesting that sonography is somewhat more sensitive than CT in detecting the septa of a cystic lesion[5,16] and is thus superior to CT in displaying the morphology of cystic hepatic lesions.

Although MRI can show small differences in tissue and provide further information concerning the nature of fluid in the cyst, and is therefore extraordinarily helpful in characterizing hepatic lesions. In our case, MRI showed an irregular cystic occupying lesion with separations. However, biliary cystadenocarcinoma could not be diagnosed in our hospital due to an insufficient recognition of it. In order to understand its enhancing characteristics and make a correct diagnosis, CEUS was performed with Philips iU22, which showed hyper-enhancement of the cystic wall with internal septations and intra-cystic solid portions in the arterial phase and reached its peak intensity in late arterial phase. However, the enhancement was washed out progressively and depicted as hypo-enhancement in the portal and late phases. An initial diagnosis of biliary adenocarcinoma was made by comparing the results in our study with published enhanced CT characteristics, which was confirmed at surgery. The hyper-enhancement in arterial phase and the hypo-enhancement in portal or late phase on CEUS in this case may indicate the malignant nature of the lesion. However, Xu *et al*[18] have reported the hyper-enhancement of the cystic wall, internal septations, and intra-cystic solid portion in the arterial phase in 1 case with benign intrahepatic biliary cystadenoma on CEUS. The enhancement was washed out progressively and depicted as hypo-enhancement in the portal and late phases, suggesting that inadequate differences in enhancement characteristics can differentiate benign intrahepatic biliary cystadenoma from cystadenocarcinoma.

In addition to the features mentioned above, Li *et al*[19] and Koffron *et al*[20] have reported increased levels of CA125 and CA19-9. The CA19-9 level was also elevated in our case. Macroscopy can discover large monolocular or multilocular cystic lesions with mural nodules projecting into the cyst in most cases. Most of the cysts are filled with a great deal of clear, yellow mucous like fluid. The cystic fluid can be coffee-colored when complicated by intra-cystic hemorrhage and bile-like if the cyst communicates with intra-hepatic bile ducts[21]. Microscopy can display moderately-differentiated adenocarcinoma with a papillary growth pattern. The papillary structure is lined by monolayer columnar or pseudo-stratified epithelial cells. The tumor cells are characterized by loss of polarity, karyomegaly, and allotypic and pathologic mitotic figures. Although percutaneous liver biopsy contributes to a definite diagnosis, it is risky to induce peritoneal implantation metastasis[22].

Biliary cystadenocarcinoma should be suspected when CT or ultrasonography reveals an elevated mass or nodule in cystic wall or in folding. However, it is extremely difficult to differentiate cystadenoma from adenocarcinoma by imaging alone. In our case, benign hepatic cyst was considered at admission. It has been reported that tumor markers, carcinoembryonic antigen and carbohydrate antigen 19-9 are increased in serum or cystic fluid of biliary cystic tumor[23-25]. However, tumor markers cannot distinguish cystadenocarcinoma from cystadenoma or both from other cystic lesions of the liver. The increased level of serum CA125 and CA19-9 contributes to the differentiation of benign from malignant tumors and is a useful index for the prognosis of biliary cystadenocarcinoma patients[23-25].

In conclusion, biliary cystadenocarcinoma is asymptomatic. When patients have a large cystic lesion in the liver, especially accompanying elevated serum CA125 and CA19-9 levels, a diagnosis of biliary adenocarcinoma can be established. Its definitive diagnosis is difficult to be made based on ultrasonography, CT and MRI findings. CEUS is useful in depicting the enhancing characteristics of cystic wall, internal septations and intra-cystic solid portions of biliary cystadenocarcinoma but cannot give a definite diagnosis. Further study is needed.

