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Guest Editorial

Medical Council of India's Amended Qualifications for Indian Medical Teachers : Well Intended, Yet Half-hearted

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The Medical Council of India (MCI) must be commended for its efforts to introduce definitive criteria for appointments and promotions for teachers in medical institutions. On June 8, 2017, the MCI issued a circular (1) to amend the Minimum Qualifications for Teachers in Medical Institutions Regulations, 1998 (hence forth Regulations, 1998) (2). The amendment clarifies the minimum qualifications required for various postgraduate teaching positions in medical colleges. It indicates MCI's sustained engagement with qualifications of teachers in medical colleges, with the aim of enhancing the quality of teaching and thereby the quality of medical professionals passing out. However, we believe that these efforts continue to be inadequate in addressing the varied issues that face medical education and the educators in India.

Some of these issues are: (i) the lack of transparency in the manner in which new medical colleges are approved, (ii) the variation in the proportion of private and public medical colleges across states, (iii) the lack of change and innovation in the undergraduate and postgraduate medical curricula to keep up with changing needs, (iv) the poor uptake of newer teaching–learning methods, (v) the poor quality of teachers in several medical colleges, (vi) methods used to assess teachers during selection and promotions, and (vii) failure to assess the impact of policy changes (such as a recent increase in the number of postgraduate seats) on the quality of medical education and training.

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In this editorial, we focus on one of these issues, ie, the appointment and promotion of teachers in medical colleges. The MCI had on September 3, 2015 (3) stated its requirements with regard to research publications for eligibility for promotion of faculty members in medical colleges. This had been critiqued (4, 5) mainly on four counts: exclusion of publications in 'electronic-only' journals from consideration for assessment of performance; awarding points only to original research articles or papers; awarding points only to first or second authors; and, the choice of indexing services for assessing the quality of a journal. While lauding the MCI's efforts towards improving the standards for teaching faculty at medical colleges in India, these critiques argued that an ill-informed framework for determining eligibility for promotion is likely be self-defeating and even harmful to the profession.

A few steps forwards and a few steps backwards

The 2017 Amendment (1) is noteworthy for it states that in order to be eligible for assessment, a paper must be published in "indexed" journals. This appears to be a step in the right direction because indexing indicates that a journal meets certain standards and quality within a specialty, specific to a particular index. However, in this amendment, the MCI has not specified any particular index(es). Thus, possibly the list of indexes previously specified in its 2015 order will continue to apply. Let us look at this issue more closely. Accepting only the MEDLINE-indexed journals would exclude some research in valuable allied fields, such as medical humanities, basic sciences and social sciences as applied to medicine. Thus, inclusion of other indexes may be useful for recognizing these diverse related disciplines beyond the pure health sciences, although with due diligence. For example, some indexes have little credibility as they are known to include pseudojournals (also known as 'predatory journals') in their listings. Hence, it is important that MCI specifies only those indexes which are reputed to have quality journals. The 2015 MCI list of eligible indexes has been faulted on this score (5). Aggarwal and colleagues (5) had suggested the following list of acceptable databases: Medline, PubMed Central, Science Citation Index, Embase/ Excerpta Medica, Scopus and IndMED. The latest amendment missed out on an opportunity to revise the list of eligible indexes.

The amendment does not specify whether or not papers published in "e-only" indexed journals are acceptable for assessment. Here too, possibly the stipulation in the 2015 order, that e-journals are not included, will continue to apply. Currently, many e-only journals (eg PLoS group, BioMed Central, etc) are comparable in quality to, and at times even better than, those published as hard copy. Their inclusion would allow a much wider range of journals for the faculty to choose from for publishing their work.

Unfortunately, as with Regulations, 1998 (2), the 2017 Amendment (1) also limits the credit for authorship to only some of those listed on the author byline. Unlike the first version in which those listed as the first and the second authors were eligible, the amendment gives the credit for a paper to only the first author and the corresponding author. As critiqued earlier, this approach inhibits collaborative research and could be counterproductive by undermining the advancement of knowledge. Some of the best research today is multidisciplinary and multi-author.

Problems untouched

The MCI regulations, even after the recent amendment (1), are problematic in other aspects too. These lay down two criteria to assess a candidate's eligibility for a particular position: duration of service and number of research publications. One would expect the parameters assessed during appointment and promotion to be aligned with the responsibilities of teachers in medical colleges, with a strong convergence suggesting appropriateness and sufficiency of the criteria. In clinical or paraclinical departments, medical teachers have three primary activities; providing clinical or laboratory/imaging service, teaching, and doing research; which vary for different specialities. However, in most medical colleges, irrespective of specialty, the research activity forms a small part of the total work of a medical teacher. Hence, any assessment of only research output without an assessment of the other

two domains does not appear to be reasonable. What about the other two activities? Provision of clinical or laboratory services and teaching are integral to the core work of a medical faculty member. The assessment, if any, of these domains is only by the years of service put in. This appears unfair. The MCI regulations should address the issue of assessing medical faculty in all the three activity domains. Undue focus on research and not on the other two domains might prove to be detrimental both to the training of medical students as well as to clinical work.

Failure of the faculty to do research is a well-documented problem in Indian medical colleges. It has been argued (6) that this is due to commercialisation of medical education in the country. However, we believe that this phenomenon is multifactorial. One major reason may be a lack of interest and training to do research on part of the teachers, or of lack of infrastructure to facilitate research on part of the institutions. Also, good research requires financial resources – and most of the institutions, whether funded publicly- or privately have no or little funds dedicated to this activity. These factors may need to be corrected first, before we can expect research to be an important criterion for assessing eligibility for appointment and promotion of medical teachers.

Lack of adequate funding too discourages the MCI's approach. India has nearly 450 medical colleges. Let us assume that each college has around 100 teachers, and that each of the nearly 45,000 teachers needs to publish a research paper every three years. This translates to around 15,000 research papers a year. In addition, around 20,000 students join a medical postgraduate course every year in the country, and each of them has to write a thesis. Let us assume the "most optimum" case scenario – that each thesis results in a paper with a student and his teacher-guide as the two eligible authors. Even with this unlikely scenario, we would require to generate at least 20,000 new research ideas every year – a formidable task. For these research works to be novel and publishable, a large proportion of these ideas would need funding – which is currently not available.

Another important consideration is whether there are sufficient peer-reviewed, "indexed" journals to publish this large body of work. The requirement to publish by teachers in Indian medical colleges and universities has seen a proliferation of "predatory journals" in India (7, 8, 9). We are not arguing that research may be altogether abolished as a criterion for eligibility and assessment of medical faculty. It is known that good research institutions globally and in India are sought after by students and patients alike, as these are considered better centres for learning and providing a better quality of care. However, whether the quality of teaching and patient care can necessarily be improved by mandating research of whatever quality remains uncertain.

Thus, it is not reasonable to make an assessment for promotion of a medical teacher solely on the basis of research activity – that too by counting the number of publications.

The way forward

It is evident from the above that the assessment of medical teachers must encompass all the three domains of their activities. Furthermore, the assessment should focus not on quantity, as is done currently by counting only years of service or number of papers, but on the quality of work in each sphere. Unfortunately, we will be told that "assessment of quality" would not be objective, and would be liable to bias and manipulation. However, this is an excuse for not doing what seems to us the right thing to do. Around the world, as in many fields in our country, employees are assessed using the so-called "subjective" criteria, with sufficient reliability. Setting up such systems – though admittedly hard – is not impossible. These will surely take time, effort and commitment to set up. But if we unquestioningly accept the simplistic tools such as publication count, we will never move to a higher plane. Hence, as a profession, we need to initiate debate for moving towards better systems of assessing quality. Such an assessment system would necessarily mean a multi-pronged evaluation – by peers, students as well as administrators.

Variation is an important rule of nature and all medical

teachers cannot be expected to have exactly the same skill set. Thus, one of them may be an excellent researcher, but not a particularly good teacher. Similarly, someone else might be better at providing a laboratory service than doing research. This is in fact desirable since it allows some persons to excel in one specific area beyond the average skill level expected, and should be encouraged. This requires that individuals with different skill sets and inclinations be provided the opportunity to do more of what they are good at and less of what they may not be so skilled at. The proportion of time spent on the three core activities referred to above could thus vary between different teachers. Thus, it would be reasonable that the assessment of medical teachers for the quality of work would be a weighted average of the quality of work in the three domains, with the weights decided by the pre-defined proportion of time spent by each teacher on activities in these domains. Clearly defining each faculty member's job description at the time of appointment or during the course of service will facilitate and/or enable such an approach to assessment.

Each core activity could be assessed using different parameters. Teaching should be assessed by the end user, ie, the student and the performance of students in an assessment should be part of the assessment of a teacher. Similarly, peers should sit in on teaching activities and provide a peer evaluation. These suggestions are neither exhaustive nor necessarily tested to be appropriate for our milieu. Hence, a constant evaluation and evolution of these methods would be essential.

Research should be evaluated but not by the number of publications. The quality of a medical faculty's research output should be assessed. This would include a peer evaluation of the individual's select few publications––a smaller number at the time of selection and an increasing number with each step in the academic ladder. For example, two best papers at the time of initial selection as a faculty member, five best at the next level and seven and ten in the further steps. As almost all medical faculty positions in India are tenured, there are few who would make the effort to write a grant application and obtain funding. Those who do so should be assessed on the quality of their grant applications or the amount of funding obtained.

How does one assess the service component of the medical faculty? This could be difficult to do but an effort should be made to use laboratory and clinical audits, and peer and patient assessment and feedback.

All this must be done transparently. The assessors, the method and process of assessment and the final evaluation must all be transparent. Anybody can make errors and hence there must be a transparent system of appeals and evaluations of appeals. Questioning a decision with sound reasoning must be permitted but the process must be free of corruption.

We are aware that some of these suggestions may appear radical in the current Indian scenario. We believe that the Indian medical education system is in urgent need of radical corrective steps, if we are to prevent it from continuing on the slippery slope that it presently is on. Minor tinkering, such as the MCI seems to be engaged in, will not do.

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Editorial – IJPP

Does Current Human Ethical Guidelines Support Translational Research in Indian Medical Systems?

Indian Council of Medical Research has recently released National Ethical Guidelines for the Biomedical and Health Research involving human participants (1). It is available in the ICMR website for public access. ICMR policy statement on Ethical Considerations Involved in Research on Human Subjects was initially established in 1980s and those guidelines were revised in 2000 as Ethical Guidelines for Biomedical Research on Human Subjects. Covering newer areas, it was again updated and brought as Guidelines for Biomedical Research on Human Participants in 2006. The current guideline has been framed to address the concerns of all stakeholders in the area of research using human subjects. The current clarity in the guidelines is expected to give support and confidence among the researchers to undertake translational research.

One must remember that the evolution of clinical research in India has created lot of interest among several investigators in private and public Institutions after the GATT agreement. Clinical research had created a massive hype amongst students due to the demand of trained manpower in the related areas. During that time, the clinical trials involved in drug discoveries and other experiments were booming in India on one hand and the lack of adequate regulations left certain concerns unaddressed on the other. Privation of clear policies and guidelines in place, non-sensitization of public towards trial related issues, media trials resulting in abrupt change in policies of the Government have resulted in tremendous pressure on the clinical research armamentarium.

Transnational biomedical research through outsourcing of clinical trials by pharmaceutical companies from industrialized nations to low and middle income nations have always raised concerns about the vulnerability of study participants (2). The dynamism in the evolution of ethical standards differs between cultures in the back drop of economy and has to be addressed time to time without affecting the fundamentals of ethics. Clinical drug research in academic institutions is mostly conducted by comparing efficacy among same class of approved compounds for the indicated disease conditions. These kinds of investigator initiated clinical studies are conducted without the support of any external sponsors (like drug companies) only for academic interest and for the larger benefit of society in the particular country to add knowledge to the existing literature.

Due to the lack of active collaboration between the pharmaceutical drug discovery programs and academia, most of the Indian academic research projects are directed to herbal isolates and extracts which are limited only to preclinical studies with notable public funding. The volume of traditional knowledge resulting in preclinical research publications are spiraling with the supporting evidences using molecular biology techniques. But, seldom they get translated into the clinical studies for their therapeutic utility. Purifying or isolating a natural compound alone cannot afford patent eligibility. Due to lack of exclusive marketing rights, getting investment from a pharmaceutical agency for translational research is very unlikely. Without the chemical derivatisation of the active compound resulting in structural change evincing a corresponding functional difference, patent rights are not possible. In this scenario, current guideline gives enough space for clinical studies in Ayurveda, Siddha, Unani and Sowa Rigpa drug formulations (Box of 7.7 of NEG 2017) (1). For conducting such academic studies, required empowerment to the institutional ethics committee has already been provided by CDSCO.

For example, launch of BGR-34 by CSIR laboratories and IME-9 by AYUSH for type II diabetes in India showed the possibility of developing the vast knowledge available in the ancient Indian medical systems like Ayurveda, Siddha and Unani can be brought into clinical use. The aforesaid formulations are the offshoot of the plants derived from Ayurvedic literature. Now, more than 40 of such formulations are available in India containing the chief ingredient '*Gymnema sylvestre'* (Gudmar). It has also been sold in online shopping websites and in pharmacies. Although, it has been known for reducing blood sugar for more than 30 years, (Khare et al 1983 [3] published in Ind J Physiol and Pharmacol), inadequate scientific evidence according to the required standards is a major stumbling block for its understanding towards pharmacology and therapeutic utility at par with allopathy (4). To our surprise in the past two years, we see more than 2 tons of Gymnema extracts (Gymnemic acid) have been imported in Indian ports and more than 4 tons were exported as extracts and formulations to various other countries and used extensively in population.

Having more than 450 medical colleges and 1100 Pharmacy colleges in India involved in drug related research indicate the availability of potential population to undertake preclinical and subsequent translational studies. It should be considered that now is the ripe time for opting to translational studies in our Indian Medical systems. One must go through NEG-2017 and GCP-ASU-2013 to understand where exactly their plant extract or ASU formulation is coming under (1 & 5). In many cases, incorporation of an ASU practitioner is insisted by the guidelines for conducting the study. With the emphasis on mainstreaming the potential of AYUSH in the National Health Policy-2017, there is an expanding scope for affordable health care promotion and cure through wholistic and perceptive approach.

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Review Article

Role of *Terminalia Arjuna* in Improving Cardiovascular Functions : A Review

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Abstract

Cardiovascular diseases are the most common cause of deaths worldwide and will become even more prevalent with the recent changes in life style, food habits and environmental pollution. Herbal medicines have been used for cardiovascular diseases and some of their derivatives have become mainstay of human pharmacotherapy. Various clinical and pharmacological studies have indicated the cardioprotective role of *Terminalia arjuna* in cardiac ailments. The present review is an effort to give a detailed survey of the literature summarizing the experimental studies of *T. arjuna* on cardiovascular system. It mainly focuses on experimental studies pertaining to various aspects of cardiovascular functions, autonomic control of myocardial functions, molecular mechanisms of its action and Cardiac histopathology.

Introduction

Cardiovascular diseases (CVD) are the number one cause of death worldwide (1). In addition to mortality, poorly managed CVD can lead to significant long-term disability from their complications. In the past quarter century, much progress has been made in understanding the molecular and cellular processes that contribute to CVD, leading to the development of effective therapies.

Natural products due to their chemical diversity are receiving increased attention from scientific and pharmaceutical communities. The newer work on medicinal plants is mostly the rediscovery of traditional effects at cellular and molecular levels (2). *Terminalia arjuna* (*T. arjuna*, -Family: Combretaceae), is an important medicinal plant widely used in medicinal formulations for several ailments. It is used in traditional medicine for treating ulcers, wound healing, and also for antibacterial, antimutagenic/anticarcinogenic, antioxidant and hypocholesterolemic activities (3-8). The use of *T. arjuna* bark in the management of cardiovascular diseases has been widely reported (8-16). This review

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is an effort to give a detailed survey of the literature summarizing the experimental studies of *T. arjuna* on cardiovascular system. It mainly focuses on experimental studies pertaining to various aspects of cardiovascular functions, autonomic control of myocardial functions, molecular mechanisms of its action and Cardiac histopathology.

Plant profile

Habitat

Terminalia arjuna is a deciduous and evergreen tree, standing 20–30 m above ground level (Fig. 1). It belongs to Combretaceae family (17-19). It is found in abundance throughout Indo-sub-Himalayan tracts of Uttar Pradesh, South Bihar, Madhya Pradesh, Delhi and Deccan region near ponds and rivers. It is also found in forests of Sri Lanka, Burma and Mauritius (20).

Ethnomedical considerations

The bark, leaves and fruits of *T. arjuna* have been used in indigenous system of medicine for different ailments (21). The bark is said to be sweet, acrid, cooling and heating, aphrodisiac, expectorant, tonic, styptic, antidysenteric, purgative and laxative. Its use has been advocated in urinary discharge, strangury, leucoderma, anaemia, hyperhidrosis, asthma and tumours. The use of bark powder as an astringent and diuretic finds mention in the literature (22). The bark powder has been attributed to possess cardioprotective properties (8-15).

Phytochemistry

From medicinal point of view bark of *T. arjuna* was considered to be the most important constituent. Hence most of the early studies were limited to bark stem of the plant. Chemical analysis of the bark showed evidence of sugar, tannins (12%), colouring matter, a glycoside, and carbonates of calcium, sodium and traces of chloride of alkali metals (23). Subsequently presence of an alkaloid as well as a glycoside was confirmed. The major chemical constituents of various parts of *T. arjuna* are shown in Table I. The glycoside was capable of increasing the force of contraction of the frog heart (24). Attempt to isolate the glycoside resulted into finding of an organic acid with a high melting point, a phytosterol, an organic ester easily hydrolysed by mineral acids, 12% tannins consisting largely of pyrocatechol tannins, large quantities of calcium and smaller amounts of aluminium and magnesium salts, colouring matter and sugar (17).

Experimental studies

Studies based on autonomic control of cardiovascular functions

The cardiovascular system is subject to precise reflex regulation so that an appropriate supply of oxygenated

Fig. 1 : *Terminalia arjuna* plant.

TABLE I: Major chemical constituents of various parts of *Terminalia arjuna* (22).

	(A) Stem bark
1.	Triterpenoids: arjunin, arjunic acid, arjunolic acid, arjungenin, terminic acid (25,26)
2.	Glycosides: arjunetin, arjunoside I, arjunoside II, arjunaphthanoloside, terminoside A (23,24).
3.	Sitosterol (24, 26).
4.	Flavonoids: arjunolone, arjunone, bicalein, luteolin, gallic acid, ethyl gallate, quercetin, kempferol, pelorgonidin, oligomeric proanthocyanidins (27,28).
5.	Tanins: pyrocatechols, punicallin, punicalagin, terchebulin, terflavin C, castalagin, casuariin, casuarinin (17, 23, 29).
6.	Minerals/trace elements: calcium, aluminium, magnesium, silica, zinc, copper (30).
	(B) Roots
1.	Sitosterol (26)
2.	Triterpenoids: arjunic acid, arjunolic acid, oleanolic acid, terminic acid (26).
3.	Glycosides: arjunoside I, arjunoside II, arjunoside III, arjunoside IV, 2,19-dihydroxy-3-oxo-olean-12-en28-oic acid28-*O*-_-d-glucopyranoside (26, 31).
	(C) Leaves and fruits
1.	Glycosides
2.	Flavonoids: luteolin (28).

blood can be reliably provided to different body tissues under a wide range of circumstances. The sensory monitoring for this critical homeostatic process entails primarily mechanical (barosensory) information about pressure in the arterial system and, secondarily, chemical (chemosensory) information about the level of oxygen and carbon dioxide in the blood. The parasympathetic and sympathetic activity relevant to cardiovascular control is determined by the information supplied by these sensors.

The autonomic nervous system modulates beat-to-beat fluctuations in heart rate (HR). It modulates the electrical and contractile activity of the myocardium via the interplay of sympathetic and parasympathetic activity. Cardiovascular autonomic neuropathy, a common form of autonomic dysfunction, causes abnormalities in heart rate control, as well as defects in central and peripheral vascular dynamics (32). Methods to quantify HR and blood pressure variability have been evaluated as indicators of sympathetic and parasympathetic modulation of the cardiovascular system in humans and in experimental models. These methods seemed to detect early autonomic dysfunction at a time when other metabolic dysfunctional changes were not clearly observed. Baroreflex sensitivity and Heart rate variability are the two frequently used parameters to assess autonomic control of cardiovascular functions.

Baroreflex sensitivity

The evaluation of baroreflex sensitivity (BRS) is an established tool for the assessment of autonomic control of the cardiovascular system. Arterial baroreceptors provide the central nervous system with a continuous stream of information on changes in blood pressure (which are sensed by the stretch receptors in the wall of the carotid sinuses and aortic arch), on the basis of which efferent autonomic neural activity is dynamically modulated. Activation of arterial baroreceptors by a rise in systemic arterial pressure leads to an increase of the discharge of vagal cardioinhibitory neurons and a decrease in the discharge of sympathetic neurons both to the heart and peripheral blood vessels. This result in bradycardia decreased cardiac contractility and decreased peripheral vascular resistance (33). Various studies demonstrating the improvement of baroreflex sensitivity with *Terminalia arjuna* are shown in Table II.

Heart rate variability

Heart rate variability (HRV) can detect cardiac autonomic impairment in individuals before traditional cardiovascular autonomic function tests (34). HRV analysis is the ability to assess over all cardiac health and the state of the autonomic nervous system (ANS) responsible for regulating cardiac activity. It

TABLE II: Experimental studies on *Terminalia arjuna* related to autonomic control of cardiovascular functions.

Study pertaining	*Plant preparation, dosage and route*	*Animal model*	*Observations*	*Interpretation*
Baroreflex sensitivity	50% aqueous ethanol extract (500 mg/kg, orally)	Anesthetized rat having diabetic cardiomyopathy. *T. arjuna* therapy started after 8 weeks of STZ and given for 30 days.	(a) Reflex bradycardia elicited by rise in arterial pressure was significantly reduced after diabetes but regained after *T. arjuna* therapy. (b) Reflex tachycardiaduring hypotension did notshow significant recovery after *T. arjuna* therapy due to depressed sympathetic activity (10).	Improved cardiovascular autonomic neuropathy in rats having uncontrolled diabetes.
	50% aqueous ethanol extract (500 mg/kg, orally)	Isoproterenol induced chronic heart failure in rats	Baroreflex sensitivityto both phenyephrine and sodium nitroprusside was significantly improved in rats with prophylactic and therapeutic treatment with *T arjuna* (13)	Improved sympathovagal balance and neurohormonal activationin CHF animals.
Heart rate variability	50% aqueous ethanol extract (500 mg/kg, orally)	Anesthetized rat having diabetic cardiomyopathy. *T. arjuna* therapy started after 8 weeks of STZ and given for 30 days.	Heart rate variability parameters i.e. SDNN, RMSSD, power in LF range, HF range, LF: HF ratio and total power was improved after *T. arjuna* treatment (36).	Improved sympathovagal balance thus improving the autonomic control of cardiovascular functions in diabetic rats.

Note: All experiments were carried out with bark constituents of *Terminalia arjuna*.

Abbreviations: Standard deviation of normal R-R intervals (SDNN), square root of mean- squared difference of successive R-R intervals (RMSSD), power in low frequency range (LF), high frequency range (HF), ISO- Isoproterenol, BRS Baroreflex sensitivity, STZ streptozotocin; CHF congestive heart failure.

reflects the heart's ability to adapt to changing circumstances by detecting and quickly responding to unpredictable stimuli (35). *Terminalia arjuna therapy* is reported to improve the altered HRV in diabetic rats (36, Table II).

Cardiovascular functions

Cardiomyopathy refers to a disease process which affects the myocardium in patients causing a wide range of structural abnormalities eventually leading to LVH (left ventricular (LV) hypertrophy) diastolic and systolic dysfunction or a combination of these (37). The systolic dysfunction is impairment in the ability of the heart to eject blood. The principle hallmark of systolic dysfunction is a depressed LV ejection fraction dysfunction. Diastole is the time period where the myocardium is no longer generating force and subsequently returns to an unstressed length and force. Diastolic dysfunction occurs when there is prolongation and slowing of this process. Experimental studies have revealed *T. arjuna* bark exerting significant inotropic and hypotensive effect, increasing myocardial contractility, coronary artery flow and protecting myocardium against ischemic damage. Table III compiles the various experimental studies on *Terminalia arjuna* related to cardiovascular system.

Molecular mechanisms affecting cardiovascular functions

Cardiovascular disease is a complex and multifactorial disease and is characterized by multiple factors. Epidemiologic studies have identified these as hyperlipidemia, hyperglycemia, inflammatory responses, coagulation factors, increased platelet activation and smoking. However, the pivotal mediator for the pathogenesis of diabetes and its cardiovascular complications is oxidative stress. Oxidative stress is the imbalance between the production of reactive oxygen and nitrogen species

TABLE III: Experimental studies on *Terminalia arjuna* related to cardiovascular system.

Study pertaining	*Plant preparation, dosage and route*	*Animal model*	*Observations*	*Interpretation*
Hypotensive actions	Aqueous and alcoholic bark extract, i.v., intra cerebral andIntravertebra	Dog, in vivo	Dose-dependent decrease in blood pressure (38).	Dose-dependent hypotension and decrease in heart rate were attributed to principlesof the extract acting centrally.
	Aqueous extract of the bark, intravenously	Dog, in vivo	Dose-dependent fall in blood pressure (39).	The vasorelaxant effect of *T. arjuna* extract could contribute to the fall in BP.
	Aqueous and tannin containing fractions 10–20 mg/kg	Rat	Lowering of blood pressure and heart rate (40).	The blockade by propranolol of the hypotension produced by *T. arjuna* indicates that the extract might contain active compound(s) possessing adrenergic β2-receptor agonist action and/or that act directly on the heart muscle.
	Aqueous extract of bark, 40 mg/kg, i.v.	Dog, in vivo study	Sustained fall in blood pressure (41).	The vasorelaxant effect of *T. arjuna* extract could contribute to the fall in BP.
	Intravenous administration of 70% alcoholic extract (5-15 mg/kg)	Anaesthetized dogs.	Dose-dependent hypotension (42).	
	50% aqueous ethanol extract (500 mg/kg). Therapy started after 8 weeks of STZ and given for 30 days.	Anesthetized rat having diabetic cardiomyopathy.	Did not improve the fall in systolic, diastolic and mean BP observed in diabetic rats (10).	50% aqueous ethanol extract has no effecton BP.
	50% aqueous ethanol extract	Isoproterenol induced chronic heart failure in rats	No effect (12).	
Effect on heart rate	50% aqueous ethanol extract (500 mg/kg, orally)	Anesthetized rat having diabetic cardiomyopathy. *T. arjuna* therapy started after 8 weeks of STZ and given for 30 days.	Did not improve the fall in heart rate observed in diabetic rats (10).	50% aqueous ethanol extract has no effecton heart rate.
	50% aqueous ethanol extract (500 mg/kg, orally)	Isoproterenol induced chronic heart failure in rats	No effect (12).	
	Aqueous and alcoholic bark extract, i.v., intra cerebral and Intravertebral	Dog, in vivo	Dose-dependent decrease in heart rate (38).	Decreased heart rate attributed to principles of the extract acting centrally.
	Aqueous as well as alcoholic bark extract	(a) Isolated frog atria; (b) isolated rat atrium; (c) isolated perfused rabbit heart/both *in vivo* and *in vitro*	Reduction in heart rate (39).	
Cardiac index	50% aqueous ethanol extract (500 mg/kg, orally)	Isoproterenol induced chronic heart failure in rats	Therapeutic and prophylactic treatment with *T. arjuna* showed significant improvement in cardiac index (12).	Improved cardiac performance reflecting cardiop rotective effect.
Cardiac haemo-dynamics	Aqueous bark extract	Isolated frog heart	Rate and force of heart contraction increased in both experiments (23).	The positive inotropic effect of the aqueous extract was proposed to be mediated via

	Aqueous bark extract	Isolated rabbit heart, isolated frog heart	Increased heart rate and force of contraction and finally stoppage of heart (20).	an action on β_1-adrenoceptors and was likely to be due to the release of noradrenaline from the sympathetic nerve endings.
	Water soluble portion of total alcoholic extract of the bark	Isolated frog heart	Increase in heart rate, amplitude and cardiac output (43).	Increased cardiac performance due to positive ionotropic and chronotropic effects.
	Aqueous extract of bark powder in doses of 30 mg/kg	Isolated rat atria	Substantial inotropic effect (45).	
	Aqueous extract of bark powder in doses of 30 mg/kg	Isolated rat atria	Positive inotropic effect which is abolished by propanolol and cocaine (46).	
	Aqueous extract, 150 mg/kg orally 10 days	Rats treated with aqueous extract and then subjectedto isoproterenol necrosis.	Reduction in heart rate and myocardial necrosis (47).	
	Aqueous and organic extracts of *T. arjuna*	Adult rat ventricular myocytes	The aqueous extract, not organic extracts, of *T. arjuna* exerted positive inotropy, accelerated myocyte relaxation and increased caffeine-induced contraction concentration-dependently (48).	Induced cardiotonic action via enhancing sarcoplasmic reticulum function, a unique action minimizing the occurrence of arrhythmias, making it a promising and relatively safe cardiotonic.
	Aqueous extract	Frogs heart in situ, hypodynamic frogs heart in situ and isolated perfused rabbits heart	Increased force of contraction (49).	
	Alcoholic extract of the bark	Dog	Enhances auricular and ventricular contraction (44).	Help in strengthening the heart muscles.
	50% aqueous ethanol extract (500 mg/kg,orally)	Isoproterenol induced chronic heart failure in rats.	Restoration of LV (dP/dt) max, LV (dP/dt) max/P LV(dP/dt) min and restoration of elevated LVEDP due to ISO challenge (12).	Both therapeutic and prophylactic treatments with *T. arjuna* caused overall enhancement of myocardial contractility and relaxation suggesting improvement in left ventricular dysfunction caused by ISO in CHF rats. Reduction in LVEDP implies that there is an increase in blood flow through the subendocardial region of the heart that bears the maximum brunt of the ischaemic insult due to ISO challenge.
	50% aqueous ethanol extract (500 mg/kg, orally)	Anesthetized rat having diabetic cardiomyopathy. *T. arjuna* therapy started after 8 weeks of STZ and given for 30 days.	Improved LVP, LV dP/dt max, LV dP/dt max, ratio of LV dP/dt max and LVP. LVEDP restored to normal (11).	An overall enhancement of myocardial contractility and relaxation, suggesting improvement in left ventricular dysfunction caused by STZ.
Effect on coronary flow	Aqueous extract 1–1024_g injected in the tube	Isolated rabbit heart, Langendorff's preparation	Increase in coronary flow (50).	Increased coronary flow makes it a good choice for CHF patients
	Aqueous extract	Isolated perfused rabbits heart	Increased coronary flow 3.4% at 400 µg only. (49).	

Note: All experiments were carried out with bark constituents of *Terminalia arjuna*.
Abbreviations: CHF – Congestive heart failure; LVP - Left ventricular functions; LVEDP- Left ventricular end diastolic pressure; LV (dP/dt) max- maximal rate of rise of left ventricular pressure; LV (dP/dt) min- maximal rate of fall of LVP (D); LV (dP/dt) max/LVP- maximal rate of rise of LVP divided by LVP; ISO- Isoproterenol; STZ streptozotocin.

(ROS and RNS) and antioxidant capacity (51). Proinflammatory cytokines specially, interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α), are capable of modulating cardiovascular function (52). TNFa has been implicated in the development of left ventricular dysfunction, left ventricular remodelling, increased cardiac myocyte apoptosis, the development of anorexia and cachexia, reduced skeletal muscle blood flow and endothelial dysfunction, activation of the inducible form of nitric oxide synthase (iNOS), b receptor uncoupling from adenylate cyclase, and other effects (53). Emerging evidence confirms the pivotal role of hyperlipemia, mainly elevated blood cholesterol, particularly LDL cholesterol and VLDL cholesterol in the development of atherosclerosis-related disease (54).

Dysfunction of the vascular endothelium is an early finding in the development of cardiovascular disease. Endothelin 1 has also been demonstrated to be associated with increased oxidative stress and endothelial dysfunction in humans (55). Apart from its direct vasomotor activity, ET-1 has been implicated in inflammatory processes within the vascular wall. Specifically, ET-1 in subnanomolar concentrations has been demonstrated to activate macrophages, resulting in the release of proinflammatory and chemotactic mediators, including TNF-a, IL-1, IL-6, and IL-8 which are of importance in the atherosclerotic process. Table IV compiles the various biochemical studies on *T. arjuna* related to molecular mechanisms affecting the cardiovascular system.

Cardiac histopathology

Histopathological examination of normal cardiac

TABLE IV : Experimental studies on *Terminalia arjuna* related to molecular mechanisms affecting cardiovascular functions.

Study pertaining	*Plant preparation, dosage and route*	*Animal model*	*Observations*	*Interpretation*
Antioxidant activities	*Terminalia arjuna* in the doses of 30 mg per tablet in amultimineral herbal formulation, abana, administered orally as a suspension.	Rats subjected to myocardial ischemia induced byisoproterenol and treated with abana.	The reversal of cardiac injury enzyme and improved heart mitochondrial uptake (56).	Enhanced the antioxidant defense against ISO-induced myocardial infarction in rats and exhibited cardioprotective property.
	Terminalia arjuna extract in doses of 5 mg/kg	In vitro study	Ameliorate glycation of Hb and exerts antioxidant effects (57).	Antioxidant effect leading to cardioprotection.
	Arjunolic acid derived from *Terminalia arjuna* bark extract 15 mg/kg, given intraperitoneally.	Rats subjected to isoproterenol induced myocardial necrosis	Arjunolic acid treated rats had significant diminished levels of cardiac injury enzymes and raised SOD, CAT, GPx and myeloperoxidase (58).	Cardioprotection of arjunolic acid pre and post treatment could possibly be due to the protective effect against the damage caused by myocardial necrosis.
	Alcoholic extract of *T. arjuna* (6.75 mg/kg)	Isoproterenol induced myocardial injury in rats	Increase in endogenous anti oxidants (GSH, SOD and Catalase) (59).	Prevents the myocardium rom isoproterenol induced of myocardial ischemic reperfusion injury by augmenting endogenous antioxidant compounds of the rat heart.
	Oral administration of *T. arjuna* for 12 weeks	Rabbit heart	In vivo ischemic reperfusion injury induced oxidative stress, tissue injury of heart and hemodynamic effects were prevented (60).	One or more of the constituents have cardiotonic (glycosides) or free radicle scavenger (tannins, flavones) properties.
	T. arjuna 90 mg/kg single dose	Male Wistar rats	Cardiac lipid peroxidation was reduced (61).	Detoxification of reactive oxygen species.
	Dried pulverized bark of *T. arjuna* (500 mg/kg)	Wistar Albino rat	Increase in baseline contents of thiobarbituric acid reactive substance (TBARS), SOD, GSH and CAT levels (62).	Better cardioprotection against oxidative stress associated with myocardial ischemic reperfusion injury.

	Aqueous extract of *Terminalia arjuna* bark 50 mg/kg orallyfor 1 week	Mice challenged with carbon tetrachloride, 1 ml/kg body weight liver and renal enzyme markers assessed	*T. arjuna* prevented the rise in liver injury enzymes, i.e., SGPT, ALP and TBARS and increased the levels of SOD, CAT, and GSH. Results were comparable to vitamin C group mice (63).	Protect the oxidant damage to the liver and kidney following carbon tetrachloride challenge in mice, indicating its endogenous antioxidant activity.
	Ethanolic extract of *Terminalia arjuna* bark in 400 mg/kg, post orally for 28 days	Single injection of *N*-nitrosodiethylamine-induced liver cancer in male rats treated with *Terminalia Arjuna.*	Decreased levels of lipid peroxidase and near normal levels of antioxidant enzymes-SOD, CAT and glutathione peroxidase (64).	Improved the antioxidant defenses thus preventing the free radical-mediated damage.
	Treatment with arjunolic acid (20 mg/kg) four days prior to Sodium arsenite intoxication	Experimental Mice	Decreased oxidative stress (65).	
	Ethanol extract of *T. arjuna*	Sodium floride induced oxidative stress in murine heart	Treatment prior to Sodium floride administration decreased oxidative stress (66).	
	Butanolic fraction of *T. arjuna*	Doxorubicin induced cardiotoxicity in male Wistar rats	Reduced oxidative stress (67).	Cardioprotection against oxidative stress
	Aqueous extract of *T. arjuna* (5 mg/kg)	Rats on Isoprenaline	Prevented isoprenaline induced oxidative stress and decline in antioxidant levels (68).	
	50% aqueous ethanol extract (500 mg/kg, orally)	Isoproterenol induced chronic heart failure in rats	Reduction in MDA level and significant improvement in GSH and SOD activity, thus maintained the rats at near normal status (12).	Reduces oxidative stress there by preventing the generation of free radicals.
	50% aqueous ethanol extract (500 mg/kg, orally)	Rats having diabetic cardiomyopathy. *T. arjuna* therapy started after 8 weeks of STZ and given for 30 days.	Markedly prevented all the STZ-induced alterations in the levels of MDA, SOD, CAT & GSH and maintained the rats at near normal status (10).	Improved the antioxidant defenses thus preventing the free radical-mediated damage.
Lipid lowering effects	*Terminalia arjuna* bark powder 250 mg/kg administered orally twice daily	Rabbits rendered hypercholesterolaemic by diet richin cholesterol	(a) Reduction in total cholesterol and triglycerides; (b) increase in HDL-cholesterol (4).	Lipid lowering effect leading to decreased risk of cardiovascular disease.
	T. arjuna (100 mg/kg, b.w.)	Triton and cholesterol fed rats	Lowering in lipids and protein levels of β-lipoproteins followed by an increase in high density lipoprotein-cholesterol (69).	
	Ethanolic extract of bark in 100–500 mg/kg dose orally	Rabbit fed high fat diet, in vivo study	(a) Reduces hyperlipidemia; (b) no change in HDL-chol (5).	
	Ethanolic extract of the *Terminalia arjuna* Wight & Arn., *Terminalia bellerica* Roxb. and *Terminalia chebula* Willd. administered orally	Rabbits fed hypercholesterolaemic diet and treated with respective *Terminalias* separately/ concurrently and sacrificed at the end of the experiment	*Terminalia arjuna* proved to be most potent hypolipidaemic agent, raised HDL-chol. and inhibited aortic atherosclerosis (70).	
	Treatment with arjunolic acid (20 mg/kg) four days prior to Sodium arsenite intoxication	Experimental Mice	(a) fall in the level of total cholesterol, triglycerides, and LDL-C (b) increased the level of HDL-C (65).	

	Ethanolic fraction of *T. arjuna* (100 and 200 mg/kg body weight)	Rabbits fed with high fat diet	(a) Decreased TC, LDL and TG levels and increases HDL (b) Lessens atherosclerotic lesion in aorta (71).	
	50% aqueous ethanol extract (500 mg/kg, orally) level of HDL-C (12).	Isoproterenol induced chronic heart failure in rats	(a) Fall in the level of total cholesterol, triglycerides, LDL-C, VLDL-C (b) Increased level of HDL-C [12].	
	50% aqueous ethanol extract (500 mg/kg, orally)	Rats having diabetic cardiomyopathy. *T. arjuna* therapy started after 8 weeks of STZ and given for 30 days.	(a) fall in the level of total cholesterol, triglycerides, and LDL-C (b) increased the level of HDL-C (10).	
Effects on inflammatory markers	50% aqueous ethanol extract (500 mg/kg, orally)	Rats having diabetic cardiomyopathy *T. arjuna* therapy started after 8 weeks of STZ and given for 30 days.	Metabolic changes induced by hyperglycemia lead to dysregulation of cytokine control, increasing their levels by an oxidative mechanism. *T. arjuna* reduced the raised serum TNF-α and IL6 to near normal levels in STZ-treated rats (10).	Anti-inflammatory activity of the bark extract
	50% aqueous ethanol extract (500 mg/kg orally)	Isoproterenol induced chronic heart failure in rats	Prophylactic and therapeutic treatment with *T. arjuna* reduced the elevated serum TNF-α to near normal levels in CHF rats (12).	Anti-inflammatory activity of the bark extract correlated with reduced myocardium Injury.
	Terminalia arjuna back powder (400 mg/kg, orally)	Albino rats	Reduced Formalin induced paw edema (72).	Anti-inflammatory activity.
Effects on Endothelin 1 levels	50% aqueous ethanol extract (500 mg/kg, orally)	Rats having diabetic cardiomyopathy. *T. arjuna* therapy started after 8 weeks of STZ and given for 30 days.	*T. arjuna* reduced the serum ET-1 to near normal levels in STZ-treated rats (11).	Improved the function of vascular endothelium thereby decreasing the pro inflammatory mediators adding to cardioprotective effect.
Effect on aortic prostaglandins	Bark powder 500 mg twice daily, orally in suspension form for 90 days.	Rabbit, in vivo study	Aortic ring PGE2 levels increased in rabbits receiving *Terminalia arjuna* (73).	Increased blood flow leading to improvement in cardiac functions.
Myocardial injury marker CK-MB	50% aqueous ethanol extract (500 mg/kg, orally)	Isoproterenol induced chronic heart failure in rats	Prophylactic and therapeutic treatment with *T. arjuna* almost restored the ISO-induced alterations of serum CK-MB to normal levels (11).	Indicates its action on maintaining membrane integrity thereby restricting the leakage of enzymes.
	Butanolic fraction of *T. arjuna*	Doxorubicin induced cardiotoxicity in male Wistar rats	Cotreatment with Doxorubicin reduced serum CK-MB levels (67).	

Note: All experiments were carried out with bark constituents of *Terminalia arjuna*.

Abbreviations: ALP, alkaline phosphatase; CAT, catalase; chol, cholesterol; CK-MB creatinine kinase- MB; ET1 – Endothelin 1; GPx, glutathione peroxidase; GSH, Glutathione reductase; Hb, haemoglobin; HDL, high density lipoprotein; IL6, interleukin 6; iNOS Inducible nitric oxide synthase; ISO Isoproterenol; LDL, low density lipoprotein; LPS, liposacharide; MDA malondialdehyde; MPO, myeloperoxidase; NO nitric oxide; PGE2, prostaglandin E2; SGPT, serum glutamic pyruvic transaminase; SOD, superoxide dismutase; STZ streptozotocin; TBARS, thiobarbituric acid reactive substances, TC, - total cholesterol; TG, triglyceride; TNF-á, tumour necrosis factor-á; VLDL, Very low density lipoprotein.

tissue demonstrates normal myofibrillar structure with striations, branched appearance and continuity with adjacent myofibrils. Cardiac tissues having cardiomyopathy shows widespread alterations in myocardial structure with subendocardial necrosis and myovacuolations. Treatment with *T. arjuna*, is seen to preserve myocardium and demonstrated marked improvement in various myocardial injuries (Table V).

Conclusion

The efficacy of *Terminalia arjuna* as a cardioprotective agent, a potent anti inflammatory and antioxidant preventing LDL cholesterol oxidation and its potential to reduce atherogenic lipid levels have been amply demonstrated in various experimental studies. Its molecular actions in different cells of the cardiovascular system are also reported. Its role in improving the autonomic control plays an important part in improving cardiovascular functions. This herbal drug with multiple beneficial effects without causing side effects can modulate the existing treatment strategies. However, there are some identified lacunae, like standardization of the 'drug', toxicity studies along with pharmacological interactions with other drugs and large multicentre randomized clinical trials, before its use by modern medicine is acceptable (14).

TABLE V : Histopathological studies of cardiac tissue on *Terminalia arjuna* therapy.

Study pertaining	*Plant preparation, dosage and route*	*Animal model*	*Observations and Interpretation*
Cardiac histopathology	Arjunolic acid derived from *Terminalia arjuna* bark extract 15 mg/kg, given intraperitoneally	Rats subjected to isoproterenol-induced myocardial ischemia and administered arjunolic acid both pre and post isoproterenol administration	Preserved myocardium thus confirming cardioprotection (58).
	Alcoholic extract of *T. arjuna*	Isoproterenol induced myocardial ischemic reperfusion injury in rats	Preserved myocardium (59).
	Treatment with arjunolic acid (20 mg/kg) four days prior to Sodium arsenite intoxication	Experimental Mice	Reduces injury due to arsenic administration and helps to maintain normal cardiac architecture (63).
	Butanolic fraction of *T. arjuna*	Doxorubicin induced cardiotoxicity in male Wistar rats	Cotreatment with Doxorubicin reduced histological alterations due to Doxorubicin (67).
	50% aqueous ethanol extract (500 mg/kg, orally)	Isoproterenol induced chronic heart failure in rats	Marked improvement in ISO-induced subendocardial necrosis, capillary dilatation and leucocyte infiltration confirming its cardioprotective actions (12).
	50% aqueous ethanol extract (500 mg/kg, orally)	Rats having diabetic cardiomyopathy. *T. arjuna* therapy started after 8 weeks of STZ and given for 30 days (11).	Marked improvement in STZ-induced subendocardial necrosis and vacuolation of the myocytes. Thus alleviated the STZ-induced cardiac injury, confirming its cardioprotective actions.

Note: All experiments were carried out with bark constituents of *Terminalia arjuna*.
Abbreviations: ISO- Isoproterenol, STZ streptozotocin.

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Original Article

Dynamics of Heart Rate Responses to Exercise in Normotensive Men

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Abstract

We evaluated cardiovascular responses to exercise in normotensive men according to resting systolic arterial pressure (SAP). Healthy men were split into two groups: G1- Subjects with at rest SAP between 120 and 130 mmHg (n=15) and G2- Subjects with at rest SAP < 120 mmHg (n=20). These conventions were necessary in order to check the recovery of the autonomic nervous system after exercise. Subjects performed physical exercise on a treadmill with intensity equivalent to 60% of V_{max}. HR (heart rate) variability was recorded in the following stages: at rest, the 10-minute periods before exercise, during exercise and the 60 minutes periods after exercise. During recovery from exercise G2 presented delayed recovery compared to G1 based on SDNN, RMSSD, pNN50, RRTri, HF, LF, SD1 and SD2 indices. HR recovery in the 3rd minute was higher in G1. Body mass index was greater in G1. In conclusion, normotensive men with SAP below 120 mmHg established delayed HRV recovery following an acute bout of aerobic exercise compared to normotensive men.

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Introduction

Cardiovascular responses during recovery from exercise are necessary in sports and exercise physiology to understand the mechanisms by which the autonomic nervous system (ANS) responds to

exercise. These mechanisms provide information on the autonomic function of the patient to the medical practitioner (1).

Heart rate recovery (HRR) is a simple and non-invasive method that analyses heart rate autonomic recovery after exercise (2). An alternative meticulously documented method that evaluates heart rate control is heart rate variability (HRV) (3), which expresses fluctuations in consecutive heart beats.

Studies reveal that a high HRV reflects the robustness of cardiovascular regulation controlling heart rate through forward and feedback modulations. HRV indicates the status of cardiovascular homeostatic mechanisms acting through sympathetic and parasympathetic branches of the ANS (4).

Previous studies established that reduced HRV reflects sympathetic hyperactivity (5), malignant ventricular arrhythmias (6), cardiomyocyte damage (7), coronary constriction (8) and mortality risks (5). Intrinsic deficiency causes the individual to be less able to endure a physiological disturbance, predisposing them to risks from all causes of death (5).

Exercise is a physiological stimulus that can tolerate physiological changes through shifting the autonomic balance (9). During aerobic exercise —in the first few minutes, vagal withdrawal occurs and then later with elevated exercise intensity, sympathetic activation ensues. Directly after termination of exercise vagal re-entry followed by sympathetic withdrawal; renewal of the autonomic balance is permissible (10).

Studies reveal that cardiac autonomic restoration is necessary for cardiovascular well-being, since a delay in vagal reactivation and a persistence of sympathetic activation may increase cardiac ectopic movement in the post-exercise period, increasing the risk of cardiovascular pathologies (11).

Studies with seemingly healthy subjects within normal arterial pressure range, a change in cardiovascular responses and HRV, signifying possible autonomic imbalance, could support the identification of risk factors for cardiovascular pathology and anticipate likely causes of these pathologies. Consequently, allowing the progression of therapies for restoration of the autonomic imbalance.

Regarding the 2007 Practice Guidelines for the Management of Arterial Hypertension (12), classification of blood pressure in adults based on systolic arterial pressure (SAP) is optimum for SAP lower than 120 mmHg, normal for 120 to 129 mmHg and higher than normal for 130 to 139 mmHg. Yet, the expected threshold level for hypertension is variable and reliant on the cardiovascular risk of each separate subject (12).

Further research is imperative using a healthy cohort that excludes cardiorespiratory diseases, and could conceivably contribute to the identification of pathological predispositions even with normal cardiovascular values. So, considering that the hemodynamic response to the exercise can provide evidence that is undetected in the resting state (13), the objective was to study cardiovascular responses to exercise in normotensive men with unalike resting SAP.

Methods

Study population

This is a prospective analytical study. The subjects participating in the study were healthy males - all non-smokers, aged between 18 and 35 years old, and were divided into two groups: G1-Subjects with SAP between 120 mmHg and 130 mmHg (n=15) and G2-Subjects with SAP between 110 mmHg and 120 mmHg (n=20). All subjects were physically active, performing moderate to intense physical activity at least 1 hour for 3 days per week, but not athletic. Subjects were informed about the procedures and the objectives of the study and gave confidential written informed consent. All procedures were approved by the Ethics Committee in Research of the Faculty of Sciences of the Universidade Estadual Paulista (No. CEP-2011-385), and were in accordance with Resolution 466/2012 National Health 10/10/1996. We excluded subjects with body mass index (BMI)

>30 kg/m^2; at rest SAP>130 mmHg and at rest diastolic arterial pressure (DAP) >90 mmHg; resting heart rate (HR) beyond 100 beats per minute, cardiovascular, respiratory and neurological disorders. Volunteers under medication(s) that influence HRV were also excluded.

Initial evaluation

Baseline information was collected: age, gender, mass, height and body mass index (BMI). Mass was determined using a digital scale (W 200/5, Welmy, Brazil) with a precision of 0.1 kg. Height was determined using a stadiometer (ES 2020, Sanny, Brazil) with a precision of 0.1 cm and 220 cm of extension. BMI was calculated as mass/height^2, with mass in kilograms and height in meters.

Cardiovascular variables

Measurements of blood pressure were monitored by the same evaluator indirectly using a stethoscope (Littmann, St. Paul, USA) and aneroid sphygmomanometer (WelchAllyn, Germany) on the left arm. The subjects were seated according to the criteria established by the VI Brazilian Guidelines on Hypertension (13). HR was obtained by a heart rate monitor – Polar RS800CX (Polar Electro, Kempele, Finland) (14). HRR was computed as the difference between the heart rate at the cessation of exercise and heart rate at 1 min (HRR1) or 3 min (HRR3) after termination of the exercise (1).

HRV analysis

We enforced directives from the Task Force publication (15). Instantaneous RR intervals (RRi) were logged with a digital telemetry system, consisting of a transmitter placed on the patient's chest and a HR monitor (Polar® RS800CX; Polar Electro Oy, Kempele, Finland). This system detects ventricular depolarization, corresponding to the R wave on the electrocardiogram, at a sampling rate of 1kHz and has been previously validated (16). They were downloaded to the Polar Precision Performance program (v.3.0, Polar Electro, Finland). The software enabled the visualization of HR and the extraction of a cardiac period (RR interval) file in "txt" format. After digital filtering accompanied with manual filtering for the elimination of premature ectopic beats and artefacts, 256 stationary RR intervals were necessary for the data analysis. Only series with sinus rhythm exceeding 95% were included in the study (17). HRV was examined in the period before the exercise (M1); during exercise (M2: 15-20 min during exercise; M3: 25-30 min during exercise) and after the period of acute exercise (M4: 5-10 minutes after exercise cessation; M5: 15-20 minutes after exercise cessation; M6: 25-30 minutes after exercise cessation; M7: 35-40 minutes after exercise cessation; M8: 45-50 minutes after exercise cessation; M9: 55-60 minutes after exercise cessation). All indices were evaluated using 256 fixed successive and stationary RR intervals.

Time and frequency domain indices of HRV

For HRV analysis in the frequency domain we approved the spectral components of low frequency (LF: 0.04 to 0.15 Hz) and high frequency (HF: 0.15 to 0.40 Hz) in absolute units (ms^2) and LF/HF ratio. The spectral analysis was calculated with the Fast Fourier Transform (FFT) algorithm (18).

Time domain analysis was monitored by the SDNN (average standard deviation of normal RR intervals), pNN50 (percentage of adjacent RR intervals lasting more difference than 50 ms) and RMSSD (square root of the average square differences between normal adjacent RR intervals).

HRV analysis was also performed by geometrical methods in time domain: RRtri (Triangular index), TINN (triangular interpolation of NN interval histogram) and Poincaré plot (SD1 and SD2). The RRtri was computed from the construction of a density histogram of RR intervals, which contains the horizontal axis of all possible values of RR intervals measured on a discrete scale with 7.8125 millisecond boxes (1/128 seconds) and on the vertical axis, the frequency with which each occurred. The union of points of the histogram columns produced a triangular shape. The RRtri was obtained by dividing the total number of RR intervals used to construct the histogram by their modal frequency — the RR interval value that most frequently appeared on RR.

The TINN comprises of the measure of the base of a triangle. The method of least squares is enforced to determine the triangle. The RRtri and the TINN express the overall variability of RR intervals (15). The Poincaré plot is a map of points in Cartesian coordinates, constructed from the values of RR intervals obtained, where each point is represented on the x-axis (horizontal) by the previous normal RR interval, and on y-axis (vertical), by the following RR interval. For quantitative analysis of the plot, an ellipse was fitted to the points of the chart, with the center determined by the average RR intervals, and the SD1 indexes were calculated to measure the standard deviation of the distances of the points to the diagonal y = x, and SD2 measures the standard deviation of the distances of points to the line y = –x + RRm, where RRm is the average of RR intervals. The SD1 is an index of immediate recording of the variability of fluctuations and represents parasympathetic modulation, whilst the index SD2 represents HRV in long-term records, and reflects Conconithe overall variability (19). For analysis of linear indices in the frequency and time domain we used the Kubios HRV® analysis software (20).

Aerobic potency measurement

For exercise intensity training, we applied 60% of V_{max} found in the progressive test through Conconi threshold, which has been proposed to estimate the anaerobic threshold for identifying the HR deflection point (HRDP) using a progressive test with the use of the D_{max} method (21).

Next, the subjects endured a systematic progressive treadmill test (TPEE; Inbrasport ATL 2000) with initial speed of 8 km / hour which incremented 1 km / hour every 2 minutes until exhaustion or onset of clinical changes that prevented the continuity of test, such as dizziness, shortness of breath or “air hunger” (22, 23). The inclination of the treadmill remained fixed at 1%, since this condition reflects more precisely the energy cost of running outdoors. We recognized volunteers that reached up to 90% of maximal HR (24).

For the identification of HRDF, the matched HR and speed points were plotted. Next the values were adjusted by means of a third-degree polynomial function and a linear equation of the first degree, which are data derived from each individual. Then, the difference of the values of HR obtained through the aforesaid equations were calculated and a curve was calculated with these values. We considered the PDFC as the highest value before a change of direction in the curve (24).

Exercise protocol

Data collection originated in the same soundproofed room for all subjects. The temperature was between 21°C and 25°C and the relative humidity between 50% and 60%. Subjects were instructed not to ingest alcohol, caffeine or other ANS stimulants for 24 hours before the evaluation. Data sets were collected on an individual basis, continuously between 18:00 and 21:00 to standardize circadian influences. All procedures necessary for the data collection were explained to each subject discretely, and the subjects were instructed to remain at rest and avoid speaking during the collection.

Volunteers performed physical exercise on a treadmill with intensity of 6.0 km/hour + 1% slope in the first five minutes for physically “warming up”, followed by 25 minutes with intensity equivalent to 60% of V_{max} according to the Conconi threshold with identical slope.

Statistical analysis

Standard statistical techniques were enforced for the calculation of means and standard deviations. Normal Gaussian distribution of the data was verified by the Shapiro-Wilk goodness-of-fit test (z value >1.0) (25). To compare variables between groups, for parametric distributions we computed non-paired Student t-test and for non-parametric distributions we applied Mann-Whitney test.

To equate variables in the exercise protocol (control at rest, during exercise and post-exercise) two-way repeated measures analysis of variance was applied, then the Bonferroni post-test for parametric distributions and Friedman followed by Dunn’s test for non-parametric distributions. Differences were

considered significant when the probability of a Type I error was less than 5% ($p<0.05$).

To assess correlation between HRV indices and HRR we enforced Pearson correlation coefficient analysis for parametric distribution and Spearman's Rank correlation coefficient analysis for non-parametric distributions. Strong correlation was assumed for $r>0.5$, moderate correlation for r between 0.3 and 0.5.

To quantify the magnitude of difference between groups and moments, the effect size was calculated using Cohen's *d* for significant differences. Large effect sized was considered for values ≥ 0.9, medium for values between 0.9 and 0.5 and small between 0.5 and 0.25 (19). We used the Software Biostat® 2009 Professional 5.8.4 (Analysis Soft, Walnut, California, United States of America).

Results

Table I illustrates descriptive statistics regarding mass, height and BMI. We detected significant increased BMI and mass in G1. HRR in the first minute was unaffected between groups, however, HRR in the third minute was significantly faster in the G2 (Table I). As expected, at rest SAP ($p<0.0001$) and at rest DAP were significantly higher in the G1 ($p<0.0001$).

We detected that during exercise there was a time effect for all variables ($p<0.0001$) except LF/HF ratio ($p=0.481$). No group effect was achieved regarding RRTri ($p=0.196$), TINN ($p=0.422$), HF ($p=0.599$), LF/HF ratio ($p=0.401$), SD1 ($p=0.522$) and SD2 ($p=0.464$), and there was a group effect in LF index ($p=0.039$). Regarding the moment and group interaction we noted significant differences in the LF ($p=0.049$); for the other indices there were no interactions (RRTri, $p=0.496$; TINN, $p=0.211$; HF, $p=0.616$; LF / HF ratio, $p=0.409$; SD1, $p=0.438$ and; SD2, $p=0.660$) (Fig. 1).

During exercise (M2 and M3) we detected decreases in LF and HF compared to at rest (M1) in both groups and the LF/HF ratio significantly increased compared to at rest in both groups. Regarding the Poincaré plot indices, in both groups we noticed a significant reduction in SD1 and SD2 indices during exercise (M2 and M3) compared to at rest (M1) (Fig. 1).

Regarding the time domain indices during exercise (M2 and M3) we detected no group effect (Mean RR, $p=0.155$; SDNN, $p=0.464$; RMSSD, $p=0.524$; pNN50, $p=0.577$). There were no time or group interactions for mean RR intervals, $p=0.180$; SDNN, $p=0.634$; RMSSD, $p=0.438$ and; pNN50, $p=0.577$ (Fig. 2).

During recovery from exercise, there was a time effect for most indices ($p<0.0001$), except TINN ($p=0.478$) and LF/HF ratio ($p=0.138$). No group effect was detected for the indices (Mean RR, $p=0.466$; SDNN, $p=0.920$; RMSSD, $p=0.637$; pNN50, $p=0.678$; RRTri, $p=0.987$; TINN, $p=0.083$; LF, $p=0.386$; HF, $p=0.708$; LF/HF ratio, $p=0.075$; SD1, $p=0.714$; SD2, $p=0.826$) and there were no time or group interactions (Mean RR, $p=0.264$; SDNN, $p=0.868$; rMSSD, $p=0.274$; pNN50, $p=0.311$; RRTri, $p=0.826$; TINN $p=0.459$; LF, $p=0.2$; HF, $p=0.543$; LF/HF ratio, $p=0.581$; SD1,

TABLE I: Descriptive statistics of mass, height and body mass index (BMI), heart rate recovery at the first (HRR1) and third minute (HRR3) of the volunteers divided by group. m: meters; bpm: beats per minute; kg: kilograms; p indicates difference betweenPre VS Post (5-10 min after exercise cessation).G1: Group with SAP > 120 mmHg; G2: Group with SAP < 120 mmHg. Mean±standard deviation (minimum-maximum).

Variables	*G1*	*G2*	*p*	*Cohen's*	*Effect size*
Age (years)	21.37±2.9 (19-24)	22.05±3.2 (18-29)	0.22	0.22	Small
Height (m)	1.76±0.05 (1.7-1.85)	1.75±0.09 (1.6-1.88)	0.26	0.13	Small
Mass (kg)	79.74±8.59 (65.7-97)	72.41±10.01 (57-89.35)	0.01	0.78	Medium
BMI (kg/m^2)	25.55±7.23 (26.25-21.36)	23.56±1.99 (19.33-26.81)	0.009	0.37	Small
HRR1 (bpm)	22.85±12.8 (2-48)	17.8±12.7 (4-57)	0.13	0.39	Small
HRR3 (bpm)	42.85±15.52 (24-87)	32.9±11.15 (21-58)	0.03	0.73	Medium

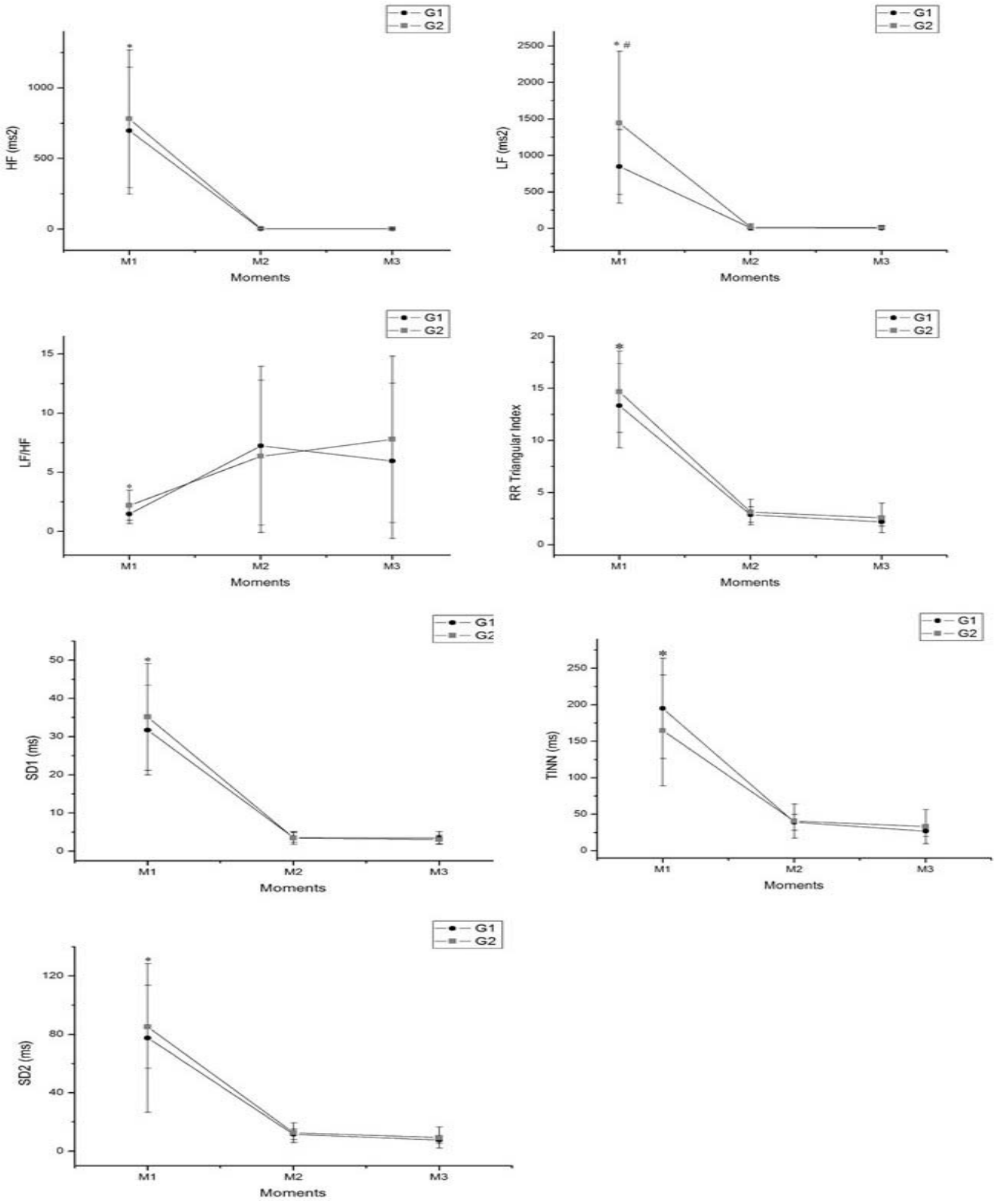

Fig. 1 : Frequency domain and geometric analysis of HRV before and during exercise. M1: Control at rest; M2: 10-15 minutes after start of exercise; M3: 25-30 minutes after start of exercise; G1: Group with SAP > 120 mmHg; G2: Group with SAP < 120 mmHg; LF: low frequency; HF: high frequency; LF/HF: low frequency/high frequency ratio; ms: milliseconds; SD1: standard deviation of the instantaneous variability of the beat-to beat heart rate; SD2: standard deviation of long-term continuous RR interval variability; TINN: Triangular interpolation of RR interval histogram; *p<0.05 Vs. M2 and M3; #p<0.05: G1 vs. G2.

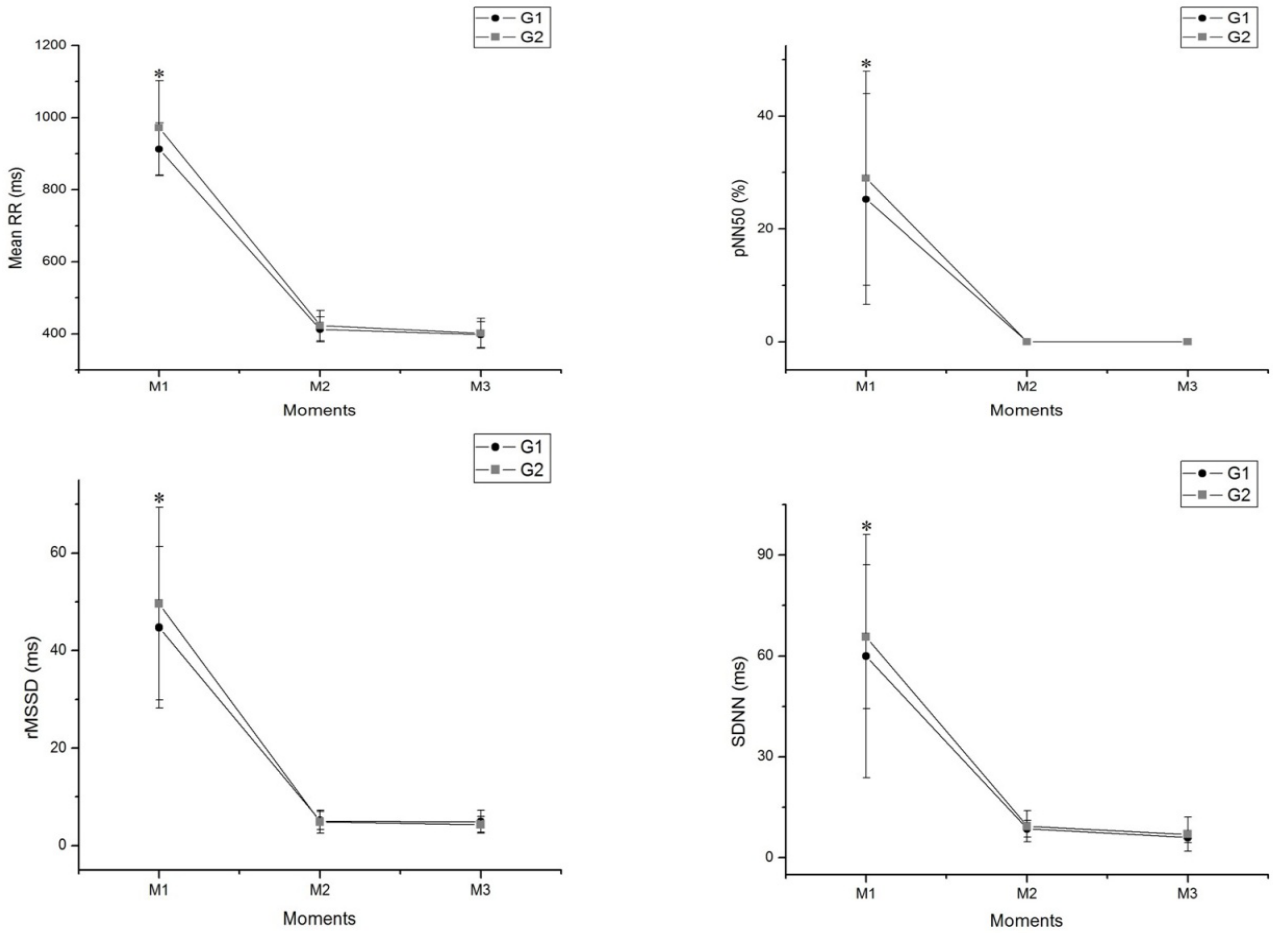

Fig. 2 : Time domain analysis of HRV before and during exercise. M1: Control at rest; M2: 10-15 minutes after start of exercise; M3: 25-30 minutes after start of exercise; G1: Group with SAP > 120 mmHg; G2: Group with SAP < 120 mmHg; pNN50: the percentage of adjacent RR intervals with a difference of duration greater than 50 ms; RMSSD: root-mean square of differences between adjacent normal RR intervals in a time interval; ms: milliseconds; SDNN: Average standard deviation of normal RR intervals; *p<0.05 Vs. M2 and M3.

p=0.452; SD2, p=0.867) (Fig. 3 and Fig. 4).

The RRTri index was lessened in M4 compared to M1 in G1 whereas in G2 it was reduced in M4 and M5 compared to M1 (Fig. 3). In both groups LF was diminished in M1 compared to M4 and M5. HF was narrowed in M1 compared to M4 and M5 in G2 and it was declined in M4 compared to M1 in G1. The LF/HF ratio was increased in M4 compared to M1 in both groups. Concerning the SD1 index, it was lessened in M4 and M5 compared to M1 in G1 while it declined in M4, M5 and M6 compared to M1 in G1. The SD2 index was diminishing in M4 and M5 compared to M1 in G2 (Fig. 3).

The mean RR interval was statistically reduced in all cases compared to M1 in G1, while in G2 it was reduced in M4, M5, M6 and M7 compared to M1. RMSSD and pNN50 indices were decreased in M4 and M5 compared to M1 in G1 and it declined in M4, M5 and M6 compared to M1 in G2. Regarding SDNN we found a reduction in M4 and M5 compared to M1 in G2 (Fig. 4).

Table II demonstrates statistical correlation of HRR1 and HRR3 with HRV indices at rest. We recognized no significant correlation in the G2. Yet, HRR3 presented significant negative correlation with RMSSD, pNN50, HF and SD1.

Table III displays correlation between BMI and cardiovascular variables before and after exercise (5 to 10 minutes after exercise cessation). Significant negative correlation of BMI with RMSSD and pNN50

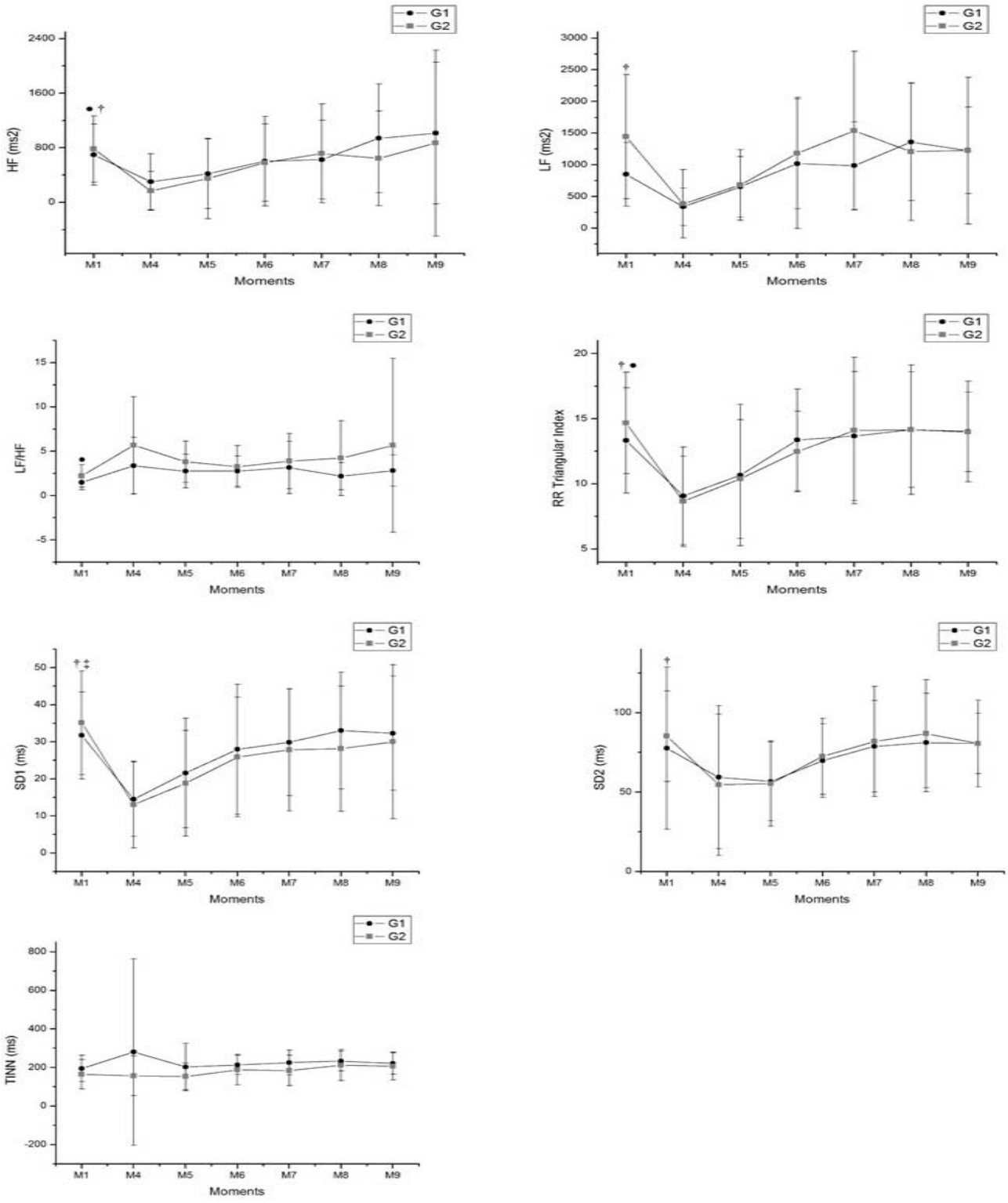

Fig. 3 : Frequency domain and geometric analysis of HRV before and after exercise. M1: Control at rest; M4: 5-10 minutes after exercise cessation; M5: 15-20 minutes after exercise cessation; M6: 25-30 minutes after exercise cessation; M7: 35-40 minutes after exercise cessation; M8: 45-50 minutes after exercise cessation; M9: 55-60 minutes after exercise cessation; G1: Group with SAP > 120 mmHg; G2: Group with SAP < 120 mmHg; LF: low frequency; HF: high frequency; LF/HF: low frequency/ high frequency ratio; ms: milliseconds; SD1: standard deviation of the instantaneous variability of the beat-to beat heart rate; SD2: standard deviation of long-term continuous RR interval variability; TINN: Triangular interpolation of RR interval histogram; $^{\dagger}p<0.05$ Vs. M4 and M5 in G2; $^{\%}p<0.05$ Vs. M4 in G1; $^{\dagger}p<0.05$ Vs. M4 and M5 in G1 and G2 for LF and in G2 for HF; $^{\%}p<0.05$ Vs. M4 in G1 for HF (ms2) and LF/HF ratio.

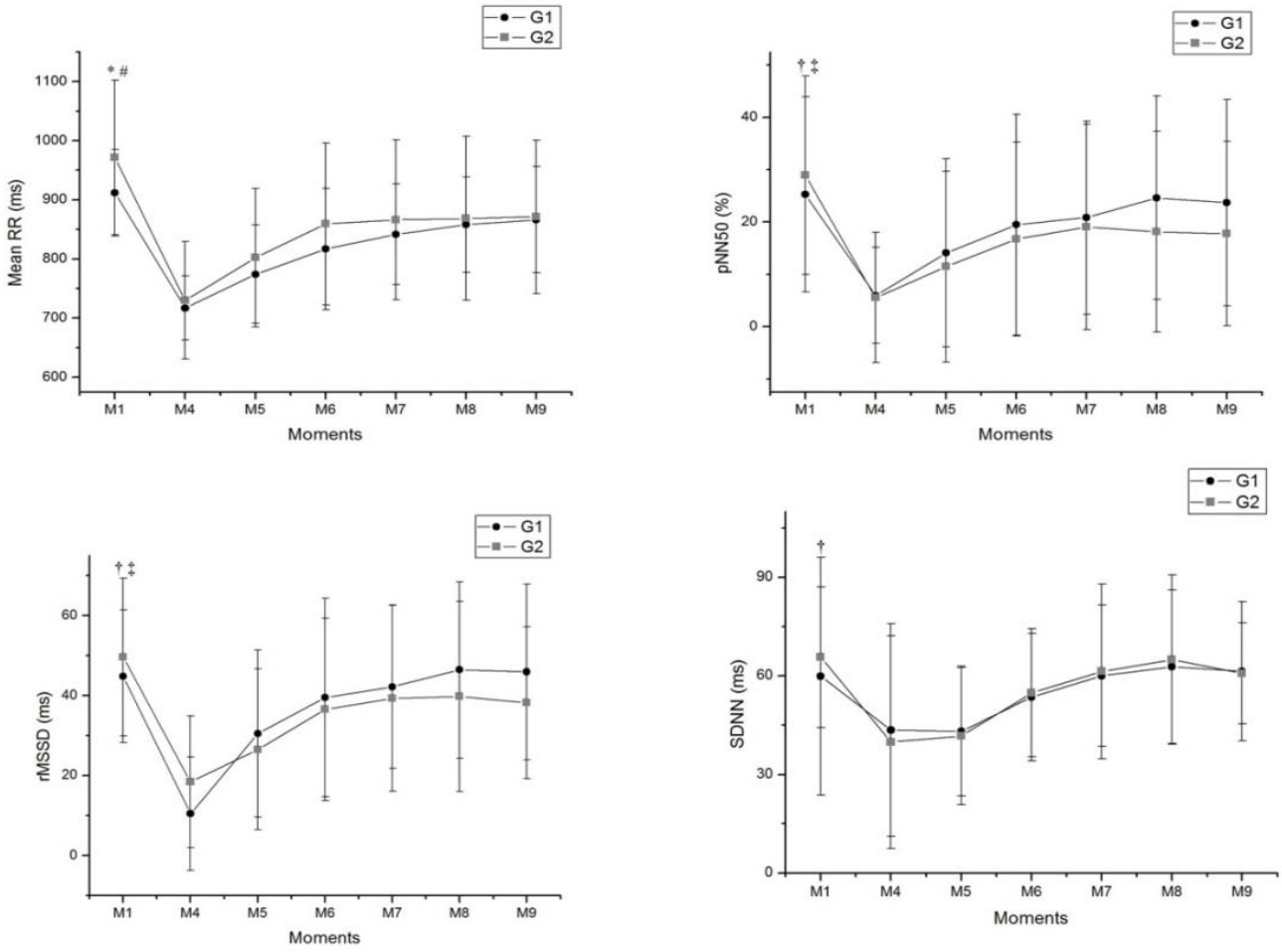

Fig. 4 : Time domain analysis of HRV before and after exercise. M1: Control at rest; M4: 5-10 minutes after exercise cessation; M5: 15-20 minutes after exercise cessation; M6: 25-30 minutes after exercise cessation; M7: 35-40 minutes after exercise cessation; M8: 45-50 minutes after exercise cessation; M9: 55-60 minutes after exercise cessation; G1: Group with SAP > 120 mmHg; G2: Group with SAP < 120 mmHg;*p<0.05 Vs. M4, M5, M6, M7, M8 and M9 in G1; #p<0.05 Vs. M4, M5, M6 and M7 in G2; †p<0.05 Vs. M4 and M5 in G2 for SDNN and in G1 for RMSSD and pNN50; ‡p<0.05 Vs. M4, M5 and M6 in G2 for RMSSD and pNN50; %p<0.05 Vs. M4 in G1.

TABLE II : Correlation of HRR1 and HRR3 with HRV indices at rest and during recovery from exercise. G1: Group with SAP > 120 mmHg; G2: Group with SAP < 120 mmHg.

G1	*HRR1*		*HRR3*	
HRV Index	*r*	*p*	*r*	*p*
RMSSD	–0.52	0.057	–0.58	0.02
pNN50	–0.47	0.09	–0.54	0.04
HF	–0.47	0.08	–0.55	0.04
SD1	–0.51	0.58	–0.58	0.02
G2	*HRR1*		*HRR3*	
HRV Index	*r*	*p*	*r*	*p*
RMSSD	–0.35	0.13	–0.34	0.051
pNN50	0.08	0.7	–0.3	0.19
HF	–0.044	0.85	–0.41	0.07
SD1	–0.004	0.98	–0.35	0.13

TABLE III : Correlation between BMI and cardiovascular parameters at rest (pre) and during recovery from exercise (post; 5–10 min after exercise cessation). G1: Group with SAP > 120 mmHg; G2: Group with SAP < 120 mmHg.

	G1		*G2*	
Variable	*r*	*p*	*r*	*p*
RMSSD pre	–0.64	0.01	0.07	0.74
pNN50 pre	0.48	0.08	–0.06	0.77
HF pre	0.38	0.18	0.12	0.6
SD1 pre	0.45	0.1	–0.035	0.88
RMSSD post	0.32	0.26	–0.55	0.01
pNN50 post	0.31	0.26	–0.53	0.01
HF post	0.48	0.08	–0.36	0.12
SD1 post	0.32	0.26	–0.4	0.051
HRR1	0.3	0.28	–0.12	0.6
HRR3	0.19	0.49	–0.22	0.34

after exercise was observed in the G2. BMI was negatively correlated with at rest RMSSD in the G1.

Discussion

We predicted estimating the HRV responses to exercise in normotensive physically active men divided into two groups (below 120 mmHg and between 120 and 130 mmHg). From our data, males with reduced SAP achieved delayed HRV recovery from exercise and decreased HRR compared to normotensive men with higher SAP. HRR was unconnected with rest parasympathetic heart rate regulation, BMI was higher in men with lower SAP and it was negatively correlated with rest vagal control in the identical group, which advocates that the slower recovery of HRV is attributable to increased BMI in the population being studied.

In this study, HRR in the first minute was unchanged between groups. Yet, HRR in the third minute was higher in the normotensive men with higher SAP. This reveals that the subjects with higher SAP experienced better autonomic read justment after exercise.

HRR is split into two stages; the first 60 seconds corresponds to the fast recovery, indicating an immediate and rapid reduction in heart rate. The slow stage includes the period after the first minute (26). Increased HRR is associated to enhanced physical fitness. Prior studies have accomplished faster HRR in physically trained individuals and athletes compared to sedentary control subjects (27, 28).

Furthermore, the slow phase was reported to be influenced by exercise intensity (27). Considering that we investigated a moderate intensity exercise, we are unable to extrapolate our data to further intense exercises.

Based on statistical analysis, normotensive men with lower SAP presented delayed recovery of mean RR interval, SDNN, pNN50, RMSSD, HF, RRTri, SD1 and SD2 indices from exercise compared to the group with higher at rest SAP. In this state, we specify that the parasympathetic modulation of HR is involved in the delayed recovery of HRV from exercise in the volunteers with lower SAP.

Impaired responses of parasympathetic HR regulation to exercise were testified in subjects with cardiovascular disorders and a predictor of mortality (29, 30). In a study by Cole et al. (29), it was reinforced that HRR could be enforced as an indicator of vagal heart rate modulation. The authors conveyed decreased values in patients with cardiovascular disorders. Myers et al. (30) assessed the association between cardiovascular parameters in response to exercise and prediction with cardiovascular risk. The authors revealed that reduced HRR was related to cardiovascular risk — the lower the HRR, the higher the cardiovascular risk.

We anticipated that the sympatho-vagal balance would be involved in the variance between both groups. Nonetheless, based on our data, there was no group effect regarding LF/HF ratio during recovery from exercise. This outcome excludes the involvement of the sympatho-vagal balance component of heart rate modulation in the delayed HRV recovery after aerobic exercise.

To confirm whether HRR was associated with at rest parasympathetic heart rate modulation we began a statistical correlation between the variables. The group with higher SAP offered negative correlation with all parasympathetic HRV indices, indicating that increased HRR in the third minute was associated with lower HRV in normotensive men with higher SAP.

Contrariwise, the slower recovery of HRV from exercise in men with lower SAP does not guarantee that this population endures risk factors to developing cardiovascular disorders. The individuals with higher SAP had elevated HRR, which issymptomatic of improved adjustment of the ANS in response to exercise (1). From both groups all volunteers were healthy, physically active and had no medical history of cardiorespiratory illnesses.

A point to highlight is concerning BMI and body mass. In this study, both variables were significantly higher in men with lower SAP, and this was accompanied by delayed HRV during recovery from

exercise. BMI was associated with HRR after exercise in healthy adults. The volunteers were divided into normal BMI, overweight and obese (31). It was noticed that BMI was inversely related with HRR after exercise. But it was recognized that subjects with impaired HRV recovery presented diminished exercise capacity than those with faster recovery.

The aforesaid study supports our data and may clarify our results, since the group with lower SAP presented higher BMI and slower HRR. It is rational to suggest that BMI was involved in the delayed recovery of HRV after exercise.

Also, we commenced a statistical correlation between BMI and cardiovascular parameters before and after exercise to further examine the role of BMI. In the group with lower SAP we detected that BMI inversely influenced parasympathetic heart rate modulation during recovery from exercise. As an alternative, BMI negatively influenced at rest HRV in the group with higher SAP. Together, it is proposed that BMI influences HRV during recovery from exercise in male normotensive subjects with lower SAP.

Therefore as a principle outcome, normotensive subjects with lower SAP (less than 120 mmHg) presented delayed HRV recovery and slower HRR to exercise compared to normotensive subjects with higher SAP (between 120 and 130 mmHg). Based on our data, we suggest that males with SAP ranging between 120 and 130 mmHg have better autonomic adjustment after moderate exercise. Our data reveals an important issue related to at rest SAP and its influence on heart rate dynamic responses to exercise.

As a limitation of the study, while every possible attention was taken regarding the selection and filtering of HRV data, we can not exclude possible misapplications of the stationarity of heart rate fluctuations during exercise and the post-exercise phases.

Conclusion

Normotensive males with lower SAP present slower recovery of HRV during recovery from exercise compared to normotensive males with higher SAP. We conclude that this is attributable to higher BMI in this specific population.

Acknowledgements

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Conflict of interest statements

The authors declare that there is no conflict of interests regarding the publication of this article.

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Original Article

CD14 Positive Selection Displays an Edge in the Isolation of Macrophages from Induced Sputum of COPD Patients Using Immunobead Technology

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Abstract

Objectives: Macrophages plays an important role in the pathophysiology of COPD. Sputum induction is a safe and non-invasive method for evaluation of airway inflammation in COPD. The present study aims to evaluate the yield of macrophage isolation from induced sputum of COPD patients by using commercially available immunomagnetic bead based approaches.

Methods: Sputum induction was done in COPD patients (n=13). Cell pellets obtained after the processing of sputum samples (n=11) were subjected to different isolation kits. Macrophages were isolated from cell pellet using positive and negative selection strategies. CD66abce microbead kit, PAN monocyte isolation kit and CD14 microbeads were used in three different combinations for obtaining pure and enriched macrophages from sputum.

Results: The results obtained from all sets of experiments were compared and per cent purity and enrichment of macrophages was calculated. CD14 positive selection kit when used for isolation yielded maximum enrichment (> 20-folds) and yielded greater purity as compared to negative selection strategies.

Conclusion: CD14 microbeads based positive selection appeared to be the method of choice for isolating macrophages from induced sputum of COPD patients for various downstream experimental processes.

Introduction

Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory disease of the lungs to smoke, dusts and other air pollutants (1). It is characterized by increased numbers of macrophages, neutrophils and cytotoxic T-lymphocytes in airways and the lung parenchyma (2). The number of macrophages are increased in patients of COPD. Although macrophages appear to play a pivotal role in the pathophysiology of COPD as these cells may be activated by cigarette smoke to release several cytokines and chemokines (3), our present knowledge

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about the patho-physiological basis of COPD is limited (4). There are several difficulties in exploring the underlying process of COPD. The small airways and surrounding lung parenchyma which are the sites for functional inflammatory response associated with COPD are difficult to assess as they are situated in the lung periphery. Sputum induction is currently used as a direct, non-invasive method for the evaluation of airway inflammation in COPD (5). Induction of sputum is relatively safe and well-tolerated in patients with advanced stages of COPD or exacerbations (6). It provides an alternative to collecting the expectorated sputum or performing fiberoptic bronchoscopy. With sputum induction, samples can be obtained from the lower airways with minimal discomfort to the patient and is also suitable for repeated measures in most patients (7). The proportion of viable cells is higher in induced sputum as compared to the spontaneously generated sputum (8, 9). Thus, it has become a well validated research tool and is used as a diagnostic technique for evaluating a variety of indices of inflammation. It is inexpensive and is preferred by patients and thus represents an alternative to standard methods of sampling the airways (10). Although macrophages isolated from induced sputum appear to be a useful model for studying the pathophysiological basis of COPD, different macrophage subpopulations are reportedly present in the lung parenchyma (11, 12). Furthermore, sputum contains heterogeneous population of cells. To isolate the target population, remaining contaminating cells have to be removed. To this effect, it remains as yet to be examined as to which immune-epitope may be used for isolation of macrophages from sputum samples in highly precise and specific manner. In the present study, we have examined and compared among different immune-epitope based isolation of macrophages from sputum sample of COPD patients, as shown in Figure 1, and observed that CD14 based magnetic separation indeed yielded highly pure population of macrophages.

Materials and Methods

Details of the patients

COPD patients (n=13, aged 30-70 years) recruited from Department of Pulmonary and Sleep Medicine, All India Institute of Medical Sciences (AIIMS), New Delhi, were either smokers (n=5) or ex-smokers (n=8) having a smoking history of more than 10 pack-years. Patients who ceased smoking for more than 1 year were considered as ex-smokers. Table I provides the details of the patients. Stable COPD patients (stages 2 and 3) according to the GOLD guidelines (13) with an evidence of airflow limitation on spirometry FEV_1/forced vital capacity (FVC) ratio of <70% were included in the present study. Patients who had suffered from exacerbation and/or those who had taken steroids, had a history of any active inflammatory disease, had a lung disorder besides COPD were excluded from the study. Written informed consent was obtained from all patients. The study was approved by the Ethics Committee of AIIMS, New Delhi.

Sputum induction and processing

Sputum induction in patients (n=13) was performed using Ultrasonic Nebuliser, Omron NE-U17 (Omron Healthcare Co. Ltd, Japan). Only 11 subjects could successfully follow the given instructions as described previously (7). Briefly, three FEV_1 manoeuvres were measured 15 minutes after inhalation of 200 µg salbutamol. Highest value was taken as baseline. Subjects were then instructed to inhale freshly prepared hypertonic saline (4%, w/v) for a total duration of 15 minutes. In initial experiments patients could not expectorate adequate amount of sputum even after repeating the procedure three times with 2-3% (w/v) hypertonic saline and 4% hypertonic saline was found to be the optimal for induction. Again, in initial experiments with a higher concentration (5%, w/v) patients had bronchoconstriction. Hence, freshly prepared hypertonic 4% (w/v) saline was used in the present study. After 5 minutes of nebulisation, spirometric tests were performed to detect broncho-constriction and the nebulisation was continued if the FEV_1 had not fallen by more than 20%. The induction was stopped, if FEV_1 decreased by more than 20% compared with post-salbutamol baseline (14). After 5 minutes and at subsequent intervals, subjects were asked to rinse their mouth and blow their noses to avoid contamination with postnasal drip and saliva.

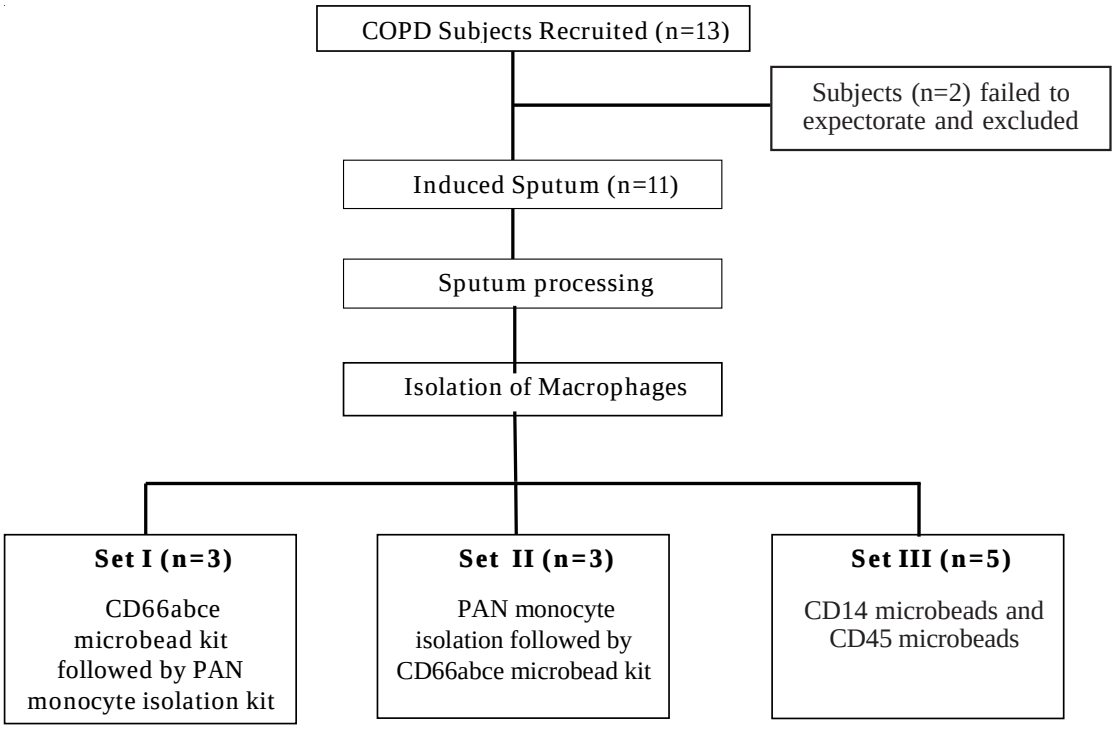

Fig. 1: Consort diagram showing various combination of kits used for experiments.

TABLE I: Showing details of COPD patients (n=13).

S. No.	Differential Leucocyte count (DLC) (%)				Pulmonary Function Test (PFT) (%)			Stages of Copd	Age	Gender	Smoking status	Pack Years	Ex–smoker (years)
	Neutro-phils	Macro-phages	Eosino-phils	Lympho-cytes	FEV1 (%)	FVC (%)	FEV1/ FVC (%)						
1	63.7	12.13	8.31	8.79	31	44	53	III	48	M	Smoker	14	–
2	71.56	9.47	6.87	4.02	37	65	53	III	69	M	Ex–smoker	12	7
3	70.78	14.61	7.97	6.64	46.24	64.16	55.41	III	53	M	Smoker	10	–
4	63.35	7.36	3.86	4.23	66	93	56.08	II	61	M	Smoker	13	–
5	62.45	11.89	5.57	6.69	33	54	44.21	III	66	M	Ex–smoker	20	4
6	51.34	4.84	6.46	5.38	56	71	61.89	II	63	M	Ex–smoker	11	3
7	80.83	6	2.26	5.55	50	58	65.72	II	65	M	Ex–smoker	10	2.5
8	88	3.84	2.59	5.56	37	56	68	III	48	M	Ex–smoker	15	3
9	74.58	6.77	6.44	12.2	51.14	81.88	65.48	II	62	M	Ex–smoker	11	2
10	84.48	4.73	3.91	5.28	45	61	62	III	61	M	Smoker	16	–
11	44.85	10.51	12.85	25.42	61	84	57.91	II	50	M	Ex–smoker	12	2
12*	69.2	11.8	7.16	10.84	35	56	53.24	III	43	M	Ex–smoker	10	5
13**	58.60	12.64	12.30	16.46	30	52	51.67	III	49	M	Smoker	11	–

* and ** shows the details of subjects not included in the study. Sputum Induction was discontinued due to complain of dizziness after two rounds of sputum induction and failure to expectorate sputum, respectively.

Then the subjects were encouraged to cough and expectorate sputum into a sterile-pot.

Collected sputum was immediately kept on ice and then processed immediately as described by Bhowmik et al. (9). To reduce the salivary contamination, sputum plugs were selected and transferred into an eppendorf tube. Since the minimum sample weighing 100 mg was sufficient to carry out further steps, no sample pooling was required. The sputum was treated with freshly prepared 0.1% (w/v) dithiotrietol (DTT) solution. The

selected plugs were treated with a volume (in microlitres) equal to two times the weight of sputum portion (in milligrams). The tube containing sputum plugs and DTT was vortex mixed and was placed in roller incubated for 15 min to ensure complete homogenisation. The liquid was further diluted with phosphate buffer saline (PBS, pH 7.2) in a volume equal to that of 0.1% DTT and vortex mixed. The suspensions were double filtered through 70 µm (Falcon cell strainer, BD sciences) and 48 µm nylon mesh (SEFAR Nitex, India) to remove mucus and debris epithelial and squamous cells. Weight of the filterate was obtained. Cell suspension was centrifuged at 480 xg for 10 min at 4°C. The supernatant was aspirated and the cell pellets were re-suspended in PBS. The cell pellet was further used for isolation of macrophages using commercially available immunomagnetic beads as described below.

Isolation of macrophages using different immune-bead kits

Various commercially available immunomagnetic microbead based kits (CD66abce microbeads, PAN monocyte isolation kit and CD14 microbeads from Miltenyi Biotech, BergischGladbach, Germany; CD14 positive selection kit and RosetteSep Human monocyte enrichment negative selection cocktail from Stem Cell Tech (Vancouver, Canada Inc.) were used for isolation of macrophages from sputum. Since these kits were primarily validated and recommended for isolation of monocytes from blood, the efficacy of kits were first checked for isolating monocytes from blood before using these kits for sputum samples. All the kits yielded satisfactory levels of enriched population of monocytes from blood, however, RosetteSep negative selection kit yielded high neutrophil contamination. CD14 microbead kit gave the maximum yield of monocytes from blood and sputum samples. As neutrophil count is generally high in the sputum samples of COPD patients, we adopted a strategy of depleting granulocytes using CD66abce microbead kit followed by PAN monocyte isolation kit. In order to compare, we have also employed only PAN monocyte isolation which is a negative selection kit as well as CD14 microbeads kit which is a positive selection kit for isolation of macrophages from sputum samples.

Isolation of macrophages using CD66abce microbead kit followed by PAN monocyte isolation kit

Target cell isolation in sputum samples was carried out as per the manufacturer's instruction. Briefly, cell pellet was re-suspended in sterile and degassed phosphate buffer saline (PBS, pH: 7.2-7.4) with 2 mM EDTA and 0.5% (w/v) bovine serum albumin (BSA) and CD66abce-biotin antibody and anti-biotin beads were added. The column was rinsed with MACS buffer before proceeding to magnetic separation. After incubation, cells were applied to MACS-MS columns that were placed in mini MACS separation unit (MiltenyiBiotec) to undertake magnetic separation. Cells in the pooled flow-through represented unlabelled cells devoid of $CD66a^+$, $CD66b^+$, $CD66c^+$ and $CD66e^+$ cells. The eluate containing the unlabelled cells was centrifuged so as to obtain the cell pellet. Supernatant was discarded and the cell pellet was further subjected to PAN monocyte isolation kit. Cells were resuspended in the MACS buffer and FcR blocking reagent and biotin-antibody cocktail was added and it was incubated 15 minutes. Cells were then re-suspended in the buffer and anti-biotin microbeads were added. After incubation, cells were proceeded to magnetic separation. Cells in the flow-through were collected as untouched macrophages. Thus, cells which were magnetically labelled by antibodies were depleted as they were retained in the MACS column. All steps were carried out at 4°C. Cytospin smears were prepared at room temperature and smears were stained as described below at each stages of the experiment to determine the percentage of inflammatory cells present.

Isolation of macrophages using PAN monocyte isolation kit followed by CD66abce microbead kit

The pellet obtained after sputum processing was subjected to PAN monocyte isolation kit. Cocktail of biotin-conjugated antibodies and anti-biotin microbeads was added and same protocol as mentioned above was followed according to manufacturer's instructions. After magnetic separation, the unlabeled cells that passed through, represented the enriched monocyte cells. The eluate

obtained was centrifuged and the pellet was further subjected to CD66abce microbead kit, as described above, in order to rid of contaminating granulocytes, if any. Cytospin smears were prepared and smears were stained as described below at each stages of the experiment to determine the percentage of inflammatory cells present.

Isolation of macrophages using CD14 microbeads

Macrophages were enriched by positive selection by using CD14 microbeads (MiltenyiBiotec, BergischGladbach, Germany) as per the manufacturer's instruction. Briefly, cells obtained from processed sputum were re-suspended in the above buffer and CD14 microbeads were added to it. After incubation, the cell suspension was loaded in MACS column placed in a MACS separator for magnetic separation. After removing the column from the magnetic-field, the magnetically labelled CD14+ cells which were stuck to the column were eluted as positively selected cell fraction by firmly pushing the plunger onto the column. Cytospin smears were prepared and smears were stained as described below to determine the percentage of inflammatory cells present.

Differential staining

Total cell counts and assessment of viability was carried out using routine procedure of trypan blue and Neubauer hemocytometer (15). Viability was found to be greater than 90% in all cases. The cell suspension was first mixed with PBS (pH 7.4) to obtain a count of 1.0×10^6 cells/ml of the suspension. 75 µl of cell suspension was used for preparing cytospin slides at 500 rpm for 4 minutes using a cytocentrifuge obtained from Medilab Solutions (Gurgaon, Haryana, India), which were then air dried and stained with Diff-Quick (Siemens. Healthcare Diagnostics Inc., Deerfield, IL, USA) for overall differential cell count using the routine procedure. Blind-fold counting of 400 non-squamous cells were performed by two investigators for all individual cytospin slides prepared at various stages of every experiment (16). Three slides were prepared at each stage of experiment. Samples containing more than 80% non-squamous cells was considered satisfactory for undertaking further steps of isolation of macrophages. Further, readings with inter-observer and intra-observer differences of $\geq 10\%$ were considered unacceptable. Using these cut-off, all reported values appeared acceptable. Removal of microbeads was not required as they did not interfere with the down-stream processes since immunobeads reportedly do not activate cells or saturate cell surface epitopes (17).

Data analysis

Total and differential cell counts were obtained from cytospin smears of cells obtained from all sets of experiments. Percentage of inflammatory cells were calculated at each stages of experiment and presented as means±SDs. Enrichment of macrophages was also calculated from each set of experiment performed as the fold increase in the percent purity. Overall enrichment was calculated by multiplication of enrichment obtained in each of the sub-steps and the results were expressed as means±SDs. Kruskal-Wallis H-test was used for comparisons of overall enrichment for strategies I, II and III, followed by Dunn's test of multiple comparison with Benjamini-Hochberg FDR.

Results

Table II shows the purity (percent) of inflammatory cells like macrophages, neutrophils and eosinophils retrieved at the end stage of every experimental approach, which were identified based on microscopic characteristics under Romanowsky staining. Table III shows the enrichment of macrophages from sputum sample of COPD patients in various sets of experiments. Percent purity of the inflammatory cells i.e., macrophages were enriched in various stages, however in one of the experiments in which CD66abce isolation was followed by PAN monocyte isolation, there was generally substantial (~50%) loss of the target cells. On the contrary, consistently high (~20-fold) degree of enrichment was seen in experiments with CD14 column. Figure 2 shows the cells obtained at various stages of experiments using the above mentioned kits.

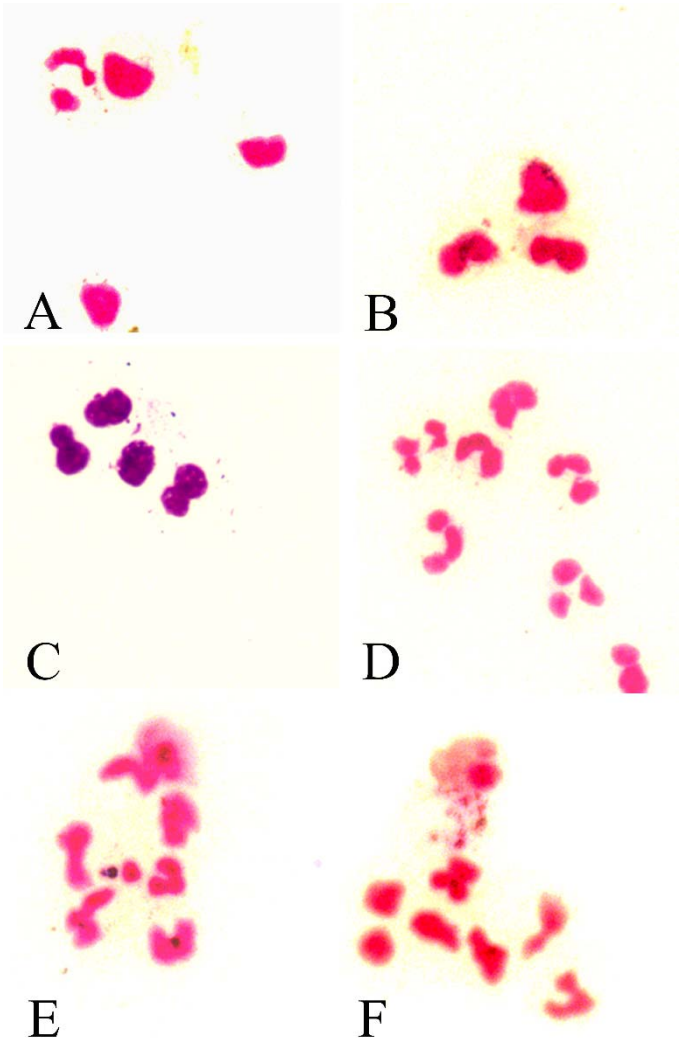

Fig. 2 : Cells obtained from sputum as seen in CD14 eluate (A), CD14 flow through (B), CD66 eluate (C), CD66 flow-through (D), PAN eluate (E) and PAN flow-through (F) stained with Giemsa. x40

TABLE II : Purity of macrophages, neutrophils and eosinophils retrieved from variousimmunobead based experiments to isolate macrophages from sputum samples.

S. No.	*Experimental details (n)*	*Cell types retrieved (Percent)*		
		Macrophages	*Neutrophils*	*Eosinophils*
1	CD66 abce kit followed by PAN Monocyte isolation kit (3)	1.9±1.6	22.8±19.8	0.1±0.1
2	PAN Monocyte isolation kit followed by CD66 abce kit (3)	4.8±8.3	–	–
3	CD14 Microbeads (5)	77.1±15.0	27.6±22.9	5.9±13.3

Values expressed as means±SDs.

TABLE III : Enrichment of macrophages observed in various sets of experimental approaches.

Procedure no.	*Details of procedure*	*Overall enrichment*
1	CD66 abce → PAN monocyte	9.4±7.7*
2	PAN monocyte → CD66 abce	0.6±1.7**
3	CD14 microbeads	20.1±4.1

Values expressed as means±SDs. Step 1 input for each procedure was 10^5 inflammatory cells in sputum, and flow through obtained from step 1 was used as step 2 input. *p<0.05, **p<0.02 as compared to procedure no. 3.

In the first set of experiments (n=3) in which CD66abce microbead kit was followed by PAN monocyte isolation kit, the total cell count ranged from 1.2 – 17.9 × 10^6 cells. PAN flow-through had high percentage (22.8%) of neutrophils followed by macrophages (2%) and very small numbers of eosinophils (0.1%). Although no frothy cell was seen in the cytospin prepared from the original cell pellet, small percentage of these cells could be seen in CD66 flow-through (2.5%) and in PAN eluate (0.8%). CD66 flow-through had 5.4% of band cells while PAN eluate had 0.002% of these cells.

In the second set of experiments (n=3) in which PAN monocyte isolation kit was followed by CD66abce isolation kit, the total cell count ranged from 2.0 – 6.9 × 10^6 cells. Macrophages obtained were only 4.8% in the final CD66 flow-through; neutrophils and eosinophils could not be seen in CD66 flow-through. Other cells such as lymphocytes (1.4%) and karyorrhectic cells (0.02%) were also seen in the cytospin prepared from CD66 flow-through. PAN eluate had neutrophils in a highly dynamic ranges (33-90%) in different experiment sets. Unexpectedly, PAN monocyte eluate also hadmonocytes. Again, in PAN flow-through, percentage of lymphocytes (31%) was higher than that of the macrophages (18%). CD66 flow-through, which was supposed to contain maximum percentage of macrophages, had only 4.8% of these cells. Small percentage (1.4%) of lymphocytes could also be seen. Mast cells (1.6%) were also present in PAN eluate and 1.9% of these cells were also present in CD66 flow-through in one set of experiment. Frothy cells were not present initially but these cells were seen in varying ranges in PAN eluate (1.2%), PAN flow-through (12.3%) and CD66 eluate (17.2%).

Karyorrhectic cells were present in small percentage in PAN flow-through (0.2%) and CD66 flow-through (0.02%). Interestingly, the mean value of overall enrichment was seen < 1, for the fact two samples out of the three failed to yield any macrophages.

In the third set of experiments (n=5) in which CD14 microbeads was used, total cell count varied from 0.9 – 7.0 × 10^6 cells. CD14 eluate had around 77.1% macrophages as expected, however, it also had 27.6% neutrophils. CD14 eluate also had a few eosinophils (1.6%). Unexpectedly, mast cells which were initially not seen were also recovered (2.2%) in CD14 eluate. CD14 flow-through contained 27.8% macrophages. Other cells such as neutrophils (71.5%), lymphocytes (3.7%) and eosinophils (2.9%) were also obtained in cytospin prepared from CD14 flow-through.

Discussion

Macrophages play a pivotal role in the pathophysiology of COPD. These are found to be elevated in the airways, lung parenchyma, Bronchoalveolar lavage fluid and sputum in COPD patients (18). Previous studies have focussed on isolation of macrophages from bronchoalveolar lavage fluid and bronchoscopy which are invasive techniques and hence cannot be used repeatedly. In COPD, there is an accumulation of airway macrophages (19) and therefore airway inflammation may be evaluated by using a safe and non-invasive method of sputum induction (5). There are only a few studies so far documenting the isolation of macrophages from induced sputum of COPD patients. In order to separate the inflammatory cells in induced sputum of COPD patients, RosetteSep technique has been used earlier for enriching macrophages from induced sputum. On the other hand, CD14 microbeads were used for separation of monocytes from blood sample (20). Per cent purity of monocytes obtained from blood sample by using CD14 microbeads, which was found to be greater than 80%, was in the line with the results obtained from study by Mayer et al. (21). In the present study, different strategies were examined and compared using microbeads for obtaining macrophages from sputum sample of COPD patients. These macrophages could be further used for various downstream experimental processes such as for ex-vivo stimulation, culture experiments, RNA or protein expression analysis.

Generally, negative selection is considered as the primary choice for isolation of macrophages in order to obtain untouched cells. However, in the present study, available negative selection kits meant for common target tissues like blood, lymph nodes and spleen did not work satisfactorily for the sputum samples. It is also difficult to design a perfect depletion cocktail to target all cells that do not carry any cluster of differentiation (CDs), also present on macrophages. The overall enrichment of macrophages obtained by using CD66abce microbead kit followed by PAN monocyte isolation kit was less than 10-fold. When the same two kits were used inversely by using PAN monocyte isolation kit first followed by CD66abce microbead kit, the mean enrichment of macrophages obtained was <1 fold. However, it is to be noted that no enrichment was obtained in two samples, resulting in fall in mean retrieval data with very high coefficient of variation (283.3%). On the other hand, positive selection kit using robust selection marker (CD14) present on macrophages (22, 23) yielded satisfactory yield of macrophages. No lymphocytes were held by CD14 column. Thus, positive selection strategy using CD14 increased the specificity and yield of macrophages by about 20-fold. Although we could satisfactorily isolate the pro-inflammatory type of macrophages which were strongly positive for CD14 cell surface marker (24, 14), the subset of macrophages with very low expression of CD14 could have been missed out. As sputum induction yields cells from lower airways, it is not possible to comment whether the macrophages obtained are alveolar or bronchial macrophages nor does it gives us any idea about these being small or large macrophages.

Cell counting was done by using haemocytometer and no other automated methods was used to keep the experiments cost effective. Previous studies have shown that manual counts are not typically higher or lower than machine counts and it is an accurate method for estimating cell numbers when compared to similar estimates determined using other methodologies (25). Though microscope counting

done by using a Neubauer chamber remains to be a gold standard for cell counting, techniques like flow cytometry improves precision and speed in retrieving CD labelled cells with fluorphore. This remains as a limitation in the present study. Moreover, it is to be noted that the serum levels of alpha 1-antitrypsin, IgG and IgA, were not checked which are differentially affected in COPD (26-30) and might have discrete interaction with different processes of macrophage isolation undertaken in the present study. This issue was not addressed in the present study.

In conclusion, sputum induction is a safe and non-invasive method for evaluation of airway inflammation. It is evident from the results of different sets of experiments of this study that CD14 microbeads based positive separation yielded maximum enrichment of macrophages from the sputum samples obtained from COPD patients as compared to other kits individually and in combinations. Thus CD14 microbeads can be used efficiently for isolating macrophages from induced sputum of COPD patients.

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Original Article

Immediate Effects of Yoga Breathing with Intermittent Breath Retention on the Autonomic and Cardiovascular Variables Amongst Healthy Volunteers

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Abstract

Background: Though breath retention is an important part of Yoga, not much is known about the physiological changes occurring following yogic breath retention. We examined the effects of 20 minutes regulated yogic breathing with intermittent breath retention (experimental session) at a frequency of 3 breaths per minute on the cardiovascular and autonomic functions.

Methods: Thirty-nine volunteers (22 females) with age-range 18 to 30 years (group mean±SD, 20.6±1.82 years) were recruited. Heart rate variability and cardiovascular variables were assessed through non-invasive blood pressure monitoring system before and after the experimental session or breath awareness (control session). The subjects were randomly assigned to either experimental or control session.

Results: There were significant reductions observed in the heart rate, stroke volume and cardiac output following the intervention. The Baroreflex Sensitivity (BRS) increased significantly following the experimental session, whereas no changes were observed following the control session. The time domain components of HRV indicated an enhanced heart rate variability following experimental session. Similar trends were observed following the control session. An increase in low frequency and decrease in high frequency components of HRV were observed following the experimental session. There was no significant change in frequency domain components following the control session.

Conclusion: The current study indicates differential autonomic modulation with enhanced BRS amongst healthy practitioners of yoga. Such yoga breathing may be useful for prevention of various metabolic disorders. The time domain components of heart rate variability suggest improvement following yoga breathing with intermittent breath retention.

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Introduction

Human respiration forms the bridge between autonomic and voluntary nervous systems since it is the only physiological system controlled by both the divisions. The impact of modulation of breathing on the autonomic and cardiovascular functions is well documented (1–4).

Breath regulation or *Pranayama* is one of the eight limbs of (*Ashtanga Yoga)* of *Patanjali* (5). Various techniques of *Pranayama* are described in *Hatha Yoga* texts (6). The texts also describe the profound effects of yoga breathing on the mind-body complex. The yoga breathing techniques include modulation of the pace of breathing, manipulation of nostrils, chanting of humming sounds, retention of breath etc. There has been growing interest in the inquiry of physiological effects of yoga and especially yogic breathing techniques in recent years (7). Several studies indicate the differential effects of various Yoga breathing techniques on autonomic functions.

In general, the practice of yoga has been found to bring balance in the autonomic functions with a trend towards parasympathetic dominance (7, 8). Various yoga breathing techniques are known to modify the cardiovascular functions (9), Baroreflex Sensitivity (10) and autonomic responses (11, 12).

Although the traditional texts of yoga emphasize on the practice of intermittent breath retention (5, 6, 13), such practice has sought very limited scientific attention. The proposed multiple health benefits of intermittent yogic breath retention include an increase in hemoglobin by increasing erythropoietin, increase in vascular endothelial growth factor leading to the formation of collaterals, reduction in blood pressure and resistance to cellular damage and thereby delayed ageing (14). A study demonstrated reduced pulse rate and increased galvanic skin resistance, following alternate nostril breathing (ANB) with intermittent breath retention (15). Another study demonstrated a significant increase in oxygen consumption while performing Ujjayi Pranayama with breath retention for a short duration. In contrast, lowered oxygen consumption was observed with prolonged breath retention (16). Since the practice of ANB and Ujjayi Pranayama are found to influence the autonomic functions even without the practice of breath retention (10, 17, 18), the effects of intermittent breath retention remain unclear.

The physiological effects of breath retention among underwater divers have been explored. The most common physiological response of the body to voluntary breath retention is to utilize the oxygen available optimally. Such response include bradycardia, reduction in stroke volume, cardiac output, and peripheral vasoconstriction. The initial phase of breath retention alters the physiology maximally, whereas the hemodynamic changes stabilize in the later part of extended breath holding (19–21). Breath retention also leads to cerebral vasodilation and increased sympathetic tone in response to hypoxia and hypercapnia (22, 23). Further, it is demonstrated that the physiological impact of breath retention depends on the psychological status of an individual (24).

Although breath retention is practiced by both underwater divers and yoga practitioners, there are fundamental differences in the way it is practiced. Amongst the underwater divers, breath retention is performed for a maximal duration following the completion of inhalation. Yoga prescribes retention in three ways – following inhalation (*antarkumbhaka*), following exhalation (*bahyakumbhaka*) and naturally occurring breath-retention (*kevalakumbhaka*). It is also prescribed to be practiced for various durations depending on the nature of the practice of *pranayama* (13, 25).

Considering the importance of breath retention in the traditional yoga texts and lack of scientific understanding of its effects, the current study was undertaken to evaluate the effect of slow yogic breathing with intermittent breath retentions on autonomic activity, cardiovascular functions including baroreflex sensitivity (BRS) through modulation of the cardiorespiratory pathways.

Methods

Participants

Thirty-Nine volunteers (17 males + 22 females) with their ages ranging from 18 to 30 years (group mean±SD, 20.6±1.82 years) were recruited for the study. They were selected from a population of 160 students, studying various long-term courses in a Yoga University situated in South India. They had experience of practicing yoga ranging from 1 to 4 years (group mean±SD, 2.92±1.75 years). Experienced yoga practitioners were chosen for the study since breath retention is an advanced yoga practice and is not recommended to be practiced by people naïve to yoga practice. Their training in yoga included understanding yoga philosophy and practice of yoga postures (*asanas*), voluntarily regulated breathing (*pranayama*) and meditation techniques. All the participants included in the study were trained in the breathing practice assessed in the present study for 20 min/day, 6 days a week, for 8 weeks prior to the assessment. This 8 weeks of supervised training was conducted to ensure uniformity of breathing practices amongst all the participants.

Sample size

The sample size was calculated based on the effect size obtained from a previous study (26) which assessed changes in blood pressure following the practice of pranayama. It was calculated using G*Power software, Version 3.1.9.2 (27), where the Power was 0.95, α = 0.05, the effect size (Cohen's *d*) was 1.018 and the recommended sample size resulted in being 31 participants in each group. Allowing a 20-30% attrition rate, we concluded to include 40 participants to the study.

The physical health of the subjects was assessed through routine clinical examination by a trained physician who otherwise had no role in the trial. The subjects with a history of any major illness in past 6 months especially any cardiac or respiratory disorders, consumption of any medications, tobacco, alcohol or substance abuse in any form were excluded from the study. The demographic data of the participants are presented in Table I.

TABLE I: Demographic data of the volunteers.

	Male	*Female*	*Total*
Sample size (n)	17	22	39
Age (years)	20.88±1.93	20.39±1.75	20.6±1.82
Height (cm)	168.65±6.92	158.56±6.25	162.85±8.19
Weight (Kg)	59.06±6.74	51.30±6.53	54.6±7.60
BMI (Kg/m^2)	20.73±1.74	20.36±1.86	20.52±1.80
Years of Yoga experience	3.82±2.67	4.00±2.86	3.92±2.75

Ethical consideration

The study was approved by the institutional ethics committee of the Swami Vivekananda Yoga Anusandhana Samsthana. A signed informed consent was obtained from all the participants.

Design

Following the 8-week training, the subjects were randomly assigned for the practice of yoga breathing with intermittent breath holding (experimental session) and breath awareness (control session). Half of the subjects had the experimental session on day 1 and control session on day 2 and for the rest, the order was reversed. The random allotment of the sessions was done using a web-based computer program (www.randomizer.org). Both experimental and control sessions lasted for 20 min each, which was preceded and followed by 5 min of assessment periods. The assessments were performed before and immediately following the experimental and control sessions. The time of the day was kept constant for each subject on both days. Female participants were assessed during the luteal phase of menstrual cycle (10 to 16 days after the onset of menstruation) to minimize the effect of menstrual cycle on autonomic functions (28).

Assessments

Electrocardiogram (ECG) and respiration were recorded using 16-channel human physiology system (PowerLab 16/35, ADInstruments, Australia) and blood pressure (BP) were monitored using Finapres Continuous Non-Invasive Blood Pressure (NIBP) Systems (Finapres Medical Systems B.V., Netherlands). The ECG was acquired using limb Lead

II system i.e., the electrodes were placed on the right arm and both legs (29). A standard finger cuff was connected to the left middle finger, in between the interphalangeal joints. Brachial correction was made at regular intervals as per the standard operating procedure of the instrument. The accuracy of NIBP by Fianpress Medical Systems has been standardized through comparable experiments with Intra-arterial blood pressure measurements (30, 31). Bitscope Easy v 2.0 software (Finapres Medical Systems B.V., Netherlands) was used for the recordings of NIBP. The digitized ECG data was analyzed offline to obtain the heart rate variability (HRV) spectrum. Respiration was recorded using a volumetric pressure transducer fixed around the trunk about 8 cm below the lower costal margin while the participants sat erect.

Heart rate variability

The ECG was recorded using a standard bipolar limb lead II configuration, which was digitized using a 16 bit analog to digital converter at a sampling rate of 1 KHz and was analyzed offline to obtain the HRV spectrum. Frequency domain and time domain analysis of HRV data were carried out using Lab Chart 8 (AD instruments, Australia) program, which uses Lomb-Scargle Periodogram algorithm.

Intervention

Experimental session

The experimental session included the regulated yogic breathing for 20 minutes incorporating phases of inhalation (*puraka*), internal retention of breath (*antarkumbhaka*), exhalation (*recaka*) and external retention of breath (*bahyakumbhaka*) in a ratio of 1:1:1:1 for 6 seconds each. The classic yoga texts suggest breath retention in varying ratios. The ratio for the intervention was chosen since it is considered ideal for subjects who are naïve to the practice of breath retention. The intervention was derived from a classical training methodology of pranayama suggested in the ancient text of Yoga (13). The intervals of 6 seconds were decided based on a previous study which used the similar duration of phases of breath retention along with *Nadisuddhi Pranayama* (15). The duration of 6 seconds was ensured through verbal cues in a pre-recorded audio track.

Control session

During the control session, the participants were seated erect, performing normal breathing with breath awareness for the same duration of 20 min in the same test environment, including the audible cues. There were no adverse events reported during either the training of participants in yoga breathing with intermittent breath retention or during the recordings.

Test conditions

The recording room in the research laboratory was sound attenuated and air-conditioned in order to avoid thermal, visual or auditory disturbance. The temperature of the recording room was maintained at 25±1°C. The relative humidity during the time of the study was on average 52%. During both practice and assessments, the participants were seated comfortably, keeping the spine erect on a soft chair with backrest.

Data extraction

The following data were extracted from the 16-channel polygraph. The heart rate in beats per minute was calculated by counting the R waves of the QRS complex in the ECG. Frequency domain and time domain analysis of HRV data were performed. The energy in the HRV series in the following specific frequency bands were studied viz., Low frequency (LF) band (0.04–0.15 Hz) and highfrequency (HF) band (0.15–0.5 Hz). According to guidelines, LF and HF band values were expressed as normalized units. The LF/HF ratio was also calculated. The following components of time domain HRV were analyzed: (i) SDNN (the standard deviation of NN intervals), (ii) the square root of the mean of the sum of the squares of differences between adjacent NN intervals (RMSSD), (iii) the proportion derived by dividing NN50 by the total number of NN intervals (pNN50). The respiratory rate in cycles per minute (cpm) was calculated by counting the total breath cycles.

Brachial artery systolic (SBP) and diastolic pressures (DBP) were extrapolated from finger arterial pressure through the use of a height correction unit and waveform filtering and level correction methods. Mean arterial pressure (MAP), SBP and DBP were expressed in mmHg. The computed measurements of Stroke volume (SV), cardiac output (CO) from the arterial BP and HR has been found reliable when compared to Modelflow-derived CO (32). The Total Peripheral resistance (TPR) estimation from the computed CO was also found to be valid (33). Another variable of interest, Baroreflex Sensitivity (BRS) was estimated from the spontaneous HRV and BP variability (BPV) measured by the Finapress method (34).

Data analysis

The data were analyzed by the statistician using Statistical Package R version 3.2.4 (www.r-project.org). Repeated measures analyses of variance (RM-ANOVA) were performed with two Within-Subjects factors, i.e., (i) Sessions with two levels; intervention and control and (ii) States with two levels, pre and post intervention.

Results

The results are presented as the group mean and standard deviation for the autonomic and the cardiovascular variables (Table II).

Following the experimental session, an increase in SDNN, RMSSD, pNN50 of the time domain variables of HRV was observed. A significant increase was also noted in LFnu, Total Peripheral Resistance and Baroreflex Sensitivity following the experimental session. We also found a reduction in HFnu, MAP, SV and CO in the experimental session. The heart rate reduced following both experimental and control sessions. After control session, an increase in RMSSD, pNN50, SBP and SV was noted. A reduction was observed in CO following the control session. No changes were observed in the frequency domain variables of HRV and Baroreflex Sensitivity following the control session. Although there was reduction noted in the respiratory rate in both groups, the changes were non-significant. Also, Pre-Post breath rates were incidentally observed to be similar for both the sessions.

Repeated measures analysis of variance

The significant changes in the components of HRV and cardiovascular variables are presented in Table III.

Post hoc analyses with Bonferroni adjustment

There was a significant reduction in HR (P<0.001,

TABLE II: Changes in the Heart Rate Variability and Cardiovascular variables before and following the experimental and control sessions.

	Experimental session		*Control session*	
	Pre	*Post*	*Pre*	*Post*
Heart rate (beats/min)	78.54±10.54	74.92±9.25***	76.67±9.58	73.94±9.29**
SDNN (ms)	64.47±6.27	74.76±29.20**	65.67±28.02	70.84±28.57
RMSSD (ms)	45.98±22.90	52.39±24.96***	49.23±23.22	54.81±23.93*
pNN50 (% units)	21.26±16.65	24.99±16.49**	25.20±17.78	30.67±17.75**
LFnU	61.72±17.55	67.51±15.77*	57.38±18.96	55.56±20.55
HFnU	38.53±17.16	32.65±15.27*	42.48±18.57	43.71±19.65
LF:HF	2.58±2.72	3.10±2.70	2.07±1.96	2.11±2.59
Respiratory Rate (cycles/min)	15.42±3.46	14.63±4.03	16.18±3.71	15.38±4.11
Systolic BP (mmHg)	102.10±12.21	101.04±11.39	104.44±10.91	106.83±11.86***
Diastolic BP (mmHg)	59.33±8.76	58.49±.30	60.88±8.46	60.61±8.23
Mean Arterial pressure (mmHg)	77.88±10.12	76.35±9.54*	79.52±9.10	79.59±8.99
Stroke Volume (ml)	68.35±15.83	66.20±15.75*	70.36±12.04	72.22±11.41***
Cardiac output (l/min)	5.27±1.20	4.88±1.05***	5.32±0.85	5.26±0.81**
Total Peripheral Resistance	0.99±0.37	1.04±0.39***	1.07±0.70	0.99±0.36
Baroreflex Sensitivity (ms/mmHg)	14.56±.55	15.81±5.71**	16.24±8.83	16.50±7.75

Repeated Measures Analyses of Variance with post hoc Bonferoni adjustment, *=p<0.05, **=p<0.01, ***=p<0.001
SDNN: standard deviation of NN intervals; RMSSD: root of the mean of the sum of the squares of differences between adjacent NN intervals; pNN50: proportion derived by dividing NN50 by the total number of NN intervals.

TABLE III: Summery of the Repeated Measures Analysis of Variance (RM-ANOVA) showing statistically significant results.

Variables	*Factor*	*F Value*	*df*	*Level of significance*
LFnu	Sessions	13.81	1, 38	0.01
HFnu	Sessions	13.61	1, 38	0.01
LF/HF ratio	Sessions	6.17	1, 38	0.05
pNN50	Sessions	5.17	1, 38	0.05
Systolic BP	Sessions	81.20	1, 38	0.001
Diastolic BP	Sessions	213.62	1, 38	0.001
Mean arterial pressure	Sessions	18.84	1, 38	0.001
Cardiac output	Sessions	612.38	1, 38	0.001
Stroke volume	Sessions	6.81	1, 38	0.05
Total peripheral resistance	Sessions	581.17	1, 38	0.001
Heart rate	States	37.96	1, 38	0.001
RMSSD	States	15.79	1, 38	0.001
pNN50	States	16.77	1, 38	0.001
SDNN	States	13.04	1, 38	0.01
Systolic BP	States	81.33	1, 38	0.001
Diastolic BP	States	732.29	1, 38	0.001
Mean arterial pressure	States	343.79	1, 38	0.001
Cardiac output	States	13.83	1, 38	0.01
Stroke volume	States	22.67	1, 38	0.001
Total peripheral resistance	States	580.45	1, 38	0.001
HFnu	Sessions x states	4.12	1, 38	0.05
Systolic BP	Sessions x states	81.31	1, 38	0.001
Diastolic BP	Sessions x states	1030.13	1, 38	0.001
Mean arterial pressure	Sessions x states	292.73	1, 38	0.001
Cardiac output	Sessions x states	14.07	1, 38	0.01
Total peripheral resistance	Sessions x states	617.01	1, 38	0.001

post hoc analyses following ANOVA), HFnu ($P<0.05$), MAP ($P<0.05$), SV ($P<0.05$), CO ($P<0.001$), whereas increase was noted in LFnu ($P<0.05$), SDNN ($P<0.001$), RMSSD ($P<0.01$), pNN50 ($P<0.01$) TPR ($P<0.001$) and BRS ($P<0.01$) following the experimental session. Reductions in HR ($P<0.01$), CO ($P<0.01$) and TPR ($P<0.001$) whereas increase in RMSSD ($P<0.05$), pNN50 ($P<0.01$), SBP ($P<0.001$) and SV ($P<0.001$) were observed following the control session.

Discussion

This study investigated the effect of yoga breathing with intermittent breath holding on frequency and time domain variables of heart rate variability (HRV) and cardiovascular functions in healthy yoga practitioners. To the best of our knowledge, this is the first attempt to scientifically explore the effects of isolated yoga breathing with breath retention among healthy volunteers. The earlier studies where yogic breath retention was used, it was in combination with other yoga breathing techniques (15, 16, 35).

HRV is the physiological phenomenon of variation in the time interval between heartbeats. It is measured by the variation in the beat-to-beat intervals (36). HRV is widely utilized to interpret the cardiac autonomic regulation following various yoga practices (7). HRV is the pattern of several overlapping oscillatory frequency components. Three components of the frequency domain analyses of HRV have been identified viz., the high frequency (0.15–0.4 Hz), low frequency (0.05–0.15 Hz), and very low frequency (0.005–0.05 Hz). In general, LF component is correlated with the activity of sympathetic and parasympathetic nervous system whereas HF component with parasympathetic activity. The physiological interpretation of VLF component is unclear (36). It is also observed that, high-amplitude peaks in the LF range during rhythmical slow breathing may reflect resonance characteristics of the cardiovascular system where respiratory sinus arrhythmia interacts with the baroreflex (37). Breathing at such resonant frequency may increase HRV and be reflected in large increases in the LF band and simultaneous decreases in the HF band. The findings of the spectral analysis of HRV of the current study indicate an increase in LF and a corresponding reduction in HF, with enhanced baroreflex sensitivity. These changes, thus, may be attributed to breathing at a very slow rate of 2.5 Hz.

The findings are similar to earlier yoga studies demonstrating an increase in LF with slow yoga breathing (38). Breathing at such slow rate imitating the resonant frequency is found to influence the heart rate and blood pressure oscillations and thus enhance the overall HRV (39) and subsequent reduction in heart rate and blood pressure (40). Yet, the blood pressure changes were nonsignificant in the current study. No changes in the frequency components of HRV were observed following the control session.

Among the time-domain variables, SDNN is an indicator of overall heart rate variability, whereas RMSSD and pNN50 are associated with vagal tone (36). The changes in the time domain components of the HRV were similar following both experimental and control sessions with an increase in SDNN, RMSSD and pNN50, thus indicating an enhanced HRV. Yet, the magnitude of change was higher following the experimental session. However, the reason for the change following the experimental session remain unclear, whether it was due to intermittent breath retention or slow breathingalone. Since, the participants were long-term yoga practitioners, and performing breath awareness during the control session, they might have entered a meditative state and thus modulating the HRV. Such enhanced HRV is common among long-term yoga practitioners (7). Similar changes in time-domain variables of HRV were observed in a previous study in participants practicing breath awareness (41).

We also found a significant increase in the Baroreflex Sensitivity following the 20 min experimental session. Our findings are consistent with earlier studies elucidating the influence of yogic breathing techniques on Baroreflex Sensitivity in healthy (10) as well as Clinical population with essential hypertension (42) and chronic heart failure (43). Such gain in the baroreflex sensitivity may also be due to slow breathing in the experimental session at the resonant frequency of about 2.5 Hz. Also, the earlier studies attribute the gain in Baroreflex Sensitivity to increased vagal tone, indicated by a gain in RMSSD and pNN50 as well as reduced heart rate. Arterial baroreceptor activity and respiratory sinus arrhythmia are interrelated (44) and therefore the increase in Baroreflex Sensitivity could be attributed to enhanced HRV following the experimental session. Jerath et. al. propose the action of inhibitory signals and hyperpolarizing current within neural and non-neural tissue activation of slowly adapting stretch receptors, responsible for modulation of the activity of the cardiorespiratory centers (45).

Reduced heart rate variability and baroreflex sensitivity is found to be a risk factor for cardiovascular diseases (46, 47), diabetes mellitus (48, 49) and various metabolic syndromes (50). The enhanced heart rate variability and baroreflex sensitivity observed following the yoga breathing assessed in the present study may indicate its role in preventing such disorders. Future studies may incorporate clinical population to assess the effect of yoga breathing with intermittent breath retention on the cardiac autonomic regulation.

We also found an increase in the total peripheral resistance and LFnu indicative of a possible sympathetic shift in the autonomic activity. These changes may be due to the very nature of the intervention, which includes focused attention on the verbal cues and constant synchronization of the breathing with it. The nature of intervention needed constant attention, which may be responsible for the selective sympathetic arousal. An earlier study of yoga breathing with breath-retention for short duration indicated an increase in oxygen consumption, which might be considered similar to the results of the present study (16). Also, intermittent hypoxia created in the experimental session would contribute to enhanced sympathetic tone (23). Despite the sympathetic arousal, the gain in baroreflex sensitivity may be attribute to inhibition of chemoreflex mechanisms due to slow breathing (51). Also, long term yoga practitioners demonstrate a generalized reduction in chemoreceptor sensitivity (52). Slow breathing possibly leads to a generalized attenuation in the excitatory pathways for respiratory and cardiovascular systems. Both respiratory and cardiovascular systems share similar control mechanisms, thus alterations in breathing may be responsible for the cardiovascular changes (53).

The reduction in cardiac output and stroke volume may be a result of body's compensatory mechanism

due to intermittent breath retention along with very slow breath. Also breathing at resonant frequency has been shown to enhance the gaseous exchange and oxygen saturation (54), thereby reducing the overall circulatory load.The changes observed in the current study following the intervention are similar to those found following voluntary breath retention in swimmers and divers, which include bradycardia, reduction in stroke volume, cardiac output, and peripheral vasoconstriction (19). However, in the current study, the practice of breath-retention was intermittent and short term instead of the maximal breath retention as practiced by the divers.

Although we could demonstrate differential changes in the autonomic and cardiovascular activity following yoga breathing with intermittent breath retention, the examination of the exact underlying mechanisms was beyond the scope of the study. Assessments during the practice of yoga breathing with intermittent retention may bring clarity on the underlying mechanisms. Lung volume and the partial pressure of CO2 (PaCO2) are known to influence the HRV spectrum in conscious subjects (55). We could not control the lung volume as well as assess the PaCO2 in the current study. Thus, future studies may be planned to assess the effects of lung volume and PaCO2 in yoga breathing techniques. Future studies may also include neuroimaging techniques focusing on the neural centers for the vagus nerve to understand the underlying mechanisms. We did not examine the long-term effects of the practice of yoga breathing with intermittent breath retention. Our study population was limited to healthy young volunteers, with training in yoga. Future studies may be designed to understand the effects of yoga breathing with breath retention in different populations and clinical setting.

Conclusion

The current study indicates differential autonomic modulation with enhanced Baroreflex Sensitivity along with selective sympathetic activation amongst healthy practitioners of yoga. The time domain components of heart rate variability suggest improvement following yoga breathing with intermittent breath retention and a similar trend following normal breathing with breath awareness. Such yoga breathing may be useful for prevention of various metabolic disorders including heart diseases and diabetes mellitus. Further studies using neuroimaging techniques in different populations could be used to understand the exact mechanisms involved in the practice of yogic breath retention and its specific effects.

Compliance with ethical standards

Disclosure of potential conflicts of interest: The authors declare no conflict of interests.

Research involving Human Subjects: The study has been approved by the institutional ethics committee and have been performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Informed consent: Written informed consent was obtained from all the participants.

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Original Article

Effects of Yoga in Type 2 Diabetes Mellitus With Hypertension : Alteration in RBC Morphology as a Marker for Oxidative Stress

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Abstract

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Purpose: Yoga is well known for improving oxygenation to the biological system and combating oxidative stress which is responsible for numerous lifestyle diseases which includes type 2 diabetes and hypertension. Therefore, the present study was conducted to evaluate the effects of yoga in modifying and improving the quality of life in type II diabetic with hypertension patients.

Methods: An interventional, prospective and open labeled study was done involving 30 patients of type 2 diabetes along with hypertension. Patients received yoga therapy for 45 days along with the standard treatment. Oxidative stress markers such as changes in RBC morphology (crenated edges and Heinz bodies), malondialdehyde levels along with fasting blood glucose levels, systolic & diastolic blood pressure, body mass index and symptoms associated with type 2 diabetes were evaluated before and at the end of the yoga therapy.

Results: Abnormal RBCs were markedly reduced as according to the severity ranking assessed after 45 days of yoga therapy. Significant reduction in the levels of malondialdehyde ($P<0.01$), blood glucose ($P<0.05$), Systolic blood pressure ($P<0.01$) body mass index ($P<0.001$) and improvement in the unpleasant symptoms were observed after yoga therapy when compared to same patients before starting yoga therapy.

Conclusion: These findings suggest that yoga intervention has therapeutic values in patients having type 2 diabetes with hypertension. This may have direct impact on the dose minimization of hypoglycemic drugs of the patient which requires further study in this area.

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Introduction

Stress can be described as a circumstance that disturbs the normal psychological or physiological functioning of a person[1]. Stress can be acute or chronic that causes stimulation of hypothalamic pituitary adrenal axis (HPA axis), sympathetic nervous system and also increases the production of reactive oxygen species (ROS) followed by release of stress hormones (2). The stress hormones especially adrenaline, glucagon, corticosteroid cause lipolysis, neoglucogenesis and increase in blood pressure which leads to metabolic syndrome (2, 3, 13, 14). ROS causes cell damage and increase the synthesis of proinflammatory and inflammatory mediators like interleukin-1, tumor necrosis factor and prostaglandins. The isoprostanes 8-iso PGF2 alpha are formed non-enzymatically from arachidonic acid directly by free radicals. This isoprostanes binds to the prostanoid receptors and cause chronic inflammation (3, 6, 8, 9, 24). When the balance between pro-oxidant and anti-oxidants is deranged due to excessive production of free radicals or low level of anti oxidants, the state is called Oxidative Stress (7).

This is responsible for insulin resistance, RBC and tissue damage in type II diabetes (3, 4). RBCs are the first cells to be affected by ROS and chronic inflammation causing damage to the cell membrane (crenated edges) and haemoglobin (heinz bodies) (2, 3, 4). The damaged RBCs are removed from the circulation by the spleen during the life span. Therefore, RBC morphology is used as a biomarker for oxidative stress in Type II diabetes.

The free radicals can also damage unsaturated fatty acids in cell membrane. Plasma lipoproteins leads to the formation of lipid peroxides and highly reactive dialdehydes that can chemically modify the proteins and nucleic acid bases. The total body radical burden can be measured from the products of lipid peroxidation (6). Malondialdehyde (MDA) is one of the most toxic byproducts of lipid peroxidation which is of major toxicological interest. The toxic byproducts formed during lipid peroxidation have effects at site away from the area of their generation hence they behave as toxic secondary messengers (9).

Yoga is an ancient discipline designed to balance physical, mental, emotional and spiritual well being in an individual. Yoga therapy has gained popularity nowadays because of its unique nature of delivering positive improvement towards numerous disorders as well as subsiding the disease progression. It includes gentle stretching of muscles and breathing exercises with wide range of classical asanas and pranayama practices. Many studies proved the pathogenic role of oxidative stress in lifelong disorders which necessitates this study to be involved with type 2 diabetes along with hypertensive patients. The aim of the study is to evaluate the impact of yoga therapy on the RBC morphology with relevance to oxidative stress. This may provide us the better understanding the molecular mechanism of how yoga therapy involved in reversing the free radical induced damages in RBC morphology.

Methodology

It was an interventional, prospective and open labeled study. The present study involved 30 Patients of either sex between the age group of 40 to 60 years old diagnosed with type 2 diabetes mellitus as well as hypertension undergoing treatment for more than 5 years as outpatients were recruited for the study after explaining the complete study purpose and procedures. Recruitment of the patients was done after Institutional Ethics committee approval (No.41102015). Informed consent was obtained from the patients who were willing to participate in the study in the prescribed format in regional language. If the patient was illiterate, left thumb impression in the presence of an impartial witness was taken. The demographic details of the patients were obtained and recorded. History of the patients was taken. Pregnant and lactating women, physically handicapped or mentally ill, patients with any advanced complications of diabetes (retinopathy and nephropathy) and those who are already practicing yoga were excluded from the study. As there was no control group included in this study because of the longer treatment status of the patients. The general & systemic examinations were carried out. 3 ml of blood was collected and transferred to EDTA coated tubes. It was centrifuged at 2000 rpm for 10 minutes at 4°C. The top yellow plasma layer was

pipetted off without disturbing the white buffy layer. The plasma was stored on ice and then transferred to a deep freezer at –80°C for estimation of the MDA levels later. The packed cells were reconstituted as 10% v/v suspension with 0.9% normal saline. A drop of this suspension was put on a glass slide under a cover slip and studied under high power microscope for assessment of morphological changes in the red blood cells.

Morphological changes in RBC's at baseline and after 45 days of yoga therapy were assessed using the following scoring pattern (4) :

No abnormal RBC/HPF = 0

10-25% abnormal RBC/HPF = 1+

25-50% abnormal RBC/HPF = 2+

50-75% abnormal RBC/HPF = 3+

>75% abnormal RBC/HPF = 4+

Presence of crenated edges with Heinz bodies in RBCs were considered as abnormal RBCs. Malondialdehyde was estimated by using the thiobarbituric acid reactive substances (TBARS) assay kit which was purchased from Caymen Chemicals, USA. The fasting blood glucose levels, body mass index, systolic and diastolic blood pressure were measured and the assessment of improvement of symptoms was done using the questionnaire as shown in the Table I.

Intervention with yoga schedule:

The yogasana schedule was designed by naturopathist involves the combination of asanas and breathing exer-cises. All the patients were trained in order to the follow the yoga schedule for 45 days. Yoga schedule starts with OM chanting (5 min) followed by naadi suthi prayanama as well as ujjai prayanama (5 min) and various asanas includes Ardha Halasana, Naukasana, Ushtrasan, Ardha pawanmuktasana, Salabasana, vakrasana, Bhujangasan, Chakrasana, katikasana and Shavasan (20 min). These asanas are selective according to the Patient condition. They were recommended to practice these asanas twice a day. They were also handed over a booklet regarding the same. If they found any difficulty in performing those asanas or if they felt any pain or injury while performing any asana, the particular asana was modified by the yoga specialist. Patients practicing yoga were asked to report once in 15 days to ensure that they were practicing the yogasana schedule regularly and that they had no difficulty in performing the asanas. The subjects were allowed to withdraw from the study at any point, if they so desired. Statistical analysis was done using Paired t test.

Results

Among 30 patients in which 6 patients were withdrawn from the study due to the reason irrelevant to yoga practice. The variation was observed in the regularity pattern among the patients. Out of 24 patients, 14 were male and 10 were female with

TABLE I : Questionnaires for assessing the improvement in diabetic complications.

S.No.	*Symptoms*	*The symptom was _____ troublesome to me.*				
		A (not at all)	*B (little)*	*C (moderate)*	*D (very)*	*E (Extremely)*
1	Lack of energy					
2	Urinary frequency					
3	Aching Intensity in Calves					
4	Dry mouth					
5	Thirst frequency					
6	Existence of irritability before meal					
7	Feeling of Numbness in Palm and feet					
8	Palpitation Frequency					
9	Sense of Fatigue					

The patients are alphabetically graded according to the severity of symptoms.

Mean±SD age of 53±9 years. RBC Morphology was improved as the damage induced by the free radical significantly declined after the yoga therapy was confirmed through the scoring patterns (Fig. 1 & 2).

Significant reduction in the levels of malondialdehyde (<0.01), fasting blood glucose (<0.05), systolic blood pressure (<0.01) and body mass index (<0.001) was observed after the yoga therapy as shown in the Table II.

The diabetic complications were progressively decreased after the yoga therapy and the significant improvement in their well being patterns was assessed through the validated self-made questionnaire (31) (Table III). Few patients even reported that they reduced the frequency of taking the hypoglycemic medications themselves, still they found good glycemic control. Data of those few patients has not been shown separately as it is beyond the scope of this study.

Discussion

As a result of chronic stress, sympathetic nervous system (SNS) activation causes the release of

TABLE II : Represents the changes in the parameters before and after the 45 days of yoga therapy.

S. No.	*Parameter*	*Before*	*After*	*P value*
1	Malondialdehyde (µM/L)	64.95±14.97	47.25±18.50	<0.01
2	Blood glucose Levels (mg/dl)	200.96±78.01	137.26±53.15	<0.05
3	Systolic blood pressure (mmHg)	141.4±10.04	135.66±9.14	<0.01
4	Diastolic blood pressure (mmHg)	93.8±12.25	89.3±7.90	0.2117
5	Body Mass Index (BMI)	23.24±2.68	22.85±2.66	<0.001

Values are expressed in Mean±SD. P value shows significant for malondialdehyde levels (0.01), blood glucose levels (0.05), systolic blood pressure (0.01) and body mass index (0.001).

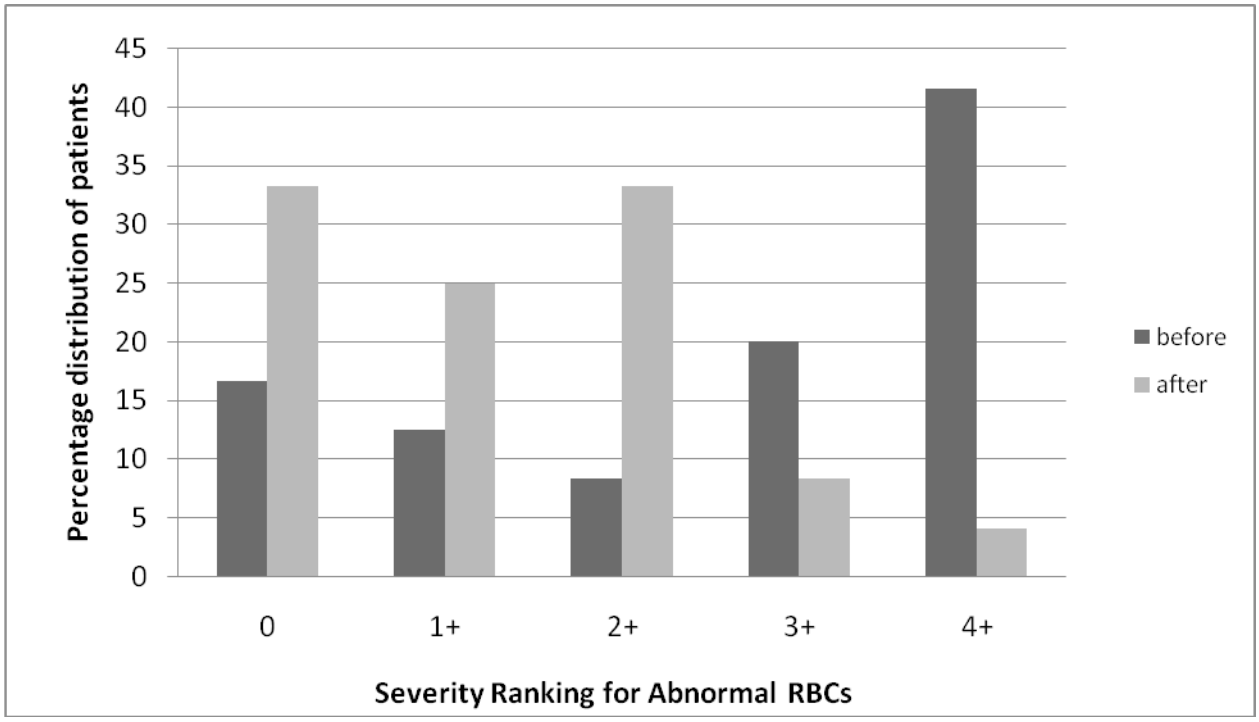
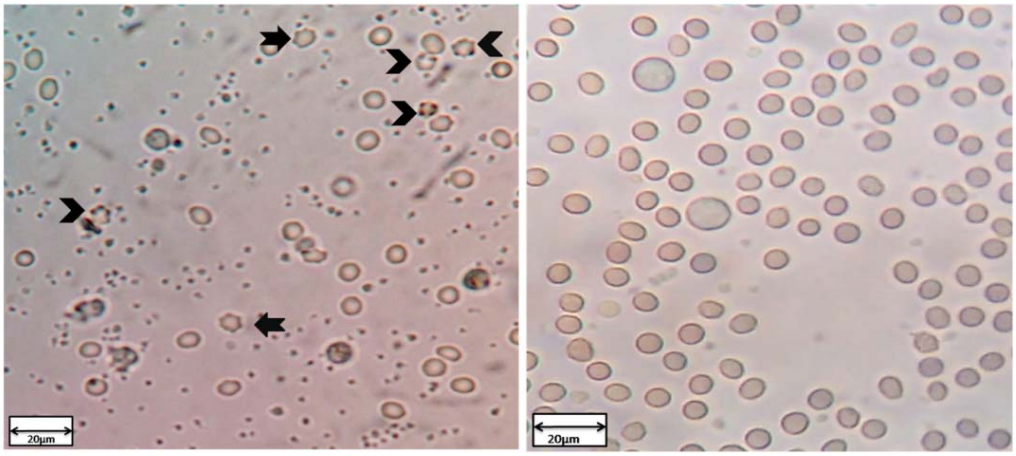


Fig. 1 : Represents the change in the severity ranking of the RBC Morphology before and after the 45 days of Yoga therapy.

TABLE III : Percentage distribution of number of patients based on symptoms severity before and at the end of 45 days of yoga therapy.

S. No.	Symptoms questionnaires	Percentage Distribution of number of patients based on the severity (%)									
		A		B		C		D		E	
		Before	After	Before	After	Before	After	Before	After	Before	After
1	Lack of energy	0	29	4	58	17	13	54	0	25	0
2	Urinary frequency	0	29	8	50	25	21	63	0	4	0
3	Aching Intensity in calves	16	17	21	33	50	17	29	0	16	0
4	Intensity of Dry mouth	27	54	15	38	27	8	31	0	0	0
5	Thirst frequency	8	29	12	46	29	25	38	0	13	0
6	Existence of Irritability before the meal	79	87	0	13	0	0	17	0	4	0
7	Feeling of Numbness or Loss of sensation in feet	29	42	12	29	25	21	17	8	17	0
8	Palpitation Frequency	46	71	0	27	29	4	17	0	8	0
9	Sense of Fatigue	50	54	17	29	21	17	0	12	0	0



(a) (b)

Fig. 2 : Shows the optical microscopy images (40x) of (a) the Heinz bodies along with crenated edges present in RBCs (b) the Normal RBCs.

➡ Indicates Crenated edges present in RBCs. ➤ Indicates Heinz bodies present in RBCs.

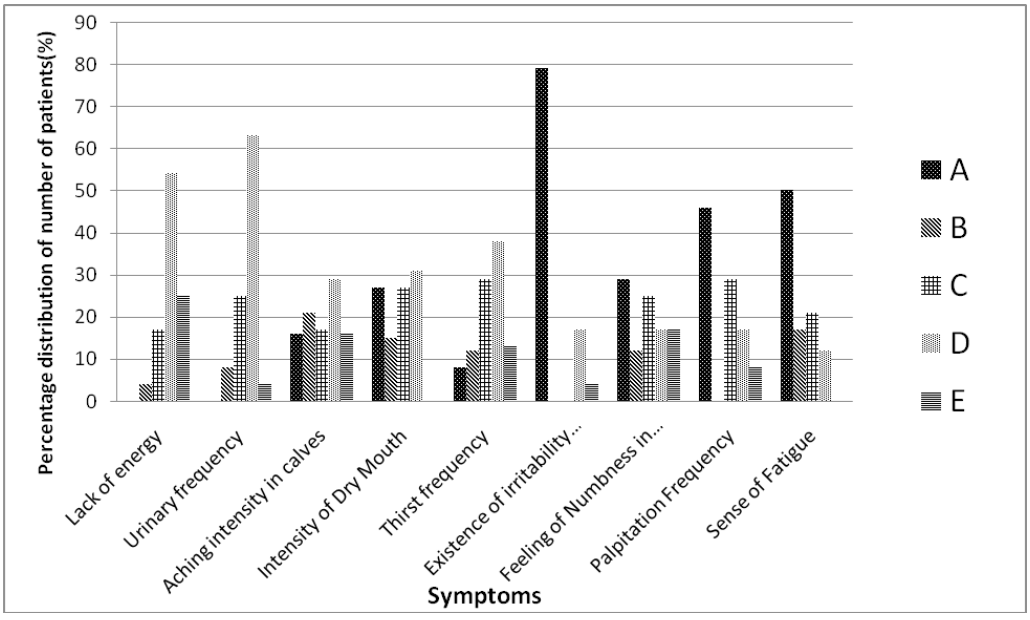


Fig. 1(a) : Represents the intensity of symptoms before the yoga therapy.

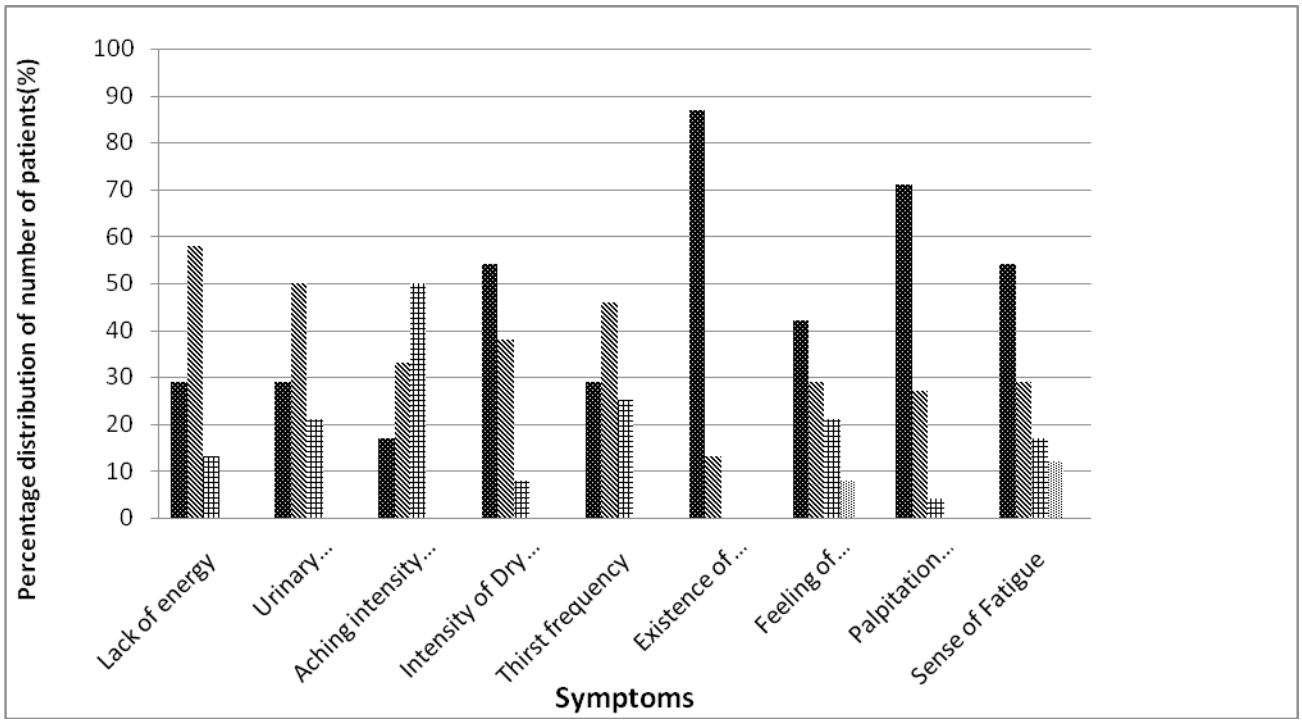


Fig. 2(b) : Represents the intensity of the symptoms after the 45 days of yoga therapy.

Noradrenaline and Epinephrine which leads to increase in heart rate, force of contraction and increased peripheral vascular resistance. In addition, SNS stimulates the release of renin which in turn increases Angiotensin II and Aldosterone secretion causing sodium and water retention. The overall effect on cardiovascular system (CVS) contributes to the increased blood pressure (11).

Low blood glucose level due to fasting is the normal stimulus for Glucagon. During periods of stress, trauma or severe exercise, the increased release of Adrenaline, stimulates the secretion of Glucagon even in euglycemic state, in anticipation of increased glucose use (11). Adrenaline and Glucagon stimulate gluconeogenesis form glycogen store in liver and adipose tissue. Adrenaline supresses the release of insulin, while glucagon antagonizes the effect of insulin, resulting in severe hyperglycemia. Glycogenolysis, gluconeogenesis and decreases utilization of glucose in muscle and adipose tissue causes Insulin Resistance (13). There is increased release of other anti-insulin hormones like corticosteroids, growth hormone which causes neoglucogenesis, decrease peripheral utilization of glucose leading to persistent hyperglycemia (14). In diabetic patients, gluconeogenesis is induced by stress hormones (Adrenaline and Glucagon) contributes to hyperglycemia. Hyperglycemia leads to changes in osmolarity of body fluids, intracellular acidosis and increased production of free radicals (ROS) (8, 9, 10).

RBCs are highly susceptible to free radical damage due to high concentration of oxygen and haemoglobin. Normal mature RBC lacks mitochondria therefore it is completely dependent on glycolysis for production of ATP which is required to meet the metabolic needs of RBCs. The circulating RBCs have effective antioxidant systems like reduced glutathione pool to protect the cell form oxidative damage. Glutathione also helps to maintain reduced state of sulphydryl groups in protein and haemoglobin in RBCs. In case of oxidative stress conditions like diabetes, the glutathione pool gets depleted exposing the RBCs to oxidative stress. Pyruvate kinase converts phospho enol pyruvate to pyruvate. This is third irreversible reaction of glycolysis producing ATP (adenosine tri phosphate). RBCs are completely dependent on this reaction for the production of ATP due to lack of

mitochondria. Decreased ATP Production causes alteration in RBC cell membrane leading to changes in the shape and flexibility of RBC's (crenated edges).

Oxidation of sulphydryl groups in protein incuding haemoglobin forms denatured proteins that forms insoluble masses of haemoglobin called Heinz bodies that attach to cell membrane. This alteration in RBCs leads to premature death and lysis resulting in haemolytic anaemia (2). The circulating inflammatory mediators like prostanoids (PGF2α) which are synthesized non enzymatically due to ROS also causes damage to RBC cell membrane and haemoglobin.

Yoga therapy which induces asanas and prayanama relieves mental stress, increase blood flow and oxygenation to all the tissues. This reduces the sympathetic over activity, release of stress hormones, production of reactive oxygen species and the synthesis of inflammatory mediators. Therefore, yoga therapy decreases insulin resistance, control hyperglycemia and correction of haemolytic anaemia.

Conclusion

It can be concluded from this study that Regular yoga practice is very effective in minimizing the oxidative stress induced damage in RBC morphology and also beneficial in improving glycemic parameter, blood pressure and body mass index with potential to minimize disease complication. This may have direct impact on the dose reduction of hypoglycemic and hypotensive drugs of the patient which requires further study. These findings suggest that yoga Intervention has certain therapeutic value in Patients with type 2 diabetes with hypertension by reducing the symptoms of the disease status effectively.

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Original Article

Estimation of Lung Functions and Risk of Developing Obstructive Sleep Apnoea in Wind Instrument Players

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Abstract

Introduction: Decreased incidence of snoring and obstructive sleep apnoea in wind instrument players has been shown by a few studies, probably due to an increased tone of respiratory muscles. Hence their lung functions were evaluated and their risk of developing Obstructive sleep apnoea was assessed.

Methodology: Test subjects (n=64) belonged to high resistance wind instrument category and controls (n=65) included subjects who did not play any form of wind instrument. Based on Berlin questionnaire subjects were divided into high or low risk. Lung functions were evaluated and statistical analysis was done using student t test and chi square test.

Results: There was no difference in MVV values (P=0.63) between the tests and controls. More number of test group subjects belonged to the low risk group as compared to the controls (P=0.000*) according to the Berlin scores. Pearson's correlation showed no association between MVV and Berlin score (r=0.062, P=0.63).

Conclusion: There is no association between improved lung functions and reduced risk of developing OSA although OSA risk is reduced in wind instrument players. Hence wind instrument playing may be considered as an option to reduce the risk or treat obstruction in sleep apnoea.

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Introduction

Obstructive sleep apnoea is a condition characterized by snoring, sleep apnoea and day time sleepiness (1, 2) and it is also associated with various disorders like stroke, hypertension, coronary artery disease

etc. (3-7). It is a common condition caused due to floppiness of the upper airway (8, 2). It is frequently suspected in many snorers but left undiagnosed and untreated (9), especially in developing countries like India because of the costs involved in the diagnostic and therapeutic procedures. But the prevalence of Obstructive sleep apnoea in India is 13.7% according to a previous study (10). Another study from India has reported a prevalence of 9.3% (11) and both the studies are from North India. According to another study the prevalence of OSA is similar in both Indian and western population (12).

It is still not clear as to what exactly leads to this floppiness. Upper airway collapsehas been postulated as a reason for OSA (8, 2). Upper airway muscles are kept patent during sleep inspite of muscle atonia which occurs in REM sleep. The pharyngeal and other upper airway muscles are found to be tonically active during sleep which helps in keeping the upper airway dilated and partially patent. The tone of these pharyngeal muscles is also modified by chemoreceptor reflex mechanisms. Snoring leads to trauma to upper airway muscles and results in denervation of these muscles thereby rendering them ineffective to respond to negative airway pressure during deep inspiration (2, 13, 14).

In one of the studies done in western countries, playing of wind instruments was used as a therapeutic measure in snorers and sleep apnoeic patients and they have shown promising results (15). The reason postulated for the decreased incidence of snoring and sleep apnoea in wind instrument players is an increased tone of respiratory muscles thereby preventing the collapse of the upper airway. Some of the studies have shown that wind instrument players have better lung functions compared to other musicians including vocalists inspite of increased incidence of chronic upper airway problems in them while other studies have reported controversial results (16-19).

Hence in this study we decided to study the lung functions in wind instrument players and then assess their risk of developing Obstructive sleep apnoea with the idea of studying the relationship between the two. The management of obstructive sleep apnoea includes surgical correction and continuous positive airway pressure (CPAP) (20). Also the diagnosis involves polysomnography which is really cumbersome for the patient. Hence OSA is left undiagnosed and untreated inspite of increasing prevalence of OSA in our community. Therefore this study hypothesis if proven can serve as a simple and cheap procedure which can be advocated to all snorers to reduce the risk of developing Obstructive sleep apnoea in them.

Methodology

This is an experimental study and was approved by the Institutional ethical committee. Subjects were mainly from villages in and around Madurai. It was decided to study 100 wind instrument players and equal number of controls at the start of the study. But due to time constraint and lack of consent from wind instrument players in and around Chennai the study was performed only in 64 subjects in test group and 65 subjects in control group. Most of the test group subjects belonged to the nathasvaram category, a high resistance wind instrument and they have been playing the instrument for nearly more than ten years. A few of them belonged to trumpet and clarinet category (Table I). Control group included subjects who did not play any form of wind instrument and singers were also excluded from the control group.

TABLE I: Type of wind instrument and number of subjects playing each.

Nathasvaram	*Trumpet*	*Clarinet*
45	10	10

n=65

All the subjects were asked to fill up the study questionnaire which also included the Berlin questionnaire after obtaining their written informed consent to take part in the study. Berlin questionnaire is a standardised questionnaire used for assessing the risk of obstructive sleep apnoea in community studies which assesses the risk based on three categories- snoring, day time sleepiness and presence of hypertension (21, 22).

Based on the scores the subjects were divided into either high risk or low risk.

High risk: Two or more categories positive

Low risk: One or no category positive

Category 1 is positive when the score is two or more

Category 2 is positive when the score is two or more

Category 3 is positive if the answer to question 10 is YES

Lung functions were evaluated using the Helios spirometer and analyzed using RMS polyrite software.

Statistical analysis was done using SPSS software version 11, Minitab and MS excel. Student t test was done to compare the means of the pulmonary function tests of the two groups and chisquare test was done using Minitab to study the difference between tests and controls' risk of developing Obstructive sleep apnoea. Box plots were done using SPSS software to analyze the distribution of data.

Null hypothesis of this study was "There is no difference between the pulmonary function tests and the risk of obtaining obstructive sleep apnoea in both the test and control groups."

Results

Of the spirometric values percentage predicted FVC, FEV1, FEV1/FVC, MVV were used for analysis. Results are presented as mean±SD. Box plots were done to analyze the distribution of these values about the mean (Figs. 1, 2). It is seen from the box plots that there is not much difference between the test and control mean values and their distribution looks similar. Statistical tests were done using student t test and p values are presented in Table-IIA. It is seen that the mean FEV1, FEV1/FVC were reduced in tests as compared to controls though statistical significance was seen only in FEV1/FVC ratio. Mean FVC value is significantly higher in tests compared to controls. MVV values were almost same in tests and controls and both of them show high scores. Subgroup analysis was also done to find out the difference between smokers and non-smokers in both the tests and controls (Table-IIB) and statistical test,

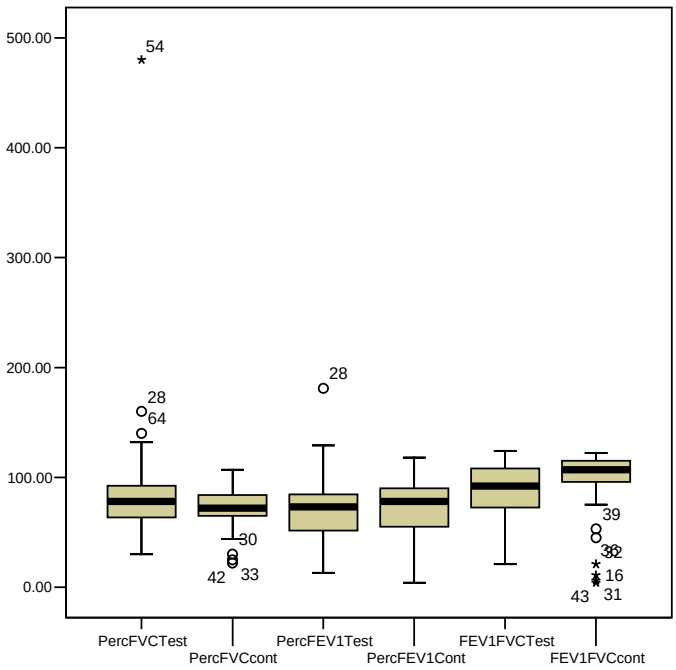

Fig. 1: Box plots showing the distribution of percentage predicted FVC, FEV1, FEV1/FVC values of both tests and controls (circles indicate the outliers).

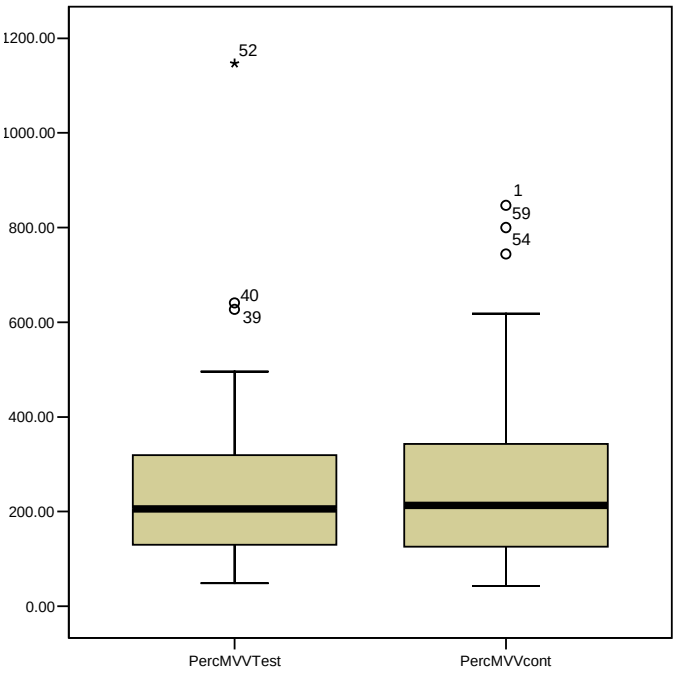

Fig. 2: Box plots showing the distribution of percentage predicted MVV values of tests and controls (circles indicate the outliers).

ANOVA with post hoc analysis (Bonferroni correction) was done but significance was obtained only between FVC in smoker-test and non-smoker test (0.007), smoker-control and non smoker-test (0.005), non smoker-test and non smoker-control (0.004). Though significance was not obtained for FEV1/FVC in smoker-test and non smoker-test (0.08) it was close to 0.05 in the post hoc test. Scoring was done using the Berlin questionnaire for the risk of developing OSA and the subjects were divided into either low risk or high risk and the results are tabulated (Table-III). It is seen that more number of test group subjects belonged to the low risk group as compared to the controls (Fig. 3 and 4). Chisquare test was

TABLE IIA : Mean PFT values of wind instrument players and controls.

Parameters	*Percent predicted FVC-test*	*Percent predicted FVC-control*	*Percent predicted FEV1-test*	*Percent predicted FEV1-control*	*Percent predicted FEV1/FVC-test*	*Percent predicted FEV1/ FVC-control*	*Percent predicted MVV-test*	*Percent predicted MVV-control*
Mean	86.7± 55.63	72.37±17.21	71.02±28.21	72.46±25.62	88.02±26.06	99.28±27.44	248.31±180.41	263.97±186.70
Student t test		0.05*		0.76		0.02*		0.63

*Indicates p<0.05; n=64 in test group and 65 in control group.

TABLE IIB : Mean PFT values of smokers and non smokers in tests and controls.

Parameter	*Test*		*Control*	
	Smokers	*Non-smokers*	*Smokers*	*Non-smokers*
FVC (ml)	75.57±23.22	111±90.12	73.37±14.23	71.2±20.33
FEV1	68.43±27	76.7±30.65	75.66±21.78	68.73±29.43
FEV1/FVC	90.82±24.44	81.85±29.01	100.46±25.56	97.9±29.87
MVV	212.4±113.63	323.7±260.39	250.14±153.04	280.1±221.27
Low Risk of developing OSA	42	17	19	17
High Risk of developing OSA	3	2	16	13

TABLE III : Number of OSA high and low risk subjects using the Berlin questionnaire.

Risk of developing OSA	*Tests*	*Controls*
Low risk	58	36
High risk	5	29

P=0.001* (n=63 in test group and 65 in control group).

TABLE IV : Number of smokers and non smokers in tests and controls.

Smoking status	*Tests*	*Controls*
Smokers	44	35
Non smokers	19	30

P=0.063 (n=64 in test group and 65 in control group).

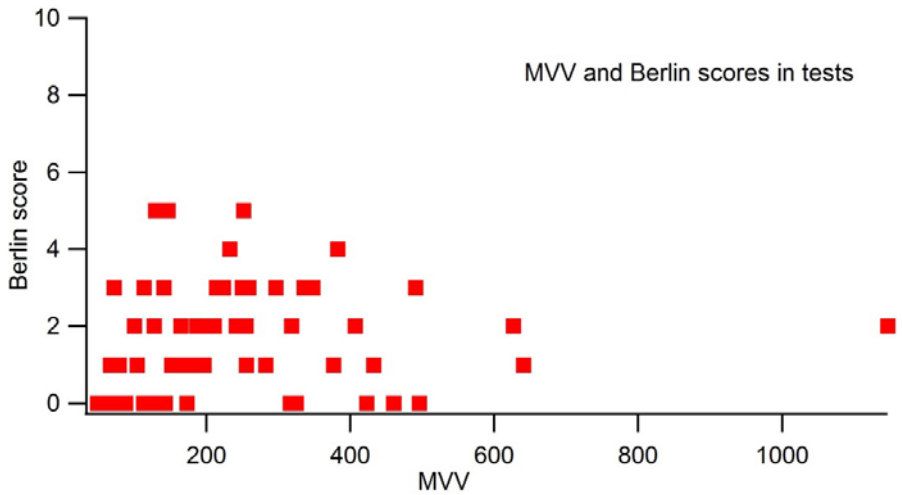

Fig. 3 : Graph showing percentage predicted Maximum voluntary ventilation against Berlin scores in test subjects.

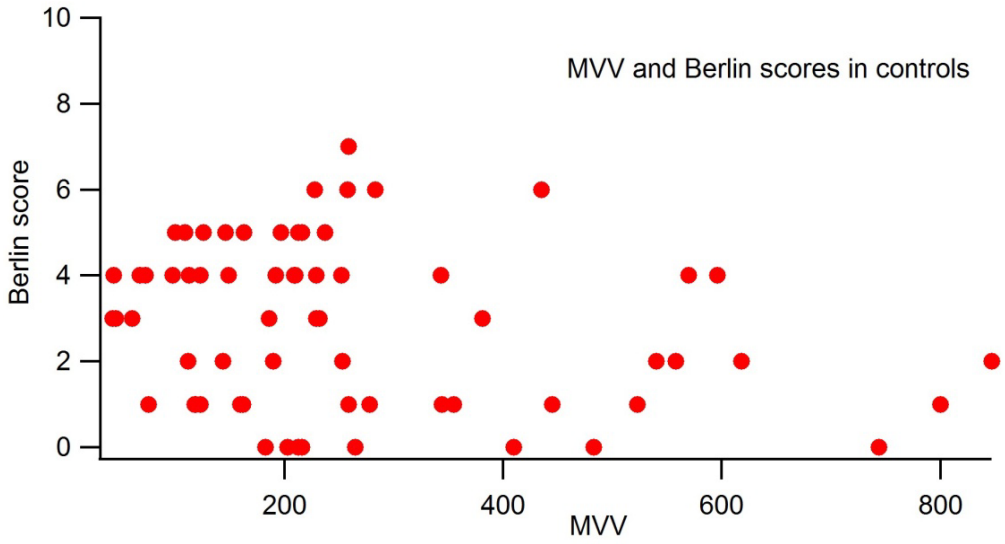

Fig. 4: Graph showing percentage predicted Maximum voluntary ventilation against Berlin scores in control subjects.

done to compare the scores of both the tests and controls (Table-III) and significant difference was observed between them (p=0.000*). Chisquare test was also done for comparing the smoking status in tests and controls and it is seen that though statistical significance (p=0.06) was not obtained there is a higher number of smokers in tests as compared the controls (Table-IV). Pearson's correlation was done to study the association between MVV which is an indicator of respiratory endurance and Berlin score and there was found to be no association (r=0.062, p=0.63) between the two. Relative risk (RR) for our study is 0.18 and the 95% confidence interval for the RR is 0.07-0.43.

Discussion

The results clearly show that wind instrument players do not have better lung functions compared to controls but they have low risk of developing obstructive sleep apnoea (Table-II and Table-III). Though they have a reduced FEV1/FVC values as compared to controls their mean values fall within normal range (Table-II). The controls show better pulmonary functions as compared to tests probably because of the less number of smokers in controls (Table-IV). Though there are studies which show conflicting results of pulmonary functions in wind instrument players majority of them have concluded there is not much difference in pulmonary functions of wind instrument players as compared to controls (16-19).

Inspite of no difference in lung functions the risk of developing OSA is reduced in wind instrument players compared to controls (Table-III) and this shows lower airways have nothing to do to decrease the risk of OSA in wind instrument players. Relative risk less than one indicates less risk of developing OSA in the wind instrument players compared to controls. There are only a few Western studies which have analysed the risk of developing OSA in wind instrument players and have shown conflicting results (23, 24). One study concluded nil association between wind instrument playing and lesser risk of OSA (23) whereas the other study showed lesser risk of OSA especially in musicians playing high resistance wind instruments (24) and this they attribute to the different sequence of movements of the muscles associated with the different types of instruments.

It has been also shown in a previous study that there is no correlation between decreased lung functions and risk of developing OSA (25). This study also shows that lung functions and OSA are not related and are independent of each other. But what could be the reason for reduced risk of developing OSA in the wind instrument players? The lower risk may be due to the fact that wind instrument players

have an increased tone of upper airway muscles as a result of training thereby preventing collapse of the upper airway (24). Studies have shown that per cutaneous electrical neuromuscular stimulation of the genioglossus resulted in increased diameter of the hypopharyngeal airway thereby reducing snoring (26, 27). Oropharyngeal exercises have been shown to improve the tone of upper airway muscles and reduce the risk of OSA (28). One of the studies also shows that a few months of training with didgeridoo wind instrument resulted in reduced OSA symptoms (15).

Therefore wind instrument playing has a lower risk of developing OSA by improving the tone of upper airway muscles.

Conclusion

There is no association between improved lung functions and reduced risk of developing OSA. At the same time OSA risk is reduced in wind instrument players as a result of increased tone of upper airway muscles. Hence wind instrument playing may be considered as an option to reduce the risk or treat obstruction in sleep apnoea.

Limitations of the study and future plans

This study couldn't exclude the smokers in both the groups because of the limited number of subjects willing to participate and also due to time constraint. Also people from different socio economic background were not studied. The sample size should have been much bigger but due to time constraint it was kept small.

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Original Article

Physical Fitness of Male Eastern Indian Judo Players

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Abstract

The present study was aimed to evaluate the fitness profile and physiological characteristics of Eastern Indian male Judo players.

State level male Judo players (n = 60, age: 22.87±1.51 yrs) were recruited from different sports academies of Kolkata, India. Sedentary control subjects (n = 60, age: 22.56±1.53 yrs) were recruited from the same area. Physical and fitness parameters (VO_{2max}, high intensity effort, agility, flexibility and body composition) were measured by standard methods.

Fitness parameters were significantly (p<0.001) better and % lean body mass (%LBM) was significantly (p<0.001) higher in Judo players whereas %fat and total fat (TF) were significantly (p<0.001) lower in Judo players than the control group.

Therefore, Judo seems to be not only a method for self defence but also it is important for promotion of health. Hence, fitness instructors may use this martial art as a beneficial mean to promote health and physical fitness.

Introduction

Sedentary life style increases the fat deposition and weight gain. It decreases physical fitness, aerobic capacity as well as bone density (1). Regular exercise improves cardiorespiratory fitness, muscular endurance, muscular strength, flexibility and also helps to maintain optimal body composition (1, 2). Judo is an Olympic event and it is one of the most popular martial art practiced worldwide (3). Regular practicing of Judo improves physical fitness in sedentary population (3, 4). Awareness about the martial arts, especially in case of Judo has increased radically in the recent past (5).

Different forms of martial arts have similar characteristics but they have different forms of actions (6). Throwing and gripping techniques are used in the high intensity sport like Judo. It is one of the

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most popular Japanese martial art in which competitors use balance and body weight, with little physical effort, to throw or grip each other in lock (7, 8, 9). Anaerobic capacity, strength, aerobic power and body composition have been considered the main characteristics developed in Canadian Judo players (10).

It is evident from earlier reports that regular practice of Judo improves one's fitness profile and also protects from potential health risks (1, 2). Fitness profile and physiological characteristics of Judo players have been reported from different countries but to our knowledge only one study has reported about the fitness profile of South Indian Judo players (3). We did not come across similar studies in eastern Indian Judo players. Hence the present study was aimed to evaluate the important fitness profile parameters in young male eastern Indian Judo players and also to compare the data with subjects leading sedentary lifestyle.

Materials and Methods

State and national level male Judo players (n=60, age: 22.86±1.51 yrs, range 21–25 yrs) with at least five years of regular involvement in high intensity Judo training were recruited from different reputed sports academies of Kolkata, India. They participated in the regular Judo training in the morning and evening sessions at least for five days a week. Sedentary control subjects (n=60, age: 22.61±1.39 yrs, range 20–25 yrs) with same socio-economic background were randomly selected from the localities where the players reside. The subjects who did not take part in any physical conditioning programme were considered as sedentary control subjects. Subjects were neither suffering from any disease nor under any medication during the study period. They had no history of major diseases, injury or bone fracture. The study was conducted at temperature ranging between 20–23°C and relative humidity ranging between 40–45%. The study was approved by the Institutional Human Ethics Committee of the Department of Physiology, University of Calcutta and conformed to the Declaration of Helsinki. Written informed consent was taken from all the subjects. The sample size was computed using PS Power and Sample Size calculation version 2.1.30 where power was set at 80 with 95% confidence interval.

Each subject reported in the laboratory at 10 am and they came to the laboratory for three times with a gap of at least 7 days in between two consecutive days of visit to ensure complete abolishment of fatigue generated due to previous day's trial. They were explained and familiarized with the experimental procedure on the first visit. The fitness profile parameters were measured during the second and third visits. Subjects took rest for half an hour on arrival in the laboratory during which the entire experimental protocol was again explained to them to allay apprehension. Body weight, height, heart rate, blood pressure, agility and high intensity effort (HIE) were measured in the second visit whereas body composition and cardiorespiratory fitness (VO_{2max}) were measured during the third visit. Body height and body mass were measured to an accuracy of ±0.50 cm and ±0.1 kg, respectively, by using a weight measuring instrument fixed with a height measuring rod (Avery India Ltd., India) with the subject standing barefoot and wearing minimum clothing. The subjects refrained from any energetic activity on the days of evaluation and took light breakfast at least 3 hrs before the test. The body surface area (BSA) and body mass index (BMI) were calculated by using the following equations: (11, 12)

$$BSA\ (m^2) = (Body\ mass)^{0.425} \times (Body\ height)^{0.725} \times 71.84$$

$$BMI\ (kg/m^2) = Body\ Mass\ (kg)/(Body\ Height\ in\ meter)^2$$

Determination of Body Composition: (13)

A skin-fold calliper with constant tension (Holtain Ltd., UK) was used to determine the body composition by using the following formulae:

$$Body\ density\ or\ BD\ (gm.cc^{-1}) = 1.10938 - 0.0008267X_1 + 0.0000016X_1^2 - 0.0002574X_2$$

(X_1 = sum of chest, abdominal and mid–thigh skinfolds, X_2 = Age in nearest yrs)

$$\%Fat = 495/BD - 450 (14)$$

Total body fat, lean body mass (LBM) and percentage of LBM (%LBM) were calculated from the following equations :

- Total Fat (TF)(kg) = %Fat/100 × Body Mass (kg)
- % Lean Body Mass (%LBM) = 100 – %Fat
- LBM (kg) = Body Mass (kg) – Total Fat (kg)

Direct estimation of VO_{2max}: (15)

The direct estimation of VO_{2max} was performed according to the protocol of Dalui and Bandyopadhyay (15). Muller's magnetic brake bicycle ergometer (Model of Max Plank Institute of Ergology, Germany) was used for this study. All the subjects were warmed up at a submaximal intensity of 75 watt for 5 minutes. Immediately after performing the warm up, the intensity was increased to the first incremental intensity of 155 watt and thereafter the intensity was increased by 25 watt every 3 min until the subject stopped due to exhaustion. In the present study, the oxygen uptake was considered maximum when peak heart rate was greater than 180 beats per min and also by levelling off, i.e., when no further increase in oxygen uptake took place despite further increase in work load.

Low resistance high velocity Collin's Triple "J Type" plastic valve was used for the collection of expired gas by open circuit method. The valve remained connected with the Douglas Bag (150 L) and the expired gas was collected at the last minute of final intensity of exercise. The volume of expired gas was measured in a wet gasometer (Toshniwal, Germany, CAT. No. CG05.10) and the aliquots of gas samples were analyzed in a Scholander micro-gas analysis apparatus following the standard procedure. The peak heart rate was recorded manually from the time taken for 10 carotid pulsations immediately following the cessation of exhaustive exercise (15).

Determination of High intensity effort (HIE): (15)

HIE was determined by 60 yard dash shuttle run test which is a steps shuttle of progressing distances. Three marker cones were placed at the yard lines 5 yards away from each other. The athlete started from one end, ran 5 yards and returned to the start point, 10 yards and back, then 15 yards and reached back the start line. A total of 60 yards was completed. It was ensured that the subject touched the line with their hand in each turn i.e. total for five times. The duration of this test was recorded in seconds.

Measurement of agility by shuttle runs method: (15)

The subject was asked to run back and forth between two parallel lines as swift as possible. Two lines were put up 30 feet apart. Two wooden blocks were kept behind one line which was opposite to the starting line. The subject started running from the starting to the other line and picked up one block and returned it to place behind the starting line, then returned again to pick up the next block, then ran back with the block to place it back across the starting line. The time taken for the entire running period was recorded in seconds by a stop watch.

Measurement of flexibility by modified sit and reach test: (15)

The subject sat on the floor with legs stretched out straight in front with shoes removed. The soles of the feet were positioned flat against the box. Both knees were locked and pressed plane to the floor. With the palms facing downwards, and the hands on top of each other or side by side, the subject was asked to reach ahead along the measuring line as far as possible. It was ensured that the hands remained at the same plane, not one reaching further forward than the other. After some practice the subject reached out and held that position for at one or two seconds while the distance was recorded. No jerky movement was allowed.

Statistical analysis

Data have been presented as mean±SD. Two-tail student's t-test was performed to test the significance of difference between mean values recorded in Judo group and sedentary control group.

Results

Values of age, body height, body weight, BMI, BSA, pre-exercise heart rate, pre-exercise systolic and diastolic blood pressure are presented in Table I. Age did not show any significant difference between the groups. Body height depicted significantly (p<0.05) higher value in the Judo players while other parameters were significantly lower in them compared to the sedentary group (Table I). Both the groups belonged to the normal range of BMI values.

The value of chest, abdominal and mid-thigh skinfolds and the sum of skinfolds were significantly (p<0.001) lower in Judo players than the sedentary group (Fig. 1).

Different components of body composition, flexibility, agility, HIE and VO_{2max} are tabulated in Table II. BD, %LBM, flexibility, agility, HIE, and VO_{2max} depicted significantly (p<0.001) higher values in Judo group, but %fat and TF exhibited significantly (p<0.001) lower values in Judo players than the sedentary group.

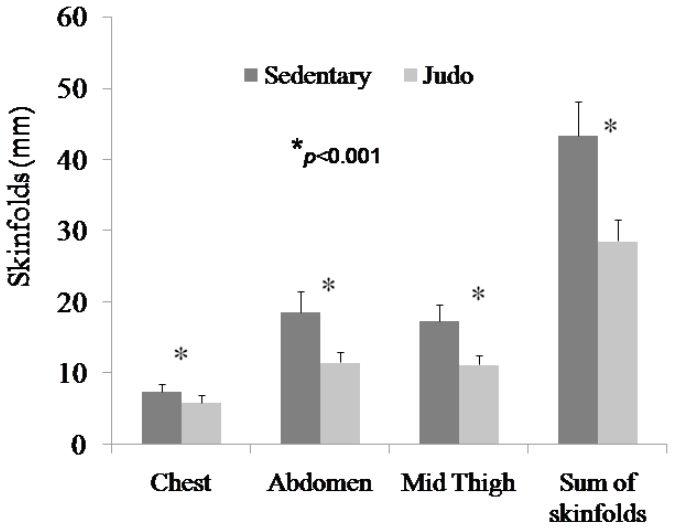

Fig. 1: Different skinfold parameters and sum of all the three skinfolds in sedentary group and Judo Players.

Discussion

The aim of this study was to evaluate the effect of Judo training on selective fitness profile parameters in Indian young male Judo players. Results demonstrated that Judo training influenced the body composition, aerobic and anaerobic power (as depicted from their

TABLE I: Physical and physiological parameters of the subjects.

	Age (yrs)	*Body height (cm)*	*Body weight (kg)*	*BMI (kg/m^2)*	*BSA (m^2)*	*Blood Pressure (mm of Hg)*		*Pre-exercise Heart Rate ($beats.min^{-1}$)*
						Systolic	*Diastolic*	
Sedentary (n=60)	22.52±1.53	164.99±2.63	61.56±3.34	22.62±1.28	1.68±0.05	118.31±4.77	78.45±8.68	84.71±5.60
Judo (n=60)	22.86±1.51	166.03±1.72*	58.93±2.83**	21.37±0.78**	1.65±0.04*	108.47±3.92**	75.43±5.4*	69.10±4.20**

Values are expressed mean±SD, *p<0.05, **p<0.001.

TABLE II: Values of body composition, Agility, flexibility and HIE and VO_{2max}.

	Body density ($gm.cc^{-1}$)	*%fat (%)*	*Total body Fat or TF (kg)*	*%LBM (%)*	*LBM (kg)*	*Agility (sec)*	*Flexibility (cm)*	*HIE (sec)*	*VO_{2max} ($ml.min^{-1}.min^{-1}$)*
Sedentary (n=60)	1.07±0.003	12.28±1.41	7.59±1.19	87.71±1.41	53.95±2.45	12.08±0.72	20.49.±4.92	9.70±0.88	41.05±4.65
Judo (n=60)	1.08±0.002**	7.84±0.90**	4.64±0.70**	92.16±1.41**	54.29±2.21	11.70±0.46**	37.01±3.23**	9.14±0.57**	53.18±3.39**

Values are expressed mean±SD, *p<0.05, **p<0.001

cardio-respiratory fitness and HIE, respectively), agility and flexibility scores in the studied population.

Subjects of both the groups were in normal range of BMI, heart rate and blood pressure (Table I). Age did not show any inter–group variation but body height, weight, BMI, BSA, heart rate and systolic and diastolic blood pressure were significantly higher in the sedentary control group. Existence of significantly lower values of body weight, BMI, BSA, heart rate and systolic and diastolic blood pressure among the Judo players might be attributed to their regular participation in the Judo training (Table I).

Judo is one of the dynamic, high intensity intermittent sport that requires complex skills and planned excellence for success (16). Muscular strength plays an important role towards the success of Judo athletes (16). Judo athletes of the present study had significantly (p<0.001) lower value of %fat than their sedentary control group as also reported in earlier studies in Tunisian Judo athletes (16). Value of %fat observed in the Judo players of the present study was lower than their Canadian (12.3%), Japanese (16.2%), Brazilian (13.7%) and North American (8.3%) counterparts (17). But Tunisian male national Judo athletes (25.1±3.7 yrs) depicted insignificant change in %fat following Judo training (18) (Table III). Body fat percentage is a key determinant of Judo performance as established in earlier studies which showed significant negative correlation between %fat and performance in different categories (state level, national level, etc.) of Judo athletes. This was most probably due to reduction in %fat is associated with a concomitant increase in aerobic capacity (3, 17).

Table III compared the values of different fitness profile parameters of Judo players reported in different populations with the values recorded in the present study.

The %LBM was significantly higher in Judo players than the control group but LBM (kg) did not show any intergroup variation. Fat mass (kg) and %fat were significantly higher in the control group. The values of fat mass, %LBM and LBM of the presently studied Judo athletes could not be compared due to unavailability of data in other populations. However, the present result supported the common belief that judo players try to maximize their lean body mass and minimize the fat mass (19).

Caucasian adult male Judo athletes had significantly higher muscle mass and lower %fat than non-athletes (20). It had been accepted that weight bearing forms of energetic exercises were associated with gaining higher muscle mass and the potentiated the benefits of %fat (20). Although higher than in the present study, one study reported that Roman Judo players had lower fat mass (7.5±3.6 kg) than the control group (11.6±5.7kg) (20). They hypothesised that the Judo training played the beneficial role to increase the muscle mass in Judo athletes (20).

TABLE III : Comparison of present data with the earlier studies.

Authors	*Population*	*%Fat (%)*	*VO_{2max} ($ml^{-1}kg^{-1}min^{-1}$)*	*HIE (sec)*	*Agility (sec)*	*Flexibility (cm)*
Ouergui et al. (2014)	Tunisian	–	58.7±5.2	–	16.2±1.0	18.2±0.5
Toskovic et al. (2002)	Spanish	–	52.8±7.9	–	–	–
Borkowski et al. (2001)	Polish	–	57.6±4.6	–		–
Taylor et al. (1981)	Canadian	–	57.5±9.5	–		–
Tumilty et al. (1986)	Australian	–	53.2±5.7	–	–	–
Kim et al. (1996)	Korean	–	62.8±5.9	–	–	–
Callister et al. (1991)	American	8.3±1.0	55.6±1.8	–	–	–
Franchini et al. (2011)	Brazilian	–	48.3±8.1	–	–	–
Taylor and Brassard (1981)	Canadian	12±3.9	–	–	–	–
Franchini et al. (2007)	Japanese	16.2±5.7		–	–	–
Franchini et al. (2007)	Brazilian	13.7±5.2		–	–	–
Saraiva et al. (2014)	Brazilian	–	–	–	–	25.83±3.07
Present study	Indian	7.84±0.90	53.18±3.39	9.14±0.57	11.70±0.46	37.01±3.23

Values are expressed mean±SD.

Judo is a suitable exercise to improve cardiorespiratory fitness (18). Toskovic et al., (20) reported that training of martial art had beneficial effect on aerobic fitness (21). Ouergui et al. (18) demonstrated significantly (p<0.001) greater value of VO_{2max} (58.7±5.2 ml $kg^{-1}min^{-1}$) of young (age 20.9±1.4 years) Tunisian Judo players than their control group as also reported in Spanish young (19.7±1.9 years) male Judoka (22). All these findings were in agreement with the present study. A comparative account of VO_{2max} values in different populations is given in table 3. Significant improvement (13.2±6.0%) in aerobic fitness was noted following 5 weeks of kickboxing training (27). Contradictory findings were also reported in some other studies that revealed insignificant change in cardiorespiratory fitness following Judo training (25, 28). Franchini et al. (17) reported that male Brazilian Judo team exhibited (age 25.6±4.0 years) medium aerobic capacity (48.3±8.0 $ml.kg^{-1}.min^{-1}$) tested by Cooper Test method and the values were lower than the presently studied Judo players (53.18±3.39 $ml.kg^{-1}.min^{-1}$). However, Brazilian national and international Judo athletes (age 22.3±3.6 years) had better VO_{2max} (63.0±10.3 $ml.kg^{-1}.min^{-1}$) than Brazilian Jiu-Jitsu players (49.4±3.6 $ml.kg^{-1}.min^{-1}$) and present study (31). Laskowski et al. (29) correlated changes in heart morphology induced by long term (10 yrs) Judo training with an increase in VO_{2max} in Polish male (age: 22.1 years) and female (age: 19.4 years) players. Bonato et al. (30) concluded that aerobic fitness of elite Italian male and female Judokas was further improved by adding 12 weeks specific aerobic training programme. French Judo athletes (age 24.4±0.9 years) had better VO_{2max} (55.0±2.9 $ml.kg^{-1}.min^{-1}$) score than the presently studied Judo athletes (31).

Cardio respiratory fitness of Olympic Italian male and female Judokas exhibited the VO_{2max} score of 47.3±10.9 and 52.9±4.4 $ml.kg^{-1}.min^{-1}$, respectively (31). The values of both the genders were lower than the present study.

However, regular practicing of other forms of dynamic martial art exercises, e.g., taekwondo, karate, etc. significantly improved the cardiorespiratory fitness (21, 31, 32). Regular involvement in Judo training probably reduced the body's %fat and increased the LBM that in turn might have helped the presently studied Judo players to achieve significantly higher value of VO_{2max} than their sedentary control subjects.

Judo is a dynamic, high intensity intermittent sport that requires complex skills and tactical quality for achievement (16, 17, 18). Maximal strength plays the beneficial role for success in Judo athletes (16, 17). Tunisian young male Judo athletes (20.9±1.4 years) had significantly (p<0.001) better speed than their control group (18). Sterkowicz et al. (28) reported significant improvement of anaerobic performance after Judo training programme. This observation was similar to the present investigation where significantly (p<0.001) higher value of HIE was depicted than their sedentary counterparts (Table II). These are due to the fact that the kickboxing is distinguished by brief high intensity techniques (punches and kicks) where the effort is possibly maintained by the Adenosine Tri Phosphate-Creatine Phosphate system (27). Specific training type and techniques probably helped the Judo players of the present study to achieve better HIE value.

Agility is the skill that promotes rapid and exact movement of the body or body parts involving majority of the muscles of that region. Besides innate capacity, training contributes a lot to improve the performance level. Both short term and long term Judo training had similar level of beneficial effect on agility, indicating that Judo training influences the agility irrespective of the training duration (19). Present study depicted significantly (p<0.001) higher agility score in Judo players than their sedentary control counterparts (Table II). This finding was similar to the findings in young (20.9±1.4 yrs) Tunisian male Judo athletes who were significantly higher agile (16.2±1.0 sec) than their control group (17.8±0.9sec) (18). Judo training comprises of frequent fast stepping and fast displacement which might have helped the Judo athletes to improve their agility in the course of training (27).

Flexibility is a very important parameter in fight training, especially since it is associated with a broader range of motion that contributes in execution of skills and reduces the risks of injury (19, 33). Flexibility depends on number of specific variables,

muscle viscosity, including distensibility of the joint capsule, adequate warm up, and compliance of various tissues such as ligaments and tendons that affect the range of motion (19). Present study depicted significantly higher value of flexibility in Judo players in comparison to their sedentary counterparts as also reported by Katralli et al. (19) and Ouergui et al. (18) in South Indian and Tunisian Judo players, respectively. Flexibility score of the presently studied male Indian Judo players (37.01±3.23 cm) was higher than the Tunisian Judo players (18.2±0.5 cm) and Brazilian Judo athletes (25.83±3.07 cm) (18, 34). More experienced Judo athletes (21.5±1.63 cm) had significantly higher flexibility score than their less experienced counterparts (16.2±3.61cm), indicating that the flexibility score of the Judo athletes was influenced by the training duration as also reported in Australian Judokas (19, 35). Katralli et al. (19) attributed such training duration related difference in flexibility score among Judo athletes to their exposure to stretching exercises during the training sessions.

The present study concluded that the Judo training had beneficial effects on fitness profile parameters. Cardiorespiratory fitness, HIE, body composition, agility and flexibility were significantly improved in the Judo athletes. These findings suggested that Judo seems to be not only a method for self defence but also a good method which can offer health promotion and a consequential exercise for improving fitness among young adults. Thus fitness instructors, sports professionals may consider the Judo training program as a beneficial form of exercise to promote physical fitness and for preventing injuries by increasing muscles' flexibility. Present data will also help the coaches to design better training regime to improve the fitness profile of Judo athletes to a greater extent.

Limitation of the study:

Although the dietary practice and fluid intake patterns influence the fitness profile parameters, but it is a shortcoming of the study that these parameters were not evaluated in the present study.

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Original Article

Association Between Neutrophil Lymphocyte Ratio and Cognitive Function in Type II Diabetes Mellitus

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Abstract

Introduction: Systemic (cerebral) inflammation has been attributed in the pathogenesis of cognitive impairment in patients with Diabetes Mellitus. Neutrophil Lymphocyte Ratio (NLR) is a reliable marker for inflammation in various diseases. Hence this study was designed to explore the role of systemic inflammation in the pathogenesis of cognitive decline in diabetes by using Neutrophil Lymphocyte ratio as an inflammatory marker.

Materials and methods: 60 Type II Diabetes Mellitus (T2DM) patients in the age group of 30-60 years were recruited. Their cognitive function was assessed using Modified Mini Mental State Examination (3MS test). HbA1c levels were determined and NLR was calculated as ratio between counts of Neutrophil and Lymphocyte.

Results: The overall prevalence of cognitive impairment among patients with T2DM was 30%. The mean age of the patients with cognitive impairment was 50.8 years. No significant correlation was observed between NLR and cognitive function scores (r = 0.078) in T2DM patients.

Conclusion: No significant association was observed between NLR and Cognitive function in Diabetic patients.

Introduction

Diabetes Mellitus refers to a group of common metabolic disorders that share the phenotype of hyperglycemia (1). It is a complex metabolic disease that affects multiple organs in the body. It is recognised as an important cause of premature death and disability. As per 2014 WHO estimate, globally 422 million adults aged over 18years were living with Diabetes. The global prevalence of Diabetes has grown from 4.7% in 1980 to 8.5% in 2014 (2). India had over 69.2 million diabetics (8.7%) as per the 2015 data (3). Diabetes can lead to many systemic complications among which, a less addressed and not as well recognised complication is cognitive dysfunction (2). Both Type 1 and Type 2 diabetes mellitus have been associated with cognitive dysfunction (4). In healthy adults, age related cognitive impairment is mostly reported after the age of 60 yrs (5). When compared with those without diabetes, people with diabetes have 1.5 and 1.6-fold greater risk of developing cognitive decline and

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dementia in future respectively (6). Many factors such as hyperglycemia, hypoglycemia, insulin resistance and genetic factors have been implicated in the pathogenesis of cognitive dysfunction in diabetes. Recently, systemic or cerebral inflammation has been suggested to play a role in the pathogenesis of cognitive decline in diabetes (7, 8). Diabetes care focuses on independent self care by the patients to achieve glycemic targets and minimize vascular complications. The intactness of important functional domains of cognition like executive function, information processing and memory are required to direct diabetes self care (7). Thus, cognitive dysfunction has an important impact on the quality of life in patients with diabetes. If left undiagnosed, it may progress to dementia.

Although many researchers have examined cognitive dysfunction in diabetes, still more studies are needed to understand the mechanisms of this complication in order to develop strategies for prevention and management. Many studies have focussed on finding the association between duration of diabetes, glycemic control and cognitive dysfunction. Only few studies have reported the role of inflammation in the pathogenesis of cognitive decline in diabetes. Diabetes is associated with chronic low grade inflammation (7, 9). Systemic inflammation can be measured by a variety of haematological markers. Among which, Neutrophil Lymphocyte Ratio (NLR) is a reliable marker for inflammation in various diseases which is cheap and readily available. NLR may be used as a prognostic marker for micro and macrovascular complications in diabetes (10, 11, 12). Considering the above factors, this study was designed to explore the role of systemic inflammation in the pathogenesis of cognitive decline in diabetes by using Neutrophil Lymphocyte ratio as an inflammatory marker. By understanding the relationship between NLR and cognition, NLR may be used as an index of cognitive impairment at its early stages of onset which will facilitate early diagnosis and appropriate management of cognitive dysfunction in diabetic patients.

Aim

To find out the association between Neutrophil Lymphocyte Ratio and cognitive function in type II Diabetes Mellitus patients.

Objectives

1. To assess cognition in Type 2 Diabetes Mellitus (T2DM) patients by using 3MS (Modified Mini Mental State Examination) test.

2. To determine NLR and HbA1c levels.

3. To find the association between NLR, HbA1c levels, duration of diabetes and 3MS scores in diabetic patients.

Methods

This is a cross sectional study. 60 patients with T2DM diagnosed according to WHO criteria were recruited from the Department of Diabetology, Government Stanley Medical College and Hospital, Chennai-1. Inclusion criteria were T2DM patients of age 30–60 yrs of both genders with minimum educational qualification of 8th STD and duration of diabetes of not less than 1 year. Exclusion criteria included patients with established diagnosis of dementia, co-morbid conditions that affect cognitive function such as neurological disorders, psychiatric disorders, history of acute / other chronic illness, patients on any other medications (Steroids, Chemotherapy, Antibiotics, Immunomodulators, Neuropsychotropic drugs or any other drugs for chronic diseases).

The study was approved by Institutional Ethical Committee. The purpose, risks and benefits of the study were explained to all the participants and Informed consent was obtained from each of them. A brief history of their education, duration of disease, current treatment and co-morbid conditions were obtained. Cognitive function was assessed using Modified Mini Mental State Examination (3MS). 3MS test is an extension of Mini Mental State Examination. When compared with MMSE, it assesses a broader variety of cognitive domains and covers a wider range of difficulty levels. It offers a brief assessment of the person's attention,

concentration, orientation to time and place, long-term and short-term memory, language ability, constructional praxis, abstract thinking, and list-generating fluency. 3MS score ranges between 0–100 (13).

Venous blood samples (about 3ml) were collected in Ethylenediaminetetraacetic acid (EDTA) tubes for determining HbA1c levels and NLR. Blood samples were analysed in the Department of Biochemistry, Govt. Stanley Medical College within 2 hours after collection. HbA1c levels were determined by high-performance liquid chromatography method using Bio-rad D-10 Hemoglobin Analyzer. Complete blood counts were determined using Sysmex Hematology Analyzer which works on the principle of Fluorescence Flow cytometry method. NLR was calculated as ratio between counts of Neutrophil and Lymphocytes.

Statistical analysis

Data were analysed using IBM SPSS, version 24. An Independent student's t-test was used to compare the means of variables. Pearson's correlation was used to find out the correlation between the variables. p value < 0.05 was considered significant.

Results

A total of 60 patients with T2DM participated in the study. 3MS test was used to assess their cognition. 3 MS score ≤ 77 was defined as cognitive impairment (14, 15, 16). HbA1c levels and NLR were determined. Patients were divided into 2 groups based on 3MS scores into those with normal cognitive function (group 1) and those with cognitive impairment (group 2). Correlations of age, duration of diabetes, HbA1c levels, NLR with 3MS scores were done in the whole group as well as in the group of patients with cognitive impairment. Data are represented as mean±standard deviation (SD).

Baseline characteristics

Baseline characteristics of diabetics and comparison of variables between patients with normal cognitive function and those with cognitive impairment are given in Table I & II respectively.

All patients were in the age group of 30–60 years. Table I shows that the mean age of the patients with cognitive impairment was 50.8 years which was higher than those with normal cognition (47.9 years) but it was not statistically significant (p = 0.5). Males represented a little more than half (54.8%) of the patients with normal cognition and females represented majority (61.1%) of patients with cognitive impairment. The mean years of education was 9.4 years in the patients with cognitive impairment which was lower when compared with those with normal cognition though it was not statistically significant (p = 0.1). No significant difference was found in the mean levels of duration of diabetes among the two groups.

Table II shows that no significant difference was found in the mean levels of HbA1c and NLR among the two groups.

Table III shows the comparison of variables between

TABLE I: Baseline characteristics of Type 2 Diabetes Mellitus patients.

S. No.	*Variable*	*All T2DM patients (n=60)*	*Group 1 - T2DM patients with normal cognitive function (n = 42)*	*Group 2 - T2DM patients with cognitive impairment (n=18)*	*p value (comparison of Group 1 and Group 2)*
1	Age (Years) mean±SD	48.6±7.8	47.9±8.3	50.8±6.3	0.5
2	Gender Males, n (%) Females, n (%)	 30, (50) 30	 23, (54.8) 19, (45.2)	 7, (38.9) 11, (61.1)	
3	Education (Years) mean±SD	10.1±2.0	10.3±2.0	9.4±2.0	0.1
4	Duration of T2DM (Years) mean±SD	5.4±4.3	5.5±4.4	5.4±4.3	0.8

TABLE II : Comparison of variables between diabetic patients with normal cognitive function and those with cognitive impairment.

S. No.	Variable	All T2DM patients (n=60)	Group 1 - T2DM patients with normal cognitive function (n = 42)	Group 2 - T2DM patients with cognitive impairment (n=18)	p value (comparison of Group 1 and Group 2)
1	3 MS score, mean±SD	80.9±9.9	86.0±4.6	68.9±8.4	0.00*
2	HbA1c levels %, mean±SD	9.1±2.2	9.0±2.3	9.2±2.2	0.8
3	NLR, mean±SD	1.7±0.6	1.7±0.6	1.6±0.5	0.6

*p value < 0.01 – highly significant, HbA1c – Glycosylated Hemoglobin, NLR – Neutrophil Lymphocyte Ratio.

males and females. No significant difference was observed in the mean levels of 3MS scores, HbA1c and NLR between males and females, though the mean value of 3MS score was slightly higher in males when compared to females. As shown in Table I, females represented majority (61.1%) of patients with cognitive impairment.

TABLE III : Comparison of variables between males and females with T2DM.

S. No.	Variable	Males (n = 50)	Females (n = 50)	p value
1	3 MS score, mean±SD	82.2±7.5	79.5±11.7	0.29
2	HbA1c levels %, mean±SD	8.7±1.9	9.5±2.5	0.16
3	NLR, mean±SD	1.8±0.6	1.6±0.6	0.14

Association between age, duration of diabetes, glycemic control, NLR and cognitive function

Age and cognitive function

Overall, no significant relationship (r = –0.031) was observed between age and 3MS test scores, though a weak negative correlation (r = –0.255) was found between age and 3MS test scores in patients with cognitive impairment.

Duration of diabetes, glycemic control and cognitive function

There was no significant relationship between duration of diabetes (r = 0.017), HbA1c levels (r = –0.140) and 3 MS test scores. Similarly, in the cognitive impairment group we observed no significant relationship between duration of diabetes (r = –0.011), HbA1c levels (r = –0.039) and 3 MS test scores. Though the correlations were in the expected direction i.e. negative correlation. Overall, poor glycemic control (HbA1c ≥ 7%) was observed in 49 patients (81.7%), among which 32.7% of the patients had cognitive impairment. Among the patients with good glycemic control (HbA1c < 7%) (n = 11), 18.2% had cognitive impairment.

Neutrophil Lymphocyte Ratio (NLR) and cognitive function

No significant correlation was observed between NLR and cognitive function scores in the cognitive impairment group (r = 0.162) as well in the whole sample of T2DM patients (r = 0.078). Overall, 23.3% (n = 14) of the patients had abnormal NLR (>2). Cognitive impairment was observed in 21.4% of patients with abnormal NLR and in 32.6% of patients with normal NLR (≤2) (n = 46).

Discussion

The main aim of this study was to find out the relationship between systemic inflammation for which NLR was used as an index and cognitive function in T2DM patients.

The following observations were demonstrated by our study. The overall prevalence of cognitive impairment among patients with T2DM was 30%, no significant correlations were observed between duration of diabetes, HbA1c levels, NLR and 3MS test scores in the whole group as well as in the cognitive impairment group, weak negative correlation between age and 3MS test scores in patients with impairment.

The mean age of the patients with cognitive impairment was 50.8 years in our study and we observed a weak negative relationship between age and cognitive function scores in T2DM patients with cognitive impairment. This shows the early onset of cognitive impairment in patients with T2DM. Few studies have demonstrated the cognitive impairment in middle-aged adults with diabetes and in early stages of diabetes (17, 18). Diabetes has been associated with 1.5 fold increase in the development of mild cognitive impairment and hence they are at a greater risk of developing dementia in the future (6).

Majority of the studies have demonstrated a strong negative relationship between duration of diabetes, glycemic control and cognitive function in diabetic patients which are different from our observations (19, 20, 21, 22). The possible reason for the difference could be the following; the mean age of T2DM patients was 48.6±7.8 years in our study, whereas the mean ages of the patients in most of the other studies were nearly or above 60 years eg. Tali Cukierman-Yaffe, 2009 and 62.5 years, Andreea M. Rawlings, 2015 and 57 years, Kristine Yaffe, 2013 and 74.2 years, Paul K. Crane, 2013 and 76 years. Age-related cognitive decline is mostly reported after 60 years of age (23). Thus, majority of the studies included older patients, and hence age-related cognitive decline could have contributed to the increased prevalence of cognitive impairment and strong negative relationship between duration of diabetes, glycemic control and cognition in those studies. Our study results were almost consistent with the results obtained by Satyajeet Roy et al (5); in their study the mean age of T2DM patients was 50 years and they observed weak negative and moderate negative relationship between HbA1c levels, duration of diabetes and cognitive function respectively.

We observed no significant correlation between NLR and 3MS test scores in diabetic patients. Overall, only 23.3% of T2DM patients had increased NLR in our study. Neutrophil Lymphocyte Ratio (NLR) is a new indicator of subclinical inflammation and recent research works have shown that raised NLR may be considered as a reliable predictor of the progression of various diseases (24, 25). NLR reflects the balance between innate neutrophilic and adaptive lymphocytic immune responses. Diabetes is associated with a low-grade chronic systemic inflammation (7). Inflammatory mediators have been found to be increased in patients with T2DM (8, 26). Many studies have showed that, NLR was increased in patients with diabetes and it has been linked to poor glycemic control, insulin resistance and cardiovascular events (10, 12, 25, 27). No study has been reported in the literature, investigating the relationship between NLR and cognitive function in diabetics. However, Riccardo et al, 2010 (8) observed significant association between elevated levels of plasma CRP, IL-6, TNF-α and poorer cognitive ability in patients with T2DM. Inflammation may have a role in the development of cognitive impairment in T2DM either by a direct effect on the brain as suggested by increased inflammation in the brain in dementia or through an influence on the development of vascular disease (7, 8).

Absence of relationship between NLR and cognitive function in patients with T2DM observed in our study could be again due to the same reason mentioned above such as, relatively young adults (mean age = 48.6 years) were included in our study and the duration of diabetes was less (mean = 5.4 years) in T2DM patients. Most of the studies that observed relationship between inflammatory mediators, cognitive function, NLR in diabetes included elderly patients (8, 12, 27). Moreover, only few patients had abnormal values of NLR (>2) in our study.

Since our main aim was to find out if NLR can be used as an index of cognitive impairment in the early stages of its onset in diabetic patients, we included relatively younger patients. Their duration of disease was less and hence only few patients had abnormal NLR. So we could not find a significant association between NLR and cognitive function in diabetic patients. In future, this study can be continued by including patients with wider age group and increasing the sample size for better understanding of the relationship between NLR and cognitive function in patients with diabetes. Further prospective studies are also required to find out the association between inflammatory markers and cognition in diabetes.

Conclusion

We observed cognitive impairment in 30% of T2DM patients suggesting its early onset and no significant correlation was observed between duration of diabetes, HbA1c levels, NLR and 3MS test scores in diabetic patients. Regular screening for cognitive decline and good glycemic control, will enable early intervention and proper management to postpone or prevent the risk of dementia in diabetic individuals.

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Original Article

Comparative Efficacy of Grotto Cream with Fucidin Cream on Normal and Diabetic Wound Models in Rats

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Abstract

Wound healing is a process of well-recognized orchestrated and predictable events, in which there are four distinct inter-related phases: haemostasis, inflammation, proliferation and remodeling. The present study was carried out to evaluate comparative efficacy of Grotto cream (combination composed of, bees wax, D-panthenol, Lavender oil, glycerin, Vitamin E, allantoin and dimethicone) with fucidin cream in normal and diabetic rats. Circular wound was excised on the dorsal side of each rat either control healthy or diabetics rats. Grotto cream or fucidin cream were topically applied to the wounds on alternating days for 21 days in normal rats and 30 days in diabetic ones. Streptozotocin (50 mg/kg b.wt) single intra-peritoneal injection was used to induced diabetic rat model. Wound contraction and epithelialization period were measured following excision wound model and were used to evaluate wound healing effect of either Grotto or fucidin cream. The obtained results indicated that topical application of Grotto creams accelerated wound healing when compared to control non treated rats. The rate of wound contraction was significantly increased on days 3–21 for normal rats and days 3 to 30 for diabetic rats in Grotto and fucidin creams treated animals respectively. The duration of wound epithelialization was decreased in wounds treated with the reference standard and Grotto creams than the vehicle-treated group. It has been concluded that Grotto cream promoted wound healing in normal and hyperglycemic rats and its effect was more efficient than wound treated with reference drug (fucidin cream).

Introduction

Wound healing is a process of well-recognized orchestrated and predictable events, in which there are four distinct inter-related phases: haemostasis, inflammation, proliferation and remodeling. A wound is defined as loss or breaking of cellular, anatomical, or functional continuity of living tissues (1). Wound healing are comprehensive process of well-recognized orchestrated and predictable events which the activity of an intricate network of blood cells, cytokines, and growth factors that leads to the restoration of the injured skin to its normal condition. The normal

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wound healing process is divided into three sequential phases: Inflammatory phase (hemostasis and inflammation), proliferative phase (granulation, contraction, and epithelialization), and remodeling phase, which organizes structure with increased tensile strength (2). The normal healing process may be impaired by several factors such as diabetes mellitus, immune disorders, ischemia, and venous stasis. Abnormalities associated with diabetic wounds which induced primarily by hyperglycemia include prolonged inflammation phase, impaired neovascularization, decreased synthesis of collagen, increased levels of proteases, and abnormal macrophage function. Moreover, diabetic wounds are also prone to infection because of impaired granulocytic function and chemotaxis (3, 4, 5).

Therefore, new era in wound healing researches are required, including new strategies to deal with this emerging issue. One of these is the use of natural products or medicinal plants to explore new therapeutic tools to be used for diabetic wound management and treatment. Grotto cream is a product composed of bees wax, D-panthenol, Lavender oil, glycerin, Vitamin E, allantoin and dimethicone.

Honey is one of the oldest known medicines. It has been valued highly in the Middle East and was in the Holy Quran since 1436 years ago. It has been used for treatment of diseases of respiratory, urinary, and digestive systems as well as skin diseases including ulcers, wounds, eczema, psoriasis and dandruff (6). Honey, an ancient remedy rediscovered during the 1990s, is now being utilized for wound care in the world as Australia (7), New Zealand (8) and in the US (9). Honey reduces inflammation, edema and exudation, promotes healing, diminishes the scar size and stimulates tissue regeneration (10-11-12). The basis of using beeswax in the mixture was derived from the observation that beeswax has antibacterial properties (13)

Panthenol, the biologically active alcohol analogue of pantothenic acid, is a pro-vitamin of the B-complex group, which is a normal constituent of skin and hair. When applied topically, it is converted to pantothenic acid, which is necessary to normal epithelial function (14). Clinical observations have reported that topically applied panthenol accelerate wound healing in burns, corneal lesions, and allergic dermatitis, with minimal risks of skin irritancy (15). Lavender essential oil is expected to have a beneficial effect on wound healing because a few evidences for its effect were already reported (16-19). Topical treatment with lavender oil on ulceration showed a significant ulcer size reduction as compared to control in both an animal experiment and a clinical study (20). In addition, Morim et al., (21) reported that wound closure progressed more rapidly as a result of topical application of lavender oil promote wound healing in the early phase by acceleration of formation of granulation tissue, tissue remodeling by collagen replacement and wound contraction through up-regulation of transforming growth factor-β (TGF-β) which are growth factors playing important roles in wound healing process such as tissue remodeling and re-epithelialization.

The aim of the present work is to evaluate the wound healing effects of the tested cream named Grottoin rats using excision wound model and comparing this activity with that of fucidin cream in normal and experimentally induced diabetic wounds in rats. The effect of grotto cream on the rate of wound healing was assessed by the rate of wound closure and period of epithelialization in normal and diabetic rats.

Material and Methods

Materials

Grotto cream was supplied by Pharma International Pharmaceutical Industries (Pico Egypt). It is composed of beeswax, D-panthenol, Lavender oil, glycerin, Vitamin E, allantoin and dimethicone while fusidic acid was obtained as 2% under trade name fucidin cream manufactured by Mina Pharm Egypt for Leo France.

Experimental animals

Wister male rats weighing 150-170 g were obtained from the Lab Animal Care Unit, Faculty of Veterinary medicine, Cairo. All animals were housed in polypropylene cages under standard experimental

conditions with 26±2°C ambient temperature and 12 h light dark cycle. The animals were fed standard diet and were provided water *adlibitum.* All studies were carried out using ten rats in each group.

Safety Evaluation (Skin Irritation Study)

To evaluate the safety of Grotto cream, skin irritation test was conducted on albino rats as per OECD guidelines number 404 (22). Twenty-four hours before application of the tested sample, back of the albino rats was shaved carefully. Grotto cream was applied on the skin patches of albino rats, and the application site in terms of erythema and/or edema was examined at 24, 48, and 72 h for changes in any dermal reactions. The irritation index was calculated to assess the irritation potential of Grotto cream and fucidin according to Draize Test (23).

In vivo Evaluation of Wound Healing Activity

Effect on Wound Healing in Normal Rats

Thirty rats were allocated into 3 groups each of 10 rats as follows :

Group 1: Control group (treated topically with cream base).

Group 2: Treated topically with *Grotto cream.*

Group 3: Treated topically with fucidin cream.

Effect on Wound Healing in Diabetic Rats

Experimental induction of diabetes was carried out by a single intraperitoneal injection of streptozotocin (50 mg/kg b·wt.) dissolved in citrate buffer (0.1 M, pH 4.5). Fasting blood glucose level was measured 3 days post injection to confirm the induction of diabetes of the tested animals. Measurement of blood glucose level was done via withdraw of blood samples from the tail vein. Rats having blood sugar level above 250 mg/dl, were selected for study. Two weeks after induction of diabetes, rats with high blood glucose level, more than 250 mg dL–1 were deemed diabetic and used for the experiment. The diabetic rats were allocated into three groups comprising ten rats in each group as given below :

Group I: diabetic control rats (treated topically with cream base).

Group II: rats treated with *Grotto cream*.

Group III: diabetic rats treated with Fucidin cream.

All animals in each group either normal or diabetic rats were anaesthetized via intraperitoneal injection of ketamine and xylazine (5 and 2 mg/kg, respectively). Skin of the dorsal area of each rat was shaved and disinfected with 70% alcohol. A uniform circular wound of approximately 100 mm^2 area was excised on the dorsal side of each rat as described by Mughrabi *et al,.* (24) Care was carried out to avoid damaging the muscle layer, and the tension of skin was kept constant during the process. The wounding day was considered as day 0. The wounds were treated with the topical application of the vehicle (cream base), reference standard or Grotto cream till the complete healing of wounds. The wounds were observed and the area of wounds was measured on 3, 6, 9, 12, 15, 18 and 21 post-wounding day for normal rats and on 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 post-wounding day for diabetic ones.

Parameters evaluated for wound healing

a. Measurement of wound contraction:

The percentage of wound contraction was assessed by tracing the wound on days 0, 3, 6, 9, 12, 15, 18 and 21 after wounding or till the wound gets healed using transparent paper and a permanent marker. The areas of wounds were measured against scale graph paper (mm^2). The rates of wound contraction were calculated (25).

$$\text{Wound contraction (\%)} = \frac{\text{Wound area on day 0} - \text{Wound area on days}}{\text{Wound area on day 0}} \times 100$$

Where n is the number of days: 3rd, 6th, 9th, 12th, 15th, 18th and 21st days.

Epithelialization period

The epithelialization period was calculated as the

duration in days required for falling of the dead tissue remnants without any residual raw wound (26).

Statistical analysis

Results were expressed as mean ± standard error of the mean (SE). Data analysis was carried out using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests. Results obtained in groups exposed to Grotto and fucidin creams were compared with those of the control groups. In addition, the results related to Grotto cream were compared with those related to fucidin cream. P<0.05 was considered statistically significant.

Results

Safety Evaluation (Skin Irritation Study)

There were no signs of redness and itching when Grotto cream or fucidin cream was applied on the shaved back of albino rats. The primary skin irritation index of the creams was calculated as 0.00. This indicates the safety of Grotto and fucidin creams.

In vivo Evaluation of Wound Healing Activity

Topical application of Grotto cream showed significant promotion of wound healing in normal rats (Table I & Fig. 1) and hyperglycemic ones (Table II & Fig. 2), as compared to their corresponding control groups. Daily topical application of experimentally induced wound with Grotto cream caused a significant reduction in wound area of both normal and hyperglycemic rats compared to their corresponding control groups. In addition, daily topical application of fucidin cream on wound induced experimentally in normal and diabetic rats produced significant reduction in the wound area compared to the wounds of the corresponding control groups.

Period of wound epithelialization was reduced in group treated with either Grotto cream or with fucidin cream than vehicle. In control rats, wound takes more than 26 days in normal rats (Table I) and more than 36 days in hyperglycemic ones (Table II) to heal completely unlike with Grotto cream in which wounds heal almost around days 16-17 in normal rats and almost around days 24-25 in hyperglycemic rats.

TABLE I: Effect of topical application of Grotto or fucidin creams on wound contraction % and period of epithelization in normal rats (n=10).

Treatment	*Wound contraction %*							*Period of epithelialization (days)*
	Day 3	*Day 6*	*Day 9*	*Day 12*	*Day 15*	*Day 18*	*Day 21*	
Control	11.2±1.04	22.8±2.13	34.2±2.7	45.5±3.4	54.4±2.17	64.9±3.8	74.8±3.4	25.3±0.57
Grotto cream	24.9±1.2*	42.7±2.1	63.2±3.7	79.4±2.9	92.7±3.7	100±0.0*	100±0.0*	17.1±0.73*
Fucidin cream	21.4±1.2*	40.5±3.7	59.8±4.2	71.5±2.9	85.9±2.17	100±0.0*	100±0.0*	18.7±0.36*

*Significantly different from the values of the control rats at P<0.05.

TABLE II: Effect of topical application of Grotto or fucidin creams on wound contraction % and period of epithelizationin diabetic rats (n=10).

Treatment	*Wound contraction %*										*Period of epithelialization (days)*
	Day 3	*Day 6*	*Day 9*	*Day 12*	*Day 15*	*Day 18*	*Day 21*	*Day 24*	*Day 27*	*Day 30*	
Diabetic control	0.0±0.0	5.4±0.04	9.2±0.47	15.7±1.27	27.8±2.17	39.0±1.23	41.2±2.28	54.7±2.49	64.2±2.48	68.8±1.44	36.4±1.57
Grotto cream	10.9±0.57*	18.7±1.61*	30.5±2.63*	41.8±3.51*	53.8±3.37*	68.4±2.11*	78.4±0.94*	93.1±1.46*	100±0.0*	100±0.0*	24.2±0.27*
Fucidin cream	12.3±0.97*	19.4±1.47*	33.1±3.47*	40.85±3.1*	52.64±1.7*	66.8±1.97*	76.4±0.67*	91.7±2.17*	100±0.0*	100±0.0*	25.6±0.49*

*Significantly different from the values of the control rats at P<0.05.

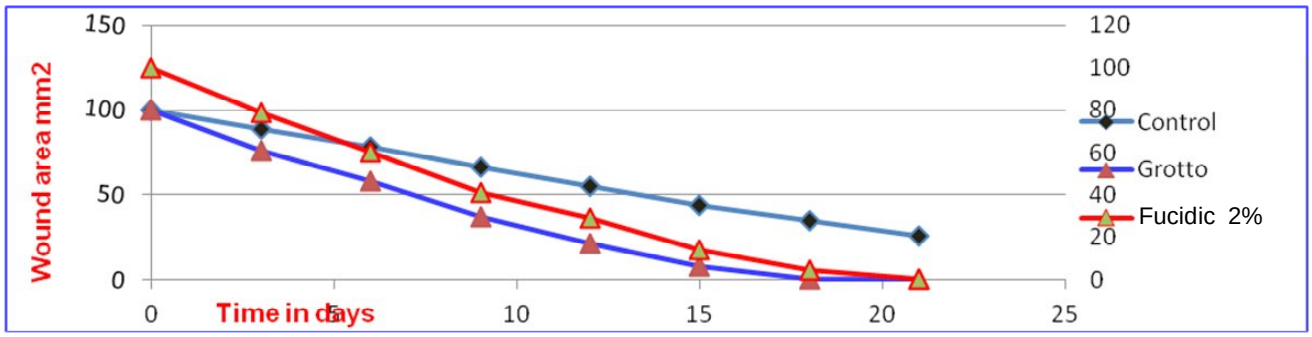

Fig. 1 : Effect of topical application of Grotto and Fucidin creams on wound area (mm^2) in normal rats (n=10).

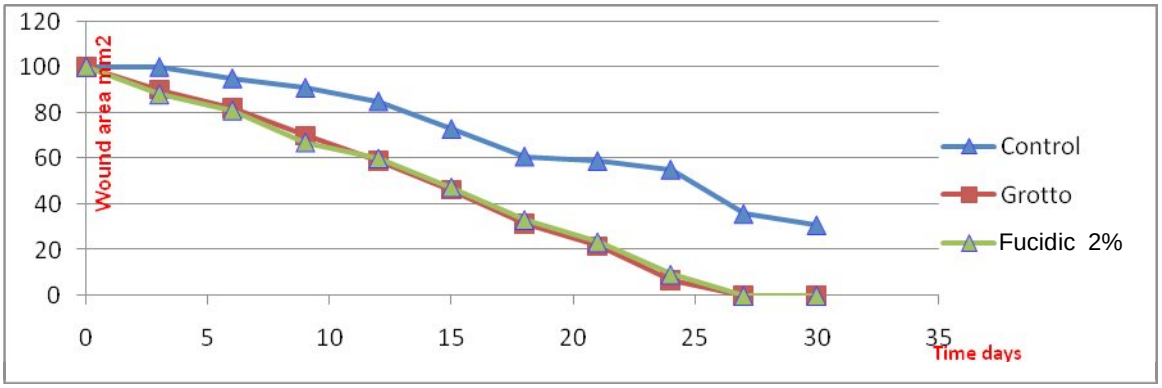

Fig. 2 : Effect of topical application of Grotto or fucidin creams on wound area (mm^2) in diabetic rats (n=10).

Discussion

Wound healing is a physiological process occurred naturally, that consists of four overlapping stages; hemostasis, inflammation, proliferation, and tissue remodeling or resolution (27-28). These processes, especially new tissue formation and tissue remodeling, consist of sequential coordinated stages including angiogenesis, cellular proliferation, collagen synthesis followed by formation of granulation tissue, matrix degradation followed by replacement of collagen, wound contraction, and scar tissue formation (29-30-31.32.33). The processes of wound healing are controlled by several factors, including cytokines, mitogens and chemotactic factors. These factors include, insulin like growth factors (IGFs), platelet-derived growth factors (PDGFs), epidermal growth factors (EGFs), and fibroblast growth factors (FGFs) which played an important role in wound healing possesses as these factors controlled cell migration and proliferation and the formation of extracellular matrix proteins, which are essential for formation of granulation tissue (34, 35, 36). The edges of excised wounds are not in contact with each other, so contraction and epithelization steps are necessary for the repairing process. The obtained results revealed that daily topical application of Grotto cream promoted contraction and reduced the period of epithelization of experimental wounds in normal and diabetic rats. Contraction of wounds area processes occurring at the healthful skin surrounding the wounds which coats or covers the naked area. These process may be attributed to the action of myofibroblasts while epithelialization or epithelial regeneration following damage, includes the proliferation and immigration of epithelial cells to the center of wounds (37). Therefore, the effect of the combination of bees wax, D-panthenol, Lavender oil, glycerin, Vitamin E, allantoin and dimethicone (Grotto cream) on the contraction and epithelialization of wounds suggest their possible promoting or enhancing effect of either component of Grotto cream on the migration and proliferation of epithelial cells,

as well as the formation and the action of myofibroblasts. In this respect, several authors reported that the use of honey and beeswax in promoting or accelerating wound healing process as measured by the thickness of granulation tissue, epithelialization of the periphery of the wound and the size of the induced wound (9, 10, 11, 12, 13). In addition, Gore and Akolekar (38) reported that, a mixture of high molecular weight alcohol which isolated from beeswax, induced a significant reduction of exudates volume of the inflammation occurred by carrageenan. Furthermore, panthenol when applied topically, is an aid of wound healing, in burns, corneal lesions, and allergic dermatitis (17) as panthenolis converted to pantothenic acid, a component of coenzyme A and holo-fatty acid synthase that is essential to normal epithelial function (14). In addition, several clinical trials suggest a beneficial effects of lavender oil on wound healing and they were reported that topical application of lavender oil on ulceration showed a significant ulcer size reduction as compared to control (18-19). Furthermore, Morim *et. al.,* (21) demonstrated that wound closure progressed more rapidly as a result of topical application of lavender oil as compared to the control, accompanied by increased expression of some growth factors (PDGF-A and EGF) which played an important role in wound healing process including tissue remodeling and re-epithelialization steps. Furthermore. Araújo, *et. al*. (39) proved the mechanism of allantion action on wound healing as allantoin occurred via the regulation of inflammatory response and stimulus to fibroblastic proliferation and extracellular matrix synthesis. In addition, Cheng-San, *et al* (40) concluded that application of silicone gel on wounds decreases inflammatory reaction and improves recovery index. Glycerine, lanolin, and dimethicone are common emollients acts by forming an oily layer on the top of the skin that traps water in the skin. The increased capability of accelerating wound healing effects with combination of beeswax, D-panthenol, Lavender oil, glycerin, Vitamin E, allantoin and dimethicone in a pharmaceutical preparation named Grotto could be explained on the basis of the anti-inflammatory effects of beeswax, an increased expression of growth factors which played important roles in wound healing process including tissue remodeling and re-epithelialization steps.

Conclusion

In conclusion, the present study indicated that Grotto cream mixture promoted wound healing in normal and hyperglycemic rats and its effect was comparable to that produced by the reference drug (fucidin cream). However, further work is required to evaluate each of the components in Grotto cream for their beneficial effects on different degree of burns.

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Original Article

Early Adaptive Changes in Transporters of Blood Retinal Barriers and Glutamate Excitotoxicity in Experimental Diabetes

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Abstract

Aim: Transporters are well studied in blood retinal barriers (BRB) to regulate transfer of respective substrates through retina. The present study was undertaken to evaluate the alteration of retinal transporters expression under hyperglycemic conditions in streptozotocin (STZ) induced diabetic rats and its functional consequences in the ocular system.

Method: Diabetes was induced in adult male wistar rats using STZ (45 mg/kg) and hyperglycemia was confirmed by measuring blood glucose. At periodic intervals, retinal function and vasculature was assessed by electroretinography and fundus imaging respectively. Gene expression analysis of 15 transporters of ABC and SLC family-importantly glutamate transporter were studied on day 10, 20, and 30. Levels of glutamate substrate in vitreous were quantified by LC-MS/MS. BRB integrity was also assessed by blood to vitreous ratio of P-glycoprotein substrate-ofloxacin.

Result: In the experimental period of 30 days, significant changes in ERG were observed at 10th day along with concordant significant fold increase in glutamate transporters expression (SLC1A1,SLC1A3) at 20th and 30th day respectively. Abc1b transporter isoform coding for P-glycoprotein was also found to be significantly upregulated at 30th day. Increased vitreous glutamate levels were accompanied by the blood to vitreous ratio of ofloxacin at 30th day.

Conclusion: This study concludes that glutamate induced retinal excitotoxicity precedes vascular complications in the very early stage of diabetes itself. Moreover, the alteration of blood retinal barrier properties in diabetic condition indicates its impact on ocular drug kinetic.

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Introduction

International Diabetes Federation (2014) reports 175 million diabetic populations progressing un-diagnosed towards diabetic complications. As endocrine disorders, diabetes represents complex pathologic state where different bodily systems are involved. Amongst the organs retina has one of the highest metabolic demands basically due to its role in phototransduction. The combination of high metabolic demand and minimal vascular supply limit the retina's ability to adapt the metabolic stress of diabetes (1). Hence efforts are needed to characterize the early diabetic changes before the onset of irreversible micro and macrovascular complications in retina.

In the retina, the importance of transporters in diabetic condition has been illustrated through GLUT1, the glucose transporter at blood retinal barrier playing a major role in the development of diabetic retinopathy (2). Also knockdown studies by Lu et al., (2013) indicate GLUT1 as a promising therapeutic target in diabetic retinopathy (3). But the change in the expression state of different transporters viz. amino acid transporters, peptide transporters have not yet been studied. Beside till date no studies have been done regarding the change in the expression level of important drug transporters viz. OCT, OAT and ABC efflux transporters in diabetic condition in eye. Diabetic induced earlier changes in the transporter expression in the blood ocular barriers are expected to play a vital role in the progression of retinopathy in diabetic conditions. Breakdown of the blood-retina barrier (BRB) is one of the most important pathophysiological aspects in the diabetic retina. The enhancement of retinal vascular permeability and vascular leakage resulting from BRB dysfunction has been observed both in patients with diabetes and diabetic animal models (4). Functional importance of transporters in cornea and retina and their modulation causing altered kinetics of their substrates from blood-to-vitreous or vitreous-to-blood has been studied with the help of externally administered xenobiotics from our laboratory (5, 6). Thus the various molecular and functional aspects of retinal transporters under the context of diabetes need to be revisited.

Therefore, the present study was conducted to understand the earlier changes in the expression of selected transporters in the retina of STZ induced diabetic rats. STZ-diabetic rats have been known to clearly manifest retinal sorbitol pathway, hyperactivity, oxidative stress, neuro retinal apoptosis and glial changes associated with diabetes, providing detailed molecular information, including acute and chronic changes in gene expression and cell signaling (7). Characterization of diabetic animal model studies have been done from a timeline of utmost 2 months during which pericyte loss (8) visual acuity damage have been noted (9). Hence an understanding of the earlier events leading to diabetic retinopathy could help in identifying an optimum time point for intervention in diabetic retinopathy. To serve these purpose, physiological parameters of fasting blood glucose & HbA1c levels and functional & vascular analysis of retina using Electroretinography (ERG) and fundus imaging were correlated with expression changes of selected transporters at time points of 10, 20, 30 days.

Materials and Methods

Streptozotocin HCl, L-glutamate, Ofloxacin were purchased from Sigma Aldrich (USA). Insulin (Humulin 70/30), xylazine and ketamine were procured from C.B Pharma, India. Sodium citrate dihydrate, citric acid, were purchased from Merck, Germany. RNA later was purchased from Ambion, USA. Glucometer was acquired from AccuCheck Active, USA. All other solvents and chemicals were of the highest analytical grade available. Primers were obtained from Oligo IDT Technologies, USA.

Induction of diabetes using streptozotocin and sampling

Male Wistar rats (225-250 g) were procured from Central Animal Facility and the study protocol was approved by the standing Animal Ethics Committee of All India Institute of Medical Sciences, New Delhi. The experiments were conducted according to Association for Research in Vision and Ophthalmology (ARVO) guidelines. The animals were housed in polypropylene cages and were maintained under standard laboratory conditions with 12 hour

light/dark cycle having free access to water and food *ad libitum.*

Diabetes was induced by streptozotocin (i.p.) in the overnight fasted rats using the vehicle containing 0.1 M citrate buffer having pH 4.5 at the dose of 45 mg/kg body weight by using the procedure adopted by Ward et al (2001). Control rats received only vehicle. Two days after the injection of streptozotocin, all rats were subjected for screening of their diabetic state by determining the blood glucose levels using calibrated glucometer (Accuchek, Roche Diagnostics, USA) by sampling blood from tail vein by prick method. Rats showing blood glucose levels more than 250 mg/dl were grouped under diabetic rats. Their body weight was measured weekly while fasting blood glucose was measured every three days.

Diabetic rats (n=30) were randomly divided into four groups. They were coded and their body weight was recorded before and end of the experiment. Group 1 served as control, Group 2, 3 & 4 were diabetic and were sacrificed after the functional investigations on the day 10, 20 & 30 respectively. After sacrificing the animals using carbon-di-oxide, blood was collected by cardiac puncture and was subjected for the analysis of glycated haemoglobin (HbA1c) by using the method of Park et al*.,* (2009) with the help of HbA1c Assay Kit (Biosystems, Spain). Two hours before sacrificing the rats, ofloxacin (35 mg/kg) was administered orally to all the groups for the calculation of blood to vitreous ratio and to evaluate the condition of blood retinal barrier permeability.

Electroretinography & Fundus Imaging

Retinal function in the diabetic model was assessed by using image guided electroretinogram (ERG) in anesthetized rats. After at least 40 minutes of dark adaptation the animals were anesthetized using ketamine (75 mg/kg B.W) and xylazine (5 mg/kg B.W). The ERG and fundus images were taken using the method described by the Aron et al., (2016) according to (International Society for Clinical Electrophysiology of Vision) ISCEV guidelines with the help of MICRON III rodent imaging system (Phoenix laboratory, USA) (10). For ERG analysis the 'a' and 'b' wave amplitude and latency were measured in all groups, using the inbuilt algorithm of Labscribe software. The oscillatory potentials were also extracted out from the obtained ERG by narrowing down the frequency bandwidth to 30-250 Hz. The fundus images were analysed in terms of tortousity index as method described previously by Liu et al. (2006) (11).

RNA Isolation and quantification of gene expression

The rats were euthanized by carbon-di-oxide and sacrificed at different time intervals of 10, 20 and 30 days. Enucleated eye balls were quickly subjected for the removal of iris and lens through transverse incision on cornea. With calibari forceps vitreous was removed followed by retina with choroid. The pooled retinal samples (n=6) were immediately placed in RNA*later* (Ambion, USA) and subsequently RNA purification was done using RN-easy Mini Kit (Qiagen, USA). The contaminating genomic DNA was digested using the RNase-Free DNase Kit (Qiagen, USA). Isolated RNA samples were quantified by using NanoDrop 1000 Spectrophotometer (ThermoScientific, USA). RNA samples of sufficient purity (A_{260}/A_{280} ratio of 1.9–2.1) were used for the synthesis of cDNA (Thermoscientific, USA). The cDNA were synthesised from an RNA concentration of 200 ng. The synthesized cDNAs were used for QPCR analysis (Thermocycler, Biorad CFX 96, USA). Relative Gene Expression Analysis was done by 2-Delta Delta C (T) method. The geometric mean of two reference genes, including phospholipase (*Ywhaz*) and *18S* was used as a normalization factor between the control (normal) and reference samples (obtained from 10^{th}, 20^{th} and 30^{th} diabetic rats). Vitreous and blood samples were stored at –80°C for the quantification of ofloxacin and glutamate levels by tandem mass spectroscopy.

Quantitative gene expression study was conducted for 15 transporters viz. *Slc1a1, Slc1a3, Slc1a6, Slc1a7, Slc6a6, Slc15a1, Slc15a2, Abcb1a, Abcb1b, Abcc1, Abcc2, Abcc3, Abcc4, Abcc5, Abcc6* (shown in Table I). The forward and reverse primers used for this study is shown in the Table I.

TABLE I : Primer sequences of different transporters along with their Ct values and reported substrates. The represented Ct is the average of threshold cycle (Ct) values of normal group from three different experimental runs. NCBI accession refers to the gene sequence used to design the primers.

Nomenclature	*Gene*	*Substrate*	*NCBI Accession No.*	*Forward Primer*	*Reverse Primer*	*Ct*
SLC6A6	*Taut*	Taurine	NM_017206.1	AGA GCA AGG GGT GGA CAT TG	GAA TGG ACC AAA AGG TGG GC	26.72
SLC1A1	*Eaat-3*	Glutamate	NM_013032	TCT CAT CTA GCT CGG CAA CC	TCT TGT GAT CCT CTT GTC CAC G	31.28
SLC1A3	*Eaat-1*	Glutamate	NM_019225	AAG TAT CAC AGC CAC AGC CG	CCC ACA GAT GTC AGC ACG A	32.32
SLC1A6	*Eaat-4*	Glutamate	NM_032065	CCT TTC CCT TCA TCG GTG G	CTC CAG GCA TCG GAA AGT GA	32.2
SLC1A7	*Eaat-5*	Glutamate	NM_001108973	CCG ACC GAT GAC ATC AAC C	ATC GCC CAG CAC GTT AAT CA	27.98
SLC15A1	*Pept-1*	β-lactam antibiotics, renin inhibitors	NM_057121	GGC CCC AAT CTA TAC CGT G	TCT CGT TAA GGG TGC TGA CG	37.51
SLC15A2	*Pept-2*	β-lactam antibiotics, renin inhibitors	NM_031672	TCA TTG TGC TTG TCG TGG C	CAT GAC GGA GAA GAT CAG GCA	N/A
ABCB1a	*Abcb1a*	Steroids, Glycosides, Glucocorticoids, Ofloxacin	NM_133401	AC TCG CAA AAG CAT CCG TG	GGA GGT ACG TCG TCA TCC AG	38.67
ABC1b	*Abcb1b*	Steroids, Glycosides, Glucocorticoids, Ofloxacin	NM_012623	CCA CAG AAG ACA AGA CCA GGA G	CTG CCA AAA GGA AAC CAT AGG C	40.02
ABCC1	*Abcc1*	Anticancer drugs, nucleoside and nucleotide analogs, leukotriene C(4)	NM_022281	TCT GAA ACG GAG AAG GAG GC	CTC TAC ACG GCC TGA ATG GG	36.11
ABCC2	*Abcc2*	Anticancer drugs, bilirubin, glucuronides	NM_012833	CCA TTG GAC TGC ACG ACC	TCA TCC TCA GAC TCC CCG AG	N/A
ABCC3	*Abcc3*	Anticancer drugs, nucleoside and nucleotide analogs, bilirubin, glucuronides	NM_080581	CTC CAT GAC CTG CGT TCA CA	AGT AAC GGC CAA AGG GAT CG	40.05
ABCC4	*Abcc4*	Anticancer drugs, nucleoside and nucleotide analogs, arsenical prostaglandins E1 and E2, cGMP	NM_133411.1	TCG GAC ACA TGG ACG ACT TG	CCA CGG CGA TCA CAC TTA CA	N/A
ABCC5	*Abcc5*	Anticancer drugs, nucleoside and nucleotide analogs, arsenical cGMP	NM_053924	TAC CCA CGA GGA GCT GAT GA	TTA ATC TCG ACC GGG GGT G	44.05
ABCC6	*Abcc6*	Anticancer drugs, nucleoside and nucleotide analogs	NM_031013	GAG GAT CAG TTT CCC GAG GC	CAT GTA GCG GCC ACA AAC AC	N/A

Quantification of ofloxacin and glutamate using tandem mass spectroscopy

Samples were thawed and the levels of ofloxacin and glutamate in vitreous and blood samples were estimated using liquid chromatography (Surveyor, Thermo, USA) coupled with tandem mass spectroscopy (4000QTrap, Absciex, USA). Briefly, the analytical separation of both the compounds were achieved using phenyl-hexyl column (Merck, Germany) using a gradient elution with water (0.1% formic acid) and methanol (0.1 formic acid) at flow rate of 200 µl/min. Sulfadimethoxine (SDM) was used as the internal standard. Analytes were quantified in multiple reaction monitoring mode and transitions 362.2/261.3, 148.1/84.2 and 311/129 were used for ofloxacin, glutamine and sulfadimethoxine respectively. All other source and compound dependent parameters were optimized using the inbuilt algorithm to get maximum ions intensity in

the analysis to reach required sensitivity.

Statistical analysis

The obtained values are represented as mean±SEM. Statistical analysis was done between normal vs 10 day diabetic ,normal vs 20 day diabetic and normal vs 30 day diabetic using unpaired t-test in Sigma Plot version 11.p value of <0.05 has been considered as statistically significant and marked with an asterisk (*). Statistical analysis of gene expression data was done using REST 2009 software.

Results

Assessment of hyperglycemia and body weight changes

Animal showing blood sugar levels more than 250 mg/dL were randomly assigned under different groups (n=6 in each group). The percentage reduction in their body weight between the start and end of the experimental period was found to be statistically significant at 20th day and 30th day. A sustained and increasing hyperglycemic condition was found in the experimental animals through the experimental period of 30 days. The increase in blood glucose levels was found to be statistically significant from 20 days. The HbA1c levels found in all the experimental groups were found to be within the range of 7.5.

Effect on Electroretinography

ERGs in different experimental groups were found to be appreciably different. Altered levels of the a-wave and b-wave for the different days were observed along with the increased amplitude of the oscillatory potentials post diabetic induction (Fig. 1A & 1B).

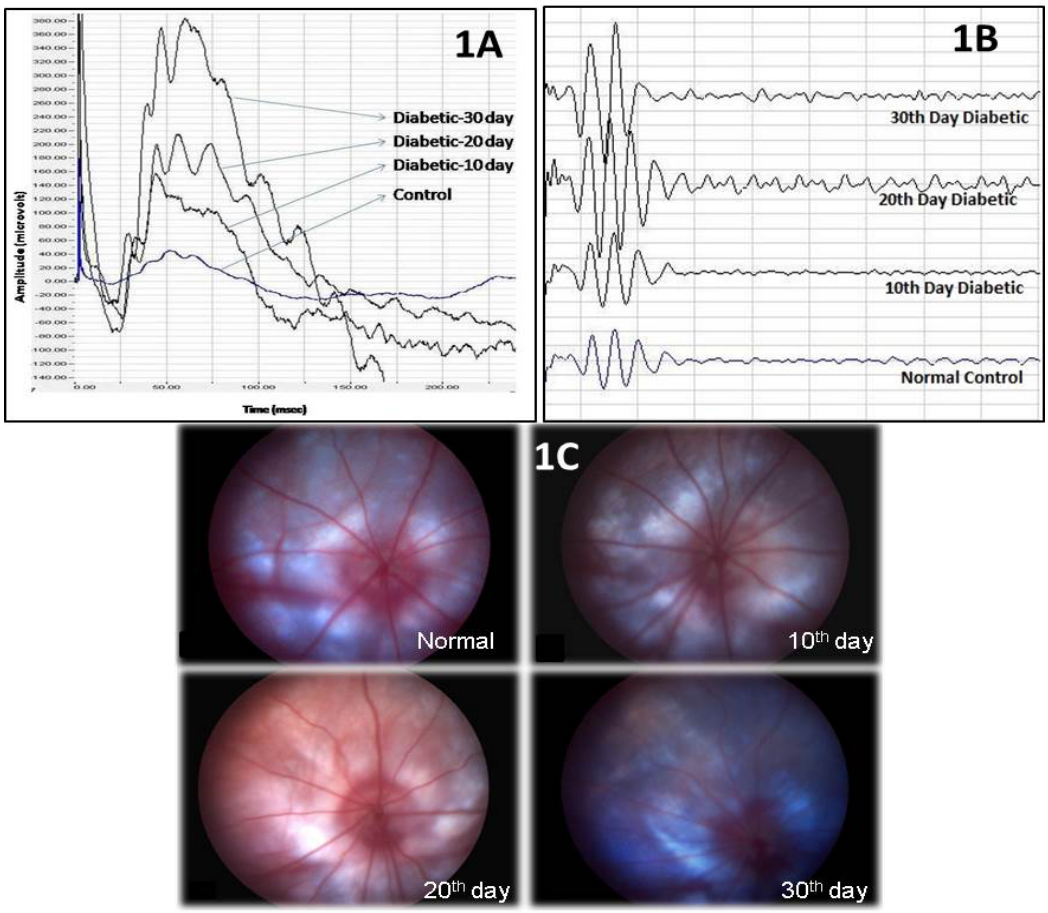

Fig. 1 : Fig. 1 depicts the diabetic changes happened in retina. Fig. 1A shows the altered electroretinography waves of 10th, 20th and 30th day when compared to normal. Same trend of altered oscillatory potentials was observed in the Fig. 1B. The representative images of retina of normal, 10th, 20th and 30th shown in Fig. 1C.

Fundus Imaging and Tortuosity Index

In the diabetic rats, along with the progression of diabetes, no significant increase in retinal tortuosity was observed through the experimental period. Representative fundus image of respective days of the experiment are shown in Fig. 1C.

Quantitative transporter gene expression analysis

Amongst the 15 transporters analysed in the samples of retina choroid, expression of 3 transporter proteins were found to be significantly altered in QPCR analysis (Table I). Glutamate transporters (Slc1a1, Slc1a3) were found to be upregulated in the retinal tissue samples. Particularly the expression of Slc1a1 was found to become more prevalent with increasing time intervals- the 20day diabetic group exhibited fold expression levels of 5 while the 30 day diabetic group exhibited fold expression levels of almost 20 (Fig. 2). While the change in fold expression levels were found to be significant for Slc1a1,Slc1a3 transporters, no significant expression changes were found in the isoforms of Slc1a6, Slc1a7 (Fig. 2).

In case of xenobiotic transporters Abc1b transporter protein coding for P-gp transporter was found to be significantly upregulated at 30th day (Fig. 3A). While other transporter proteins- Slc6a6, Slc15a1, Abcb1a, Abcc1, Abcc3, Abcc4, Abcc5 showed no significant expression changes during the experimental period (Fig. 3B & 3C). Moreover the transporters Slc15a2, Abcc2, Abcc6 were not found to be expressed in the retinal samples (Unpublished data).

Assessment of blood retinal barrier Integrity

The blood to vitreous ratio of ofloxacin and concentrations of glutamate in vitreous, blood were estimated by LC-MS/MS. The values of ofloxacin in the normal groups ranged from 4.8±0.7 and increased significantly to a value of 12.9±5.2 on the 30 day in diabetic group (Fig. 4A). Moreover the glutamate levels in the normal groups which were at a concentration of 6457.1±508 ng/ml increased significantly to a concentration of 9892±927 ng/ml in diabetic animals on 30th day. (Fig. 4B). While the glutamate levels in the blood did not vary significantly between the groups (Fig. 4C).

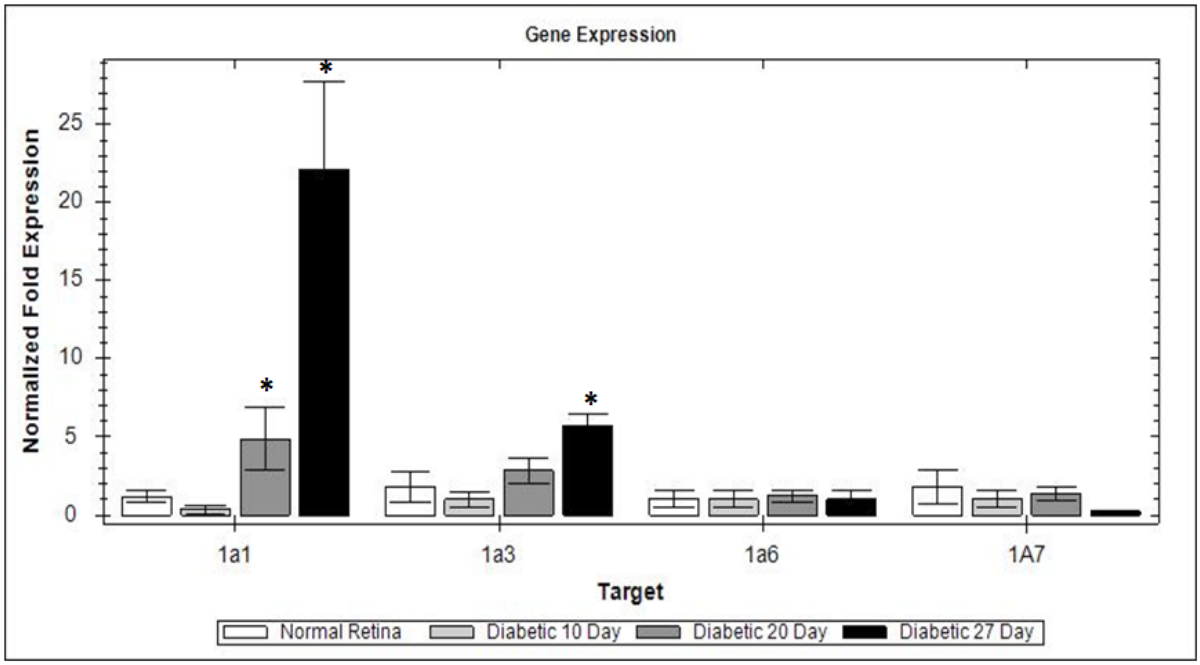

Fig. 2 : Fig. 2 depicts the gene expression of glutamate transporters at various day of normal, 10th, 20th and 30th day. Slc1a1, Slc1a3 were found to be significantly upregulated at day 30th. No significant expression changes were found in the isoforms of Slc1a6, Slc1a7. Statistical analysis was done using REST software, *p≥0.05.

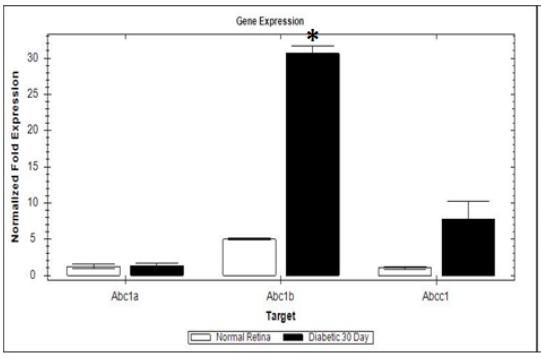

Fig. 3A

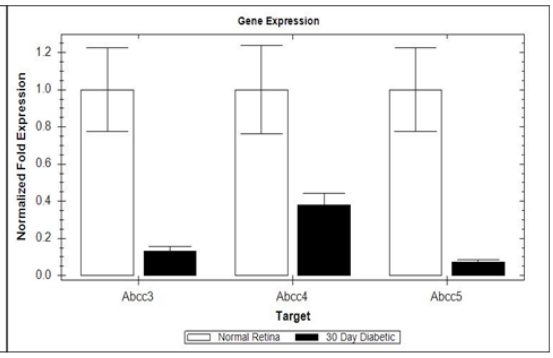

Fig. 3B

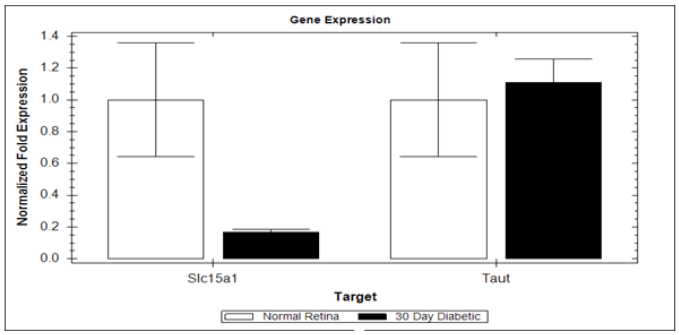

Fig. 3C

Fig. 3 : Xenobiotic transporters Abc1b transporter protein coding for P-gp transporter was found to be significantly upregulated at 30th day (Fig. 3A). While other transporter proteins-, Abcb1a, Abcc1, Abcc3, Abcc4, Abcc5 (Fig. 3B) and Slc15a1 (Fig. 3C) showed down regulation as compare at 30th day but the change was not statistically significant. Statistical analysis was done using REST software, *p≥0.05.

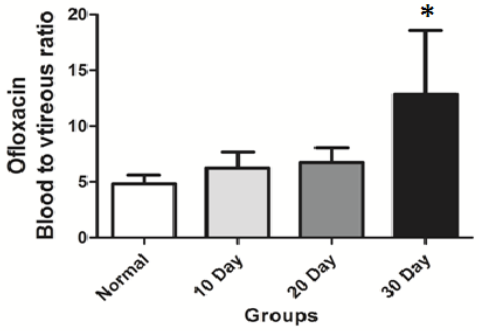

Fig. 4A

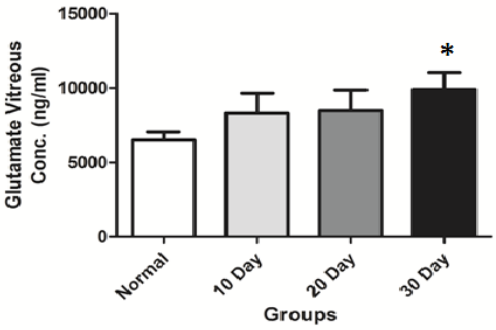

Fig. 4B

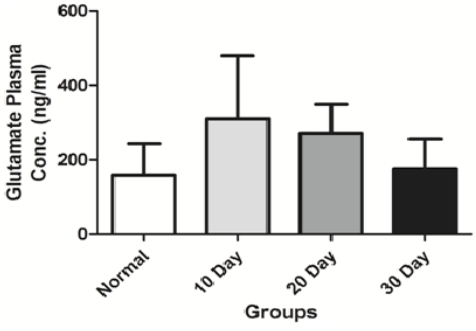

Fig. 4C

Fig. 4 : Figure 4 shows the blood to vitreous ratio of ofloxacin and concentrations of glutamate in vitreous, blood were estimated by LC-MS/MS. The ofloxacin ratio increased significantly at the 30 day in diabetic group (Fig. 4A). The glutamate levels were also significantly high at 30th day post diabetes as compare to normal (Fig. 4B). While the glutamate levels in the blood did not vary significantly between the groups (Fig. 4C). Statistical analysis was done using student t-test, *p≥0.05, **p≥0.01.

Discussion

Hyperglycemia induced early diabetic changes accompanied by the alteration in the transporter expression in the blood ocular barriers (BRB) are of interest in understanding the initiation of cascade of events leading towards neuronal dysfunction and vascular changes in the retina. Therefore, the present study was conducted to identify the earlier changes in the expression of selected retinal transporters and to correlate the changes with the retinal functions using ERG in experimentally induced diabetes in rats.

To achieve the above objective, diabetes was induced in the experimental group by using streptozotocin (45 mg/kg) as a single i.p injection to identify the earlier changes in BRB, where control rats received saline. After the induction of diabetes, only rats showed blood sugar levels more than 250 mg/dl on day 3 were included to avoid variation in their diabetic state. Along with the time, progressive loss of body weight was observed in hyperglycemic rats as compared to the control (data not shown). These rats were randomly selected for retinal function studies at different days and were sacrificed to enable the quantitative gene expression of transporters in retina choroid. Before sacrificing the rats at different days, fundus photography was documented after analysis their retinal functions by recording electroretinography (ERG) and oscillatory potentials (Fig. 1A & 1B).

Retinal vascular tortuousity has been documented as an early indicator of microvascular damage in diabetes (12, 13). In this study, fundus images of the diabetic rats revealed no significant increase in tortousity indices as compared to control implying that the vascular complications were yet to begin till the 30th day (Fig. 1C). In the blood, glycated haemoglobin has been considered as an indirect indicator of diabetic microvascular (14). Although, a positive correlation is known to exist between blood glucose levels and glycated hemoglobin with time (15), in the present study at the end of 30th day, the Hba1c was not found to increase. This could be due to the lack of complete change in RBC within the study period owing to its lifespan (rat RBCs life span is 60±3.2 days) (16).

In this study, quantitative gene expression analysis was performed on 15 transporters from two super families such as Solute Linked Carriers (Slc1a1, Slc1a3, Slc1a6, Slc1a7, Slc6a6, Scl15a1, Slc15a2) and ATP Binding Cassettes (Abcb1a, Abcb1b, Abcc1, Abcc2, Abcc3, Abcc4,Abcc5, Abcc6). Among the studied transporters, it is evident that hyperglycemia induced significant gene alteration was seen in Slc1a1 and 1a3 within the study period of 30 days (Fig. 2). More than 20 fold increase in the expression of Slc1a1 (EAAC1) and 5 fold increase in levels of 1a3 (GLAST) was seen in the rats on day 30. Slc1a1 and Slc1a3 are reported as high-affinity glutamate transporters mediating cellular uptake of glutamate by the mechanism involving co-transport of three sodium (Na+) with one proton (H+), along with the counter-transport of one potassium (K+) (17).

A significant upregulation of Slc1a3 was found at 30th day together with progressive increase in glutamate levels in the vitreous of the diabetic rats as compared to normal (Fig. 2). Slc1a3 upregulation was also reported in the study of Lau et al (2013) in diabetic Long-Evans rat retina after 4 weeks of STZ injection (18). Also corroborating evidence of increasing vitreous glutamate levels and BRB leakage at 5th week after induction of diabetes has been reported by Kusari et al (2010) (19).

This study characterized the initial excitation of ERG wave forms, oscillatory potentials and Slc1a1 transporter upregulation at 10th & 20th day respectively before the increasing vitreous glutamate levels at 30th day. An increase in vitreal glutamate levels was found without any increase in blood glutamate level pointing to an endogenous source of glutamate in the ocular system (Fig. 4B & 4C). Under conditions of severe depolarization Slc1a3 in Muller cells has been reported to undergo reversal releasing glutamate and thereby increasing extracellular glutamate concentration to excitotoxic levels (20, 21). In our study increase in b-wave pointing to an increased depolarisation of the contributing cells types such as muller cells, bipolar cells and

horizontal cells was found in the initial time points of 10th and 20th day. Thus through the experimental timeline of 30 days this study demonstrates the initial excitation of ERG wave forms, the associated upregulation of the glutamate transporters in RGC and muller cells preceding increasing glutamate levels and BRB leakage.

The results of the current study indicated that the gene coding P-gp efflux transporters- Abc1b was showing the trend of significant upregulation through the experimental period of 30 days (Fig. 3A). In contrast, blood to vitreous ratio of ofloxacin at the end of 30 days was found to be higher as compared to normal rat's eye. Both paracellular pathway involving endothelial cell tight junctions, and the endothelial transcellular pathway mediated by endocytotic vesicles (caveolae) have been reported to be affected in blood retinal barriers as far as vascular permeability is concerned (22-24). In our study the increased blood to vitreous ratio of ofloxacin found along with increased expression of the efflux transporter validates the findings of Reichel et al (2011) (Fig. 4A) (25).

In conclusion, our work has characterized the initial changes in the rat retina of streptozotocin induced model of diabetes. The raised levels of glutamate in the vitreous, increased amplitude of ERG signals, increased expression of glutamate transporter showed the glutamate excitotoxicity predominating in the diabetic retina. While the upregulation of Pgp efflux transporters in the blood ocular barriers observed along with the increased blood to vitreous ratio of its substrate suggests the alteration of blood retinal barrier integrity at initial stages. Moreover the upregulation in the P-gp isoform expression and increased blood to vitreous ratios of its substrates in diabetic groups implicates the altered ocular kinetics following hyperglycemia. Thus the importance of transporters both in the pathophysiology as well as in ocular pharmacokinetics remain a vital point to be investigated in diabetes.

Acknowledgements

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Conflict of interest :

None

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Original Article

Current Density of Voltage-gated Proton Currents Decreases During Differentiation of Human Peripheral Blood Monocytes to Macrophages in Culture

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Abstract

Voltage gated proton channels play an important role during the respiratory burst in phagocytic cells. Proton channels have been earlier described in all leucocytes, including THP-1 monocytes. In this study, proton currents in peripheral blood monocytes (PBMs) and the changes that occur in these currents during differentiation into macrophages in culture were studied. The proton currents in PBMs were similar to proton currents described in other mammalian phagocytic cells in terms of their threshold potential, zinc sensitivity, activation and inactivation profiles. The proton currents in PBMs were larger than the currents previously reported in THP-1 monocytes. There was a remarkable increase in cell size during differentiation of monocytes under appropriate culture conditions to monocyte derived macrophages. This light microscopic finding was supported by an increase in cell capacitance. The current density decreased significantly as the monocytes differentiated into monocyte derived macrophages. The results of the study indicate that differences exist between PBMs and THP-1 monocytes with respect to proton current amplitude and kinetics. Hence THP-1 cells may not be an accurate model to study electrophysiological characteristics of monocytes. Further, monocytic differentiation lead to a decrease in proton current density which could be related to functional changes that occur in these cells during differentiation.

Introduction

Voltage gated proton channels have been demonstrated in neutrophils, lymphocytes, eosinophils, basophils and alveolar epithelial cells (1-6). Phagocytes generate superoxide radicals within their phagosomes to kill invading pathogens. This process is termed the “respiratory burst”. This involves a coordinated action of an enzyme complex called NADPH oxidase, voltage-gated proton channels and several other membrane transporters including proton pumps (7). The redox reaction mediated by NADPH oxidase transfers negatively

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charged electrons out of the cytoplasm. This leads to accumulation of positively charged H^+ ions in the cytoplasm, causing membrane depolarization and intracellular acidification. Therefore, H^+ extrusion through proton channels helps to prevent depolarization of the cell during the respiratory burst and is essential to maintain the activity of the enzyme NADPH oxidase (8, 9). Proton channels are also expressed in phagosomes of granulocytes (10) which is consistent with its proposed role during the respiratory burst. Proton channels also help to prevent intracellular acidification in neutrophils during respiratory burst (11). Voltage gated proton channels present in the brain microglia contribute to neuronal death after ischemia (12).

Proton currents in peripheral blood monocytes have been studied in perforated patch configuration after stimulation with phorbol myristate acetate (PMA) and exposure to glucose. An increase in proton conductance, faster channel activation and slow deactivation were observed on activation with PMA, while glucose did not have any effect on the proton currents (13). Proton currents have been described in THP – 1 monocyte (a monocyte leukemic cell line) and after their differentiation to macrophages with PMA (14). The total current as well as current density was found to decrease after PMA induced differentiation.

THP – 1 monocytes, derived from a human monocytic leukemia cell line, are considered to be a more differentiated cell line than PBMs (15, 16). Monocyte derived macrophages are also closer in phenotype and function to tissue macrophages when compared to macrophages obtained by PMA induced differentiation (16). This study investigates the changes in proton currents during culture-induced differentiation of PBMs to macrophages.

Materials and Methods

The work was conducted at the Department of Physiology, Christian Medical College, Vellore after obtaining approval from the institutional review board.

Isolation of monocytes from peripheral blood

Peripheral blood mononuclear cells were isolated by density gradient centrifugation. 10 ml of heparinised peripheral venous blood collected from healthy human volunteers was diluted with an equal amount of RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA). The diluted blood was layered over 10 ml of Ficoll-paque (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 400 g at 20ºC for 30 min. The mononuclear cells were harvested from the interface between Ficoll and the supernatant plasma and washed twice with phosphate buffered saline (PBS) containing 2 mM EDTA. The cells were re-suspended in PBS with EDTA. Monocytes were isolated from peripheral blood mononuclear cells by magnetic-activated cell sorting (MACS) technique using anti CD 14 coated magnetic beads (Miltenyi Biotec, Aurburn, CA, USA).

Culture of monocytes

The monocytes were cultured in 35 mm tissue culture dishes at a concentration of 2×10^5 cells/ml in RPMI supplemented with 10% fetal bovine serum (FBS)(Gibco, Auckland, New Zealand), 100 U/mlPenicillin/Streptomycin, 2.5 µg/ml of Amphotericin-B at 37ºC in a humidified 5% CO_2 incubator for 3-6 days. Cells were harvested on day 3 or day 6 of culture. Harvesting was done by gentle flushing of the culture dishes with PBS containing EDTA, and the cells were washed and re-suspended in PBS. Patch clamp experiments were performed on the day of isolation, day 3 and day 6 of culture.

Patch clamp experiments

The data were acquired using the Axopatch 200B patch-clamp amplifier and digitized with the Axon Instruments Digidata 1322A A-D converter. Micropipettes were fabricated using borosilicate glass capillary tubes (Kimax Borosilicate Capillaries, Fischer Scientific, USA). Tip resistance ranged between 3-5 MΩ when immersed in bath solution.

Voltage gated proton currents were recorded in monocytes and monocyte derived macrophages in whole-cell configuration. The bath solution contained

60 mM CsCl, 100 mM HEPES, 2 mM $CaCl_2$, 1 mM EGTA and 10 mM glucose. The osmolarity of the bath solution was made up to 300 mOsm/L and pH titrated to 7.5 with 1M tetramethyl ammonium hydroxide. The pipette solution had 100 mM MES, 35 mM CsCl, 3 mM $MgCl_2$, 1 mM EGTA and 10mM glucose. The pH of pipette solution was titrated to 6.0 with concentrated methane sulphonic acid. Voltage-gated proton currents were recorded using depolarizing step voltages. All experiments were performed at room temperature (25°C).

Series resistance compensation (60-70%) was applied before each recording. Data were sampled at 10 kHz. Offline filtering of data was done when required. Igor Pro version 5.0.4.8 (Wave Metrics, Inc.) was used for data representation and offline analysis.

Statistical analysis

All data are expressed as mean±SD. Capacitance, absolute current and current densities on different days of culture were compared using Mann Whitney – U test. SPSS version 17 was used for statistical analysis. $p<0.05$ was considered to be significant. The activation time constant (τ_{act}) and the tail current time constant (τ_{tail}) were obtained by fitting raw current tracings with a single exponential curve. Clampfit version 9.2 (Axon Instruments) was used for curve fitting. As depolarizing potentials positive to +20 mV resulted in drooping of outward currents during the later phases of the voltage pulse, curve fitting was done only on the initial segment.

The Boltzmann function used to fit the conductance-voltage (g-V) relationship used the following formula,

$gH = gH.max/[1 + \exp((V_{1/2} - V)/V_{slope})]$, where gH is the conductance of hydrogen channels, gH.max is the maximum conductance, $V_{1/2}$ is the membrane voltage at which conductance is half the maximum value; and V_{slope} is the slope factor related to the steepness of the curve.

Results

Slowly activating outward currents were recorded when depolarizing pulses were applied to monocytes (Figure 1a). They were inhibited by addition of 0.5 mM $ZnCl_2$ to the bath solution (Figure 1b).

The outward currents activated at –30 mV (Figure 2a and 2b) and reversed at –70 mV (Figure 3). The conductance-voltage (g-V) relationship as shown in Figure 2b depicts an increase in conductance with membrane depolarization. Data were best fitted with a standard Boltzmann fit, commonly used to describe g-V curves.

The mean peak proton current density in peripheral blood monocytes recorded at +60 mV was found to be 113.56±37.6 pA/pF (n=21).

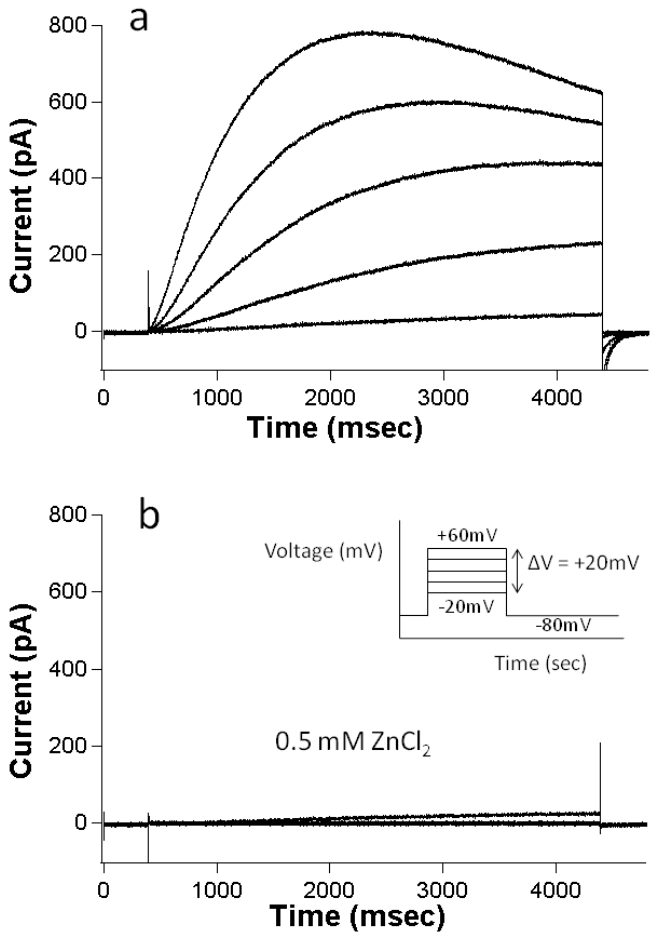

Fig. 1 : Zinc-sensitive outward currents in a human monocyte a) A family of slow-activating outward currents recorded from a human monocyte using step voltage pulses. V_{Hold} = –80mV; test potentials range from –20 mV to +60 mV at 20 mV increments. b) Recording made from the same cell after addition of 0.5 mM ZnCl2 shows inhibition of outward currents. The voltage protocol used is shown in the inset. Filtered at 1 kHz.

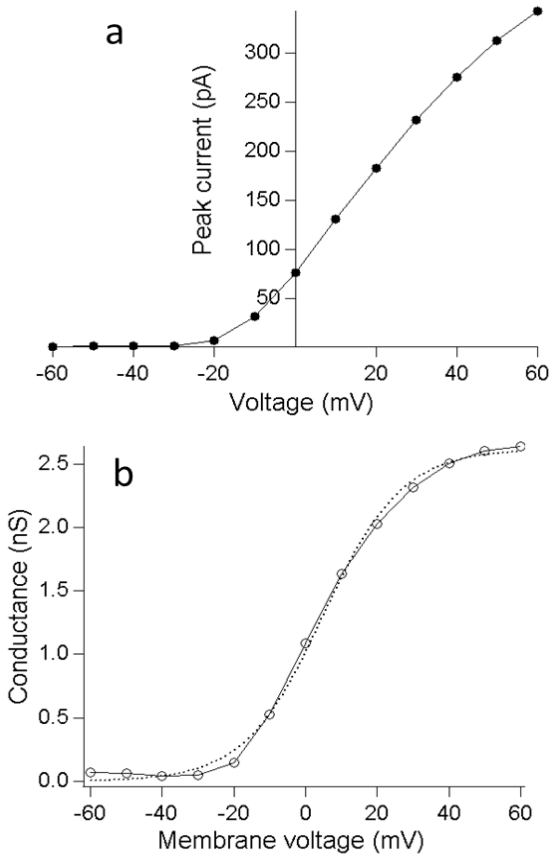

Fig. 2 : I-V and g-V relationships of proton currents recorded in human monocyte a) The current-voltage relationship of a representative proton current recording. The activation threshold of proton currents is observed to be around –30 mV. The bath pH (pHo) was 7.5 and the internal pH (pHi) was 6.0. b) The conductance-voltage relationship of the proton channels plotted using the data shown above. The solid line with markers represents the conductance as calculated from the data. The conductance increases in a sigmoid fashion with membrane depolarization. The dotted line represents asigmoid fit of the g-V curveusing Boltzmann function. Boltzmann fit parameters are: $V_{1/2}$ = 4.9±0.86 mV; V_{Slope} = 11±0.8 mV and $g_{H.max}$ = 2.6±0.06 nS.

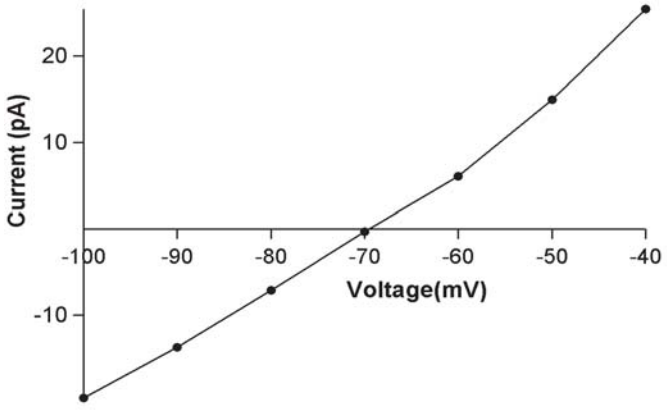

Fig. 3: Tail current-voltage relationship of proton currents. A representative tail current-voltage relationship of proton currents obtained from a human monocyte on the day of isolation, showing reversal around –70 mV. The membrane was held at –80 mV and depolarized to 0 mV. Test pulses ranging from –100 mV to –40 mV at 10 mV increments were applied.

Changes in culture

Light microscopy showed an increase in cell size as the cells differentiated in culture. Increase in cell size is accompanied by an increase in the surface area of the cell membrane. Cell membrane acts as a good capacitor due to its extreme thinness and low electrical conductivity. Therefore, an increase in the surface area of a cell would increase its electrical capacitance. This line of reasoning is supported by the increase in the cell capacitance observed on day 3 (12.3±1.4 pF) and day 6 (21.2±2.6 pF) of culture compared to the day of isolation (4.4±1.1 pF) ($p<0.001$). The increase in cell capacitance on day 6 compared to day 3 was also significant ($p=0.002$) (Fig 4).

There was an increase in the peak current amplitude recorded at +60 mV on day 3 (849±365.8 pA) and day 6 (772±180.9 pA) of culture compared to day 0(510.8±241.8 pA) (Figure 5a). However, there was a drop in the peak current amplitude on day 6 compared to day 3 which was not statistically significant.

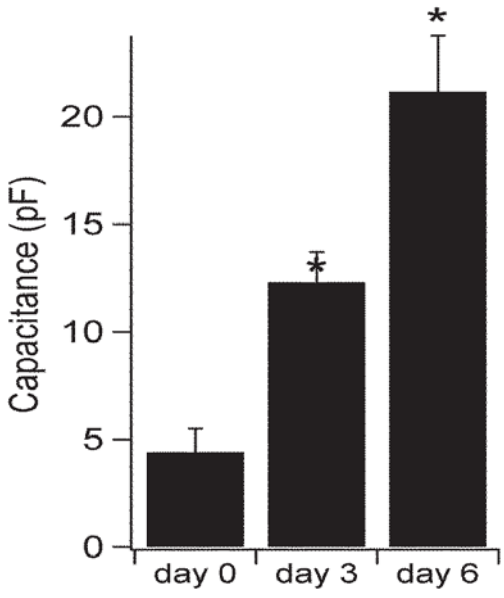

Fig. 4 : Cell capacitance during differentiation of monocytes in culture into monocyte derived macrophages. Capacitance of monocytes recorded on the day of isolation, third and sixth days during culture. The capacitance values are given as mean±SD. The cells had grown significantly larger during the culture as seen by their increase in cell capacitance. (n = 11 on day 0; n = 8 on day 3 and n = 6 on day 6). *p<0.001 with Mann-Whitney U test.

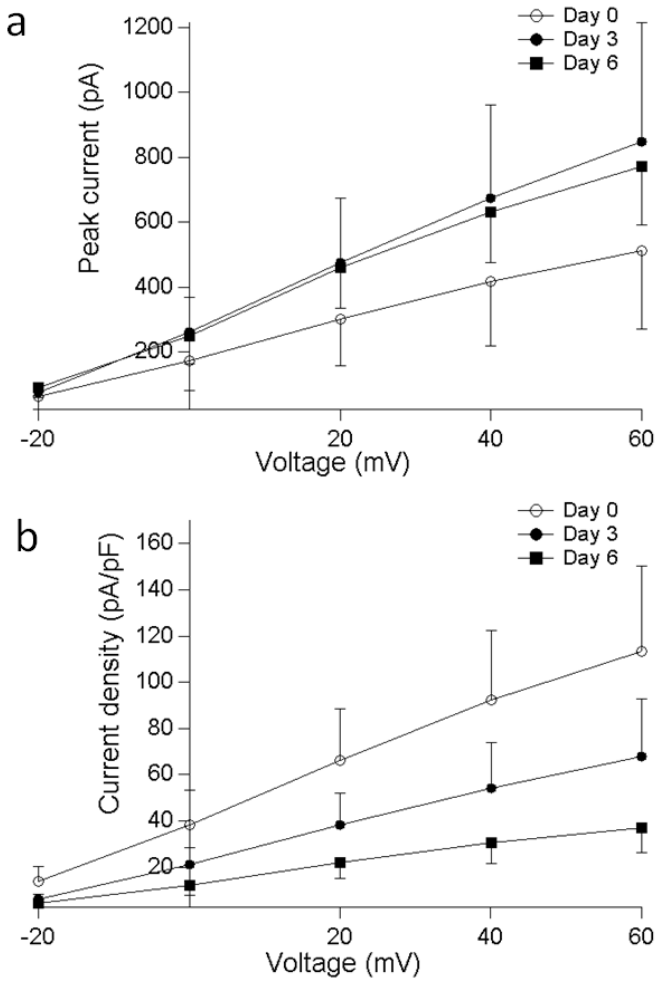

Fig. 5 : I-V relationship of proton currents in monocytes and monocyte derived macrophages a) The relationship between absolute current magnitude of proton currents and membrane voltage at day 0, 3 and 6 of culture. The currents recorded on day 3 and 6 were significantly larger than those recorded on the day of isolation. b) The relationship between proton current density and membrane voltage on three different days of culture (day of isolation, 3 and 6 days during culture). The current density showed significant reduction on day 3 and day 6 of culture as compared to the day of isolation ($p<0.05$ with Mann-Whitney U test). Proton currents were recorded with pHo = 7.5 and pHi = 6.0. (n = 11 on day 0; n = 8 on day 3 and n = 6 on day 6).

The peak current density at +60 mV was reduced to 60% and 32% on day 3 and day 6 of culture respectively, when compared to the current density on day of isolation ($p<0.05$) (Figure 5b).

Gating kinetics

The activation time constants for each voltage were obtained by fitting the raw current tracings with a single exponential curve. The activation time constants (τ_{act}) decreased progressively as the membrane voltage was stepped up to more depolarizing potentials, which indicates faster activation with depolarization (Figure 6a). The deactivation time constant (τ_{tail}) increased with depolarizing pulses showing slowing of proton channel deactivation at depolarizing voltages (Figure 6b). The activation kinetics was not significantly different before and after culture.

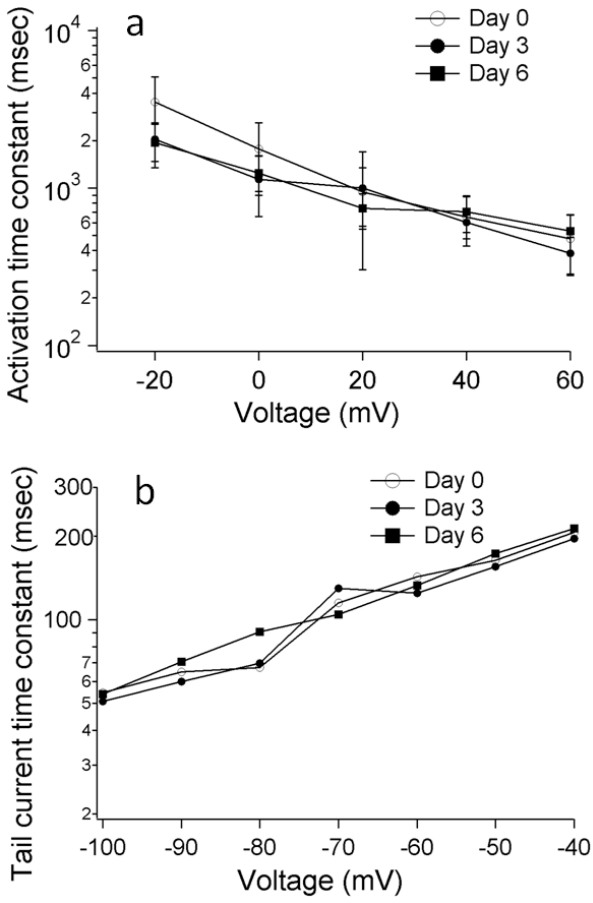

Fig. 6 : Activation and deactivation time constants of proton currents during differentiation of monocytes in culture to monocyte derived macrophages. The time constants were obtained by fitting raw current tracings with a single exponential curve. The y-axes are shown in logarithmic scale. a) The relationship between activation time constant of proton currents and membrane voltage on day 0, 3 and 6 during the culture. Data shown as mean±SD. Activation was observed to be faster with progressive membrane depolarization as shown by a decrease in activation time constant. No significant difference in channel activation was observed during culture. (n = 11 on day 0; n = 8 on day 3 and n = 6 on day 6). b) The tail current time constants plotted against membrane voltage. The figure shows 3 representative data from day 0, day 3 and day 6 of culture. Deactivation time constant increased with progressive membrane depolarization.

Discussion

Voltage gated proton currents have been previously described in THP-1 monocytes, which is considered to be a more differentiated cell line than peripheral blood monocytes (15,16). Differentiation of monocytes to macrophages is accompanied by changes in the phagocytic capacity and respiratory burst activity, which are closely related to the function of proton channels. This study aims to describe proton currents in freshly isolated human peripheral blood monocytes, and the changes that occur during their differentiation to macrophages in culture.

Proton currents in THP -1 monocytes and peripheral blood monocytes (13,14) has been described earlier and were found to be similar to proton currents described in other phagocytic cells in terms of their voltage dependence, kinetics and zinc sensitivity. In the present study, voltage-gated proton currents were studied in peripheral blood monocytes in whole-cell configuration. The threshold potential for the recorded outward currents was observed to be around –30 mV when the proton gradient between the pipette and bath solutions was 1.5 pH units with the proton gradient directed outward. This is comparable to the data previously published on proton channel activation in alveolar epithelial cells (18). The outward currents recorded were blocked by 0.5 mM Zn^{2+}, a known blocker of voltage-gated proton channels. At membrane potentials positive to +20mV, the outward currents exhibited a discernible droop towards the later phase of the voltage pulse. As the proton channels do not exhibit inactivation, the cause for the droop is probably intracellular proton depletion (19).

Proton channel conductance increased in a sigmoid fashion as the membrane voltage was progressively depolarized as seen in Figure 2b. Proton channel activation was found to occur faster with progressive membrane depolarization (Fig. 6a). Deactivation was hastened with progressive membrane hyperpolarization (Fig. 6b). Analysis of the tail current-voltage relationship showed that the reversal potential (E_{rev}) of the recorded outward currents was approximately –70 mV. The calculated H^+ reversal potential (E_H) was –90 mV (Fig. 3). This discrepancy between the observed and calculated reversal potentials could be due to the depletion of hydrogen ions on the intracellular side as a result of continuous proton efflux. This is expected to occur at the depolarizing membrane potential used to measure E_{rev}. The resultant alkalinization of the intracellular environment would shift the proton reversal potential to a less negative value as observed in the tail current-voltage relationship.

The peak proton current density at +60 mV was recorded in 21 freshly isolated peripheral blood monocytes. The peak current density was about 5 to 6 times higher in the freshly isolated human monocytes when compared to that reported in THP-1 monocytes in a previous study (14) (Fig. 5b). Though the THP-1 monocytes have many characteristics similar to peripheral blood monocytes, they are considered to be more differentiated cells than peripheral blood monocytes (15, 16). This could be the cause for the difference in density of voltage-gated proton currents between the two cell types. The activation time constant was 2 fold smaller in the peripheral blood monocytes when compared to that of THP-1 cells, which implies faster activation in PBMs.

Peripheral blood monocytes are known to differentiate into macrophages, when cultured in medium containing serum (20). Differentiation of monocytes to macrophages is associated with changes in phenotype and function (20). Changes in expression of ion channels have also been reported during differentiation. Expression of potassium channels has been found to change during differentiation of THP -1 cells into macrophages under the influence of PMA (21). Another study reports changes in density of proton currents during PMA-induced differentiation of monocytes to macrophages (14).

In the present study, monocytes were cultured for 6 days under appropriate culture conditions in the presence of FBS. Analysis of the cells under light microscopy revealed progressive increase in cell size, suggesting differentiation of monocytes into macrophages. However, this was not confirmed by functional or phenotypic assays. A significant and progressive increase in cell capacitance was noticed

during culture which corroborates the light microscopic finding of increase in cell size. The mean current amplitude increased on day 3 when compared to the day of isolation. However a small reduction in current magnitude was observed beyond day 3 in spite of a 1.7-fold increase in cell size from day 3 to day 6. The current density decreased on day 3 and day 6 of culture compared to the day of isolation at all recorded membrane voltages (Figure 5b). This suggests that density of voltage gated proton channels decreased on the monocyte membrane during their differentiation into macrophages. The results of the present study are consistent with the results of a previous study in which PMA-induced differentiation of THP-1 monocytes was accompanied by a reduction in the density of voltage gated proton currents to half (14).

The results of the present study indicate that proton current density decreases when peripheral blood monocytes differentiate into macrophages in culture. These results are comparable to the earlier reports on THP-1 monocytes. However, these findings are contrary to that found during granulocytic differentiation studied in PLB-985 cells, which was accompanied by a parallel increase in expression of Hv1 proton channels and Nox2 (an enzyme of the NADPH oxidase complex) (22). Phagocytic cells exhibit a correlation between the activities of the proton channel and the enzyme NADPH oxidase (23) that generates superoxide radicals during respiratory burst. PMA-induced activation of phagocytes activates NADPH oxidase and increases proton channel activity by enhancing their gating characteristics. Cells that express high levels of NADPH oxidase show increased gating enhancement of proton channels during PMA activation.

Differences have been reported between monocytes and macrophages in their capacity to generate superoxide radicals and efficiency to kill intracellular pathogens. The observed decrease in proton current density of monocytes upon differentiation can be explained by the comparatively lesser ability of macrophages to produce superoxide radicals (24). It suggests a probable decrease in the expression of NADPH oxidase during differentiation. However, this demands further experimentation and functional assays.

Conclusion

Differentiation of peripheral blood monocytes to macrophages in culture is accompanied by a decrease in proton current density, which could be related to functional changes accompanying differentiation. Differences exist between PBMs and THP-1 monocytes with respect to proton current amplitude and kinetics. Hence THP-1 monocytes may not be a perfect model to study electrophysiological characteristics of monocytes.

Acknowledgements

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Conflict of interest

None

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Original Article

To Evaluate the Effect of Olmesartan on Blood Glucose Levels and Blood Lipid Levels in Streptozotocin Induced Diabetic Rats

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Abstract

Experimental Approach: Wistar albino rats were randomly selected and divided into 3 groups. Diabetes was induced by injecting Streptozotocin intraperitonelly. The control group received 1% Gum acacia (oral), standard group received 0.5 mg/kg Glibenclamide (oral) and the test group received Olmesartan 3.6 mg/kg body weight (oral) from 0-28 days respectively. Fasting blood glucose was estimated on 0, 1, 3, 7, 14, 21 & 28th day by (ACCUCHECK) glucometer and fasting lipid profile by lipid screening strips on 1st and 28th day.

Key Results: The blood glucose levels in the Olmesartan group was less when compared to the control group at all the intervals but comparable with that of glibenclamide. The Olmesartan group showed improved lipid profile when compared to control group in streptozotocin induced diabetic rats.

Conclusion and Implications: Olmesartan showed hypoglycemic activity and improved lipid profile action which is comparable to standard drug glibenclamide.

Introduction

Diabetes mellitus (DM) refers to a group of common metabolic disorders that share the phenotype of hyperglycemia. DM is usually caused by a complex interaction of genetics, environmental, inflammation and autoimmune factors. The metabolic dysregulation and complications are associated with diabetes are due to glucotoxicity, lipotoxicity, formation of Advanced Glycation End Products (AGEs), Protein kinase C and Hexosamine pathway products, all these comprehensively causes secondary pathophysiologic changes in multiple organ systems

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that impose a tremendous burden on the individual with diabetes and on the health care system (1, 2, 3).

Newer targets of angiotensin receptor blockers (ARB's) in diabetes mellitus:

Angiotensin II is formed from angiotensin I in a reaction catalyzed by angiotensin-converting enzyme (ACE, kininase II). Angiotensin II is the principal pressor agent of the renin-angiotensin system, with effects that include vasoconstriction, stimulation of synthesis and release of aldosterone, cardiac stimulation, and renal reabsorption of sodium (4).

Action of angiotensin II through AT_1 receptor:

Angiotensin II acting through AT_1 receptor increases the synthesis and concentration of tumor necrosis factor α, interleukin6, IL-1, chemokine monocyte chemo attractant protein1 and nuclear factor kappa of activated B cells (NFκB) which results in inflammatory cell infiltration in β cells and is important in the pathogenesis of type 2 diabetes. These inflammatory cytokines are important in the pathogenesis of lipid metabolism also. IL-6 and IL-1 act on the liver to produce the characteristic dyslipidemia of the metabolic syndrome, with increased VLDL and decreased HDL. IL-1β is known to activate the Inhibitor of $\kappa\beta$ (I$\kappa\beta$) and induce insulin resistance. Hence, Angiotensin II through AT_1 receptor can advance the occurrence of Diabetes or insulin resistance by above said mechanisms.

Action of adiponectin and adipokines:

Obesity causes inflammation particularly central obesity (mesenteric fat) (LTB4 an inflammatory molecule) which in turn can lead to type 2 diabetes by releasing inflammatory cytokines from the mesenteric adipocytes. Extra fat particularly in the liver and mesentery activates the resistant macrophages and immune cells. When these macrophages are activated release LTB4 and other immune signaling molecules to influx of new macrophages as a positive feedback loop, the newly arriving macrophages also get activated and release more LTB4. When inflammation is chronic as in case of obesity and the LTB4 starts activating other cells Fig. 1.

Macrophages of liver, fat (more so in mesenteric fat) and skeletal muscle cells also have LTB4 receptors on their surfaces and are activated when LTB4 binds to them. In obesity these cells become inflamed and release adipokines leading to insulin resistance (5).

Adipocytes secrete a number of biological products (adiponectin, non-esterified free fatty acids, retinol binding protein 4, leptin, TNF-α and resistin). Adipocytes products or adipokines produce an inflammatory state, these adipokines modulate insulin sensitivity and cause insulin resistance in skeletal muscles and liver and may explain why markers of inflammation as IL6 and C-reactive protein are often elevated in type 2 diabetes (6). Adiponectin acts as an insulin sensitizing peptide is reduced in Diabetes

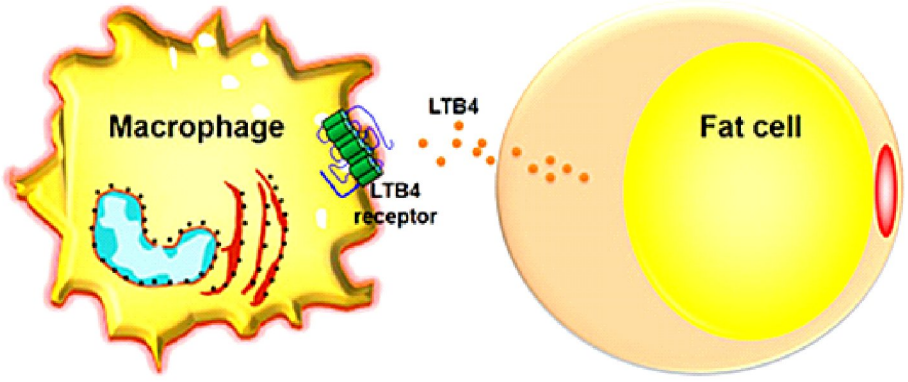

Fig. 1: Release of adipokines.

and this may contribute to insulin resistance (7).

Adiponectin belongs to the family of adipocytokines, is exclusively synthesized by white adipocytes, and is induced during adipocyte differentiation. Adiponectin plays a role in regulation of glucose metabolism, lipid metabolism, inflammation and oxidative stress. Hence, adiponectin plays a role in lowering blood glucose and blood lipid levels (8).

Adiponectin has 2 receptors

1. Adipo R1 – skeletal muscle

2. Adipo R2 – Liver

These two adiponectin receptors are predicted to contain seven transmembrane domains but to be structurally and functional distinct from G-protein coupled receptors. Expression/suppression of AdipoR1/R2 by small interfering RNA serve as receptor for globular and full length adiponectin and they mediates AMP kinase, PPAR γ and PPAR-α ligand activities as well as fatty acid oxidation and glucose uptake (9, 10).

Molecular mechanism underlying the insulin sensitizing action of adiponectin, indicates that stimulation of glucose utilization and fatty acid combustion by adiponectin, through activating AMPK (5' Adenosine Monophosphate kinase) and there by directly regulating glucose metabolism and insulin sensitivity (11).

Improved hepatic insulin sensitivity occurs, leading to postulate that the primary effects of adiponectin on muscle are to augment uptake and combustion of free fatty acids (FFAs), whereas decreased liver triglyceride content results from secondary reduction in serum FFA and triglyceride levels Fig. 2.

Hence angiotensin II activity through AT_1 receptor will decrease the production of adiponectin and increase the inflammatory adipokines by which

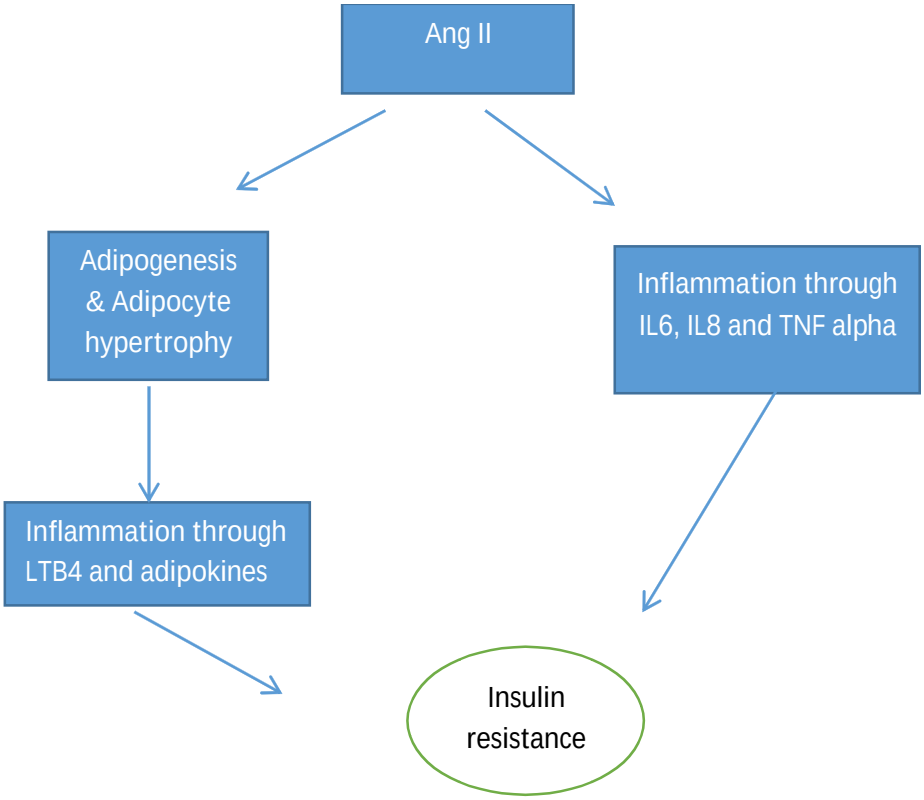

Fig. 2: Mechanism of insulin resistance.

contributing to one of the factor of insulin resistance and diabetes. Thus, Angiotensin II acting through AT_1 receptor at various places of its target like â cells, hepatic tissues, adipose tissues (skeletal muscle tissue) possibly influence aggravation or initiation of diabetic status.

Angiotensin receptors blockers reduce inflammation, regulate cell growth, apoptosis, decreasing fibrosis, decreasing collagen deposition etc. of β cell, skeletal muscle cell and adipose tissue. These are expressed because it increases adiponectin directly by inhibiting the activation of AT_1 receptors by angiotensin II and also mediated by PPAR-γ and AMP (5' Adenosine Monophosphate) activated protein kinases. Olmesartan is a partial PPAR α agonist and induces PPAR α expression. Thus there is induction of hepatic ACSL1 (Acyl CoA Synthetase Long chain) and CPT1A (Carnitine Palmitoyl Transferase). This causes significant decrease of triglyceride level.

Thus, angiotensin II receptor inhibition through Olmesartan has blood glucose lowering effect and also lowers triglyceride levels, total cholesterol levels, LDL levels and raise HDL levels, by inducing adiponectin protein expression, via PPAR γ, AMP kinases activation and decreasing the inflammatory response of IL1, IL6, TNF-α etc. Lipid lowering effect by PPAR α expression, induction of hepatic ACSL1 (Acyl CoA Synthetase Long chain), CPT1A (Carnitine Palmitoyl Transferase) and reduction in catecholamine levels (noradrenaline).

Hypothesis:

Thus it may be hypothesized that Olmesartan decrease the blood sugar level and lipid level through its activity of blocking action of angiotensin II on AT_1 receptor and promoting the activity of adiponectin and decreasing the activity of adipokines.

Methods

The study was conducted at Central Animal Facility and all animals care and experimental protocols were approved by the Institutional Animal Ethics Committee (*IAEC)*.CPSEA approval number from IAEC of: JSSMC/IAEC05/5657/DEC 2013.

Wistar albino rats of either sex of average weight 150-200 gms aged 3-4 months were used in the experiments. The rats were inbred in the central animal house, under suitable conditions of housing, temperature, ventilation and nutrition. Rats were housed two to three per stainless cage under conventional conditions. They were kept at a constant temperature of 26±2°C and relative humidity of 30-70% under a 12 h dark/light cycle. Food and water were available *ad libitum*. The rats were acclimatized to the laboratory conditions for seven days prior to test before assigning animals to treatment group. The doses of drugs were based on human daily dose converted to that of rats according to Paget and Barnes (1962). The method employed in this study to induce diabetes was chemical method using streptozotocin, given intraperitoneally. Blood glucose estimation was done by using glucometer.

Drugs and Chemicals: Glibenclamide (*Sanofi Aventi, India),* Olmesartan (*Macleods, India),* Streptozotocin *(Sisco Research Laboratories Pvt. Ltd. India).* The rats were divided into 3 groups containing six animals (n=6) in each group (control, standard and test group).

Induction of diabetes:

Following an overnight fast, 24 rats were injected intraperitonally, with freshly prepared Streptozotocin (dissolved in sodium citrate buffer) under aseptic precautions in a dose of 55 mg/kg body weight. Animals were carefully observed for first 24 hours following the injection for any evidence of allergic reactions, behavioural changes, convulsions and hypoglycemic attacks. No untoward reactions were observed in any animal.

Blood glucose level was recorded daily morning at 9.00 am for 3 days. Animals which developed stable hyperglycemia on 3rd day with blood glucose level more than 2500 mg/L were selected for the study. They were randomly grouped as Diabetic control, Diabetic standard, Olmesartan group. All the drugs were given for 28 days.

Group 1: Diabetic control: 1% Gum acacia (PO)

Group 2: Standard: 0.5 mg/kg body weight, Glibenclamide (PO)

Group 3: Olmesartan: 3.6 mg/kg body weight (PO).

- Blood was collected from 12 hr fasted rats by rat tail vein puncture method, 1hr after each dose administration of the respective drugs and fasting blood glucose was estimated by (ACCUCHECK) glucometer on 0, 1, 3, 7, 14, 21 & 28th day.
- Body weight of the individual rats was measured on the respective days before blood glucose estimation on 0, 1, 3, 7, 14, 21 & 28th day.
- Estimation of fasting lipid profile by lipid screening strips on 1st and 28th day.

Statistical analysis:

The results was analyzed Mean and standard deviations were calculated for each group. One way ANOVA was used for multiple group comparisons followed by post hoc Tukey's test for statistical significance between groups. IBM SPSS statistics ©IBM Corporation and Other(s) 