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A dynamic model of once-daily 5-aminosalicylic acid predicts clinical efficacy

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Abstract

New once daily mesalamine formulations may improve adherence to medication usage. Response to Asacol and other forms of 5-aminosalicyclic acid (5-ASA) is better correlated with tissue concentrations and best predicted by concentrations of the drug within the lumen of the colon. Our group used computer simulation to predict colonic 5-ASA levels after Asacol administration. In our study, the model simulated Asacol distribution in the healthy colon, and during quiescent and active ulcerative colitis. An Asacol dosage of 800 mg, three times a day, was compared to 2400 mg given once a day. Under ideal conditions, the predicted maximum drug in the total colon and individual colonic segments over 100 d differed by less than 3% between single and multiple doses. Despite changes in motility and defection rates, the predicted maximum and average 5-ASA concentrations in the total colon and individual colonic segments differed by less than 10% between dosing regimens. Asymmetric distribution of 5-ASA in the colon was influenced by frequency of bowel movements and colonic transit rate. In active colitis, sigmoid 5-ASA concentration becomes negligible. Our model supports once daily administration of Asacol as standard treatment for ulcerative colitis.

Key words: Ulcerative colitis; 5-aminosalicylate; Mesalazine; Asacol; Once-daily

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TO THE EDITOR

We read with a great interest the editorial by Lakatos[1] that summarizes the available literature on the short and medium term efficacy and safety of the new once-daily mesalazine formulations. Single dose regimens may improve adherence to medication usage. However, older forms of 5-aminosalicylic acid (5-ASA) may also be administered in a single daily dosage, apparently with adequate effects[2]. Most pharmacokinetic studies on

Asacol and other forms of 5-ASA are limited to data collected from serum, urine or fecal drug concentrations. However, response is better correlated with tissue than with plasma concentrations, and is best predicted by concentrations of the drug within the lumen of the colon[3,4]. A number of factors influence the concentrations of drugs in colon, such as 5-ASA. Our group[5] created a computer model to predict 5-ASA levels in colon after Asacol administration using STELLA software (Isee Systems, Inc., Lebanon NH, USA). This model divides the intestinal system into individual compartments-upper GI tract, right colon, transverse colon, descending colon and the recto-sigmoid colon, and predicts the movement of 5-ASA from one compartment to the other. Retrospective data for drug concentrations based on serum levels have been utilized[6,7]. In addition to local transfer of the drug, each colonic compartment loses a fraction of its drug concentration due to mass movements with defecation[8]. In our study, the model was run to simulate Asacol distribution in a healthy colon, and during quiescent and active ulcerative colitis. To achieve this, simulations were performed with increasing defecation rates up to 12 bowel movements daily along with variation of upper GI and colonic motility. One hundred 24 h cycles were studied. An Asacol dosage of 800 mg, three times a day, was compared to 2400 mg given once a day. Under ideal conditions, the predicted maximum drug in the total colon and individual colonic segments over 100 d differed by less than 3% between single and multiple doses. Despite changes in motility and defection rates, the predicted maximum and average 5-ASA concentrations in the total colon and individual colonic segments differed by less than 10% between dosing regimens. The model could also predict almost no drug within the lumen of the recto-sigmoid colon during severe disease activities[5].

Our model supports the once daily administration of Asacol, a concept catching on with new clinical trials. Asymmetric distribution of 5-ASA in the colon is influenced by frequency of bowel movements and the rate of colonic transit is an important factor in determining 5-ASA dosing in active ulcerative colitis.

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Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases

October 07-09
Belgrade, Serbia
The 7th Biannual International Symposium of Society of Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

October 23-27
Barcelona, Spain
18th United European Gastroenterology Week

October 29-November 02
Boston, Massachusetts, United States
The Liver Meeting® 2010--AASLD's 61st Annual Meeting

November 13-14
San Francisco, CA, United States
Case-Based Approach to the Management of Inflammatory Bowel Disease

December 02-04
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Instructions to authors

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Acknowledgments

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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

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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.0000035706.28494.09]

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

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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

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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.

The format for how to accurately write common units and quantums can be found at: http://www.wjgnet.com/wjg/help/15.doc.

Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

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.*
Biology: *H. pylori*, *E coli*, *etc.*

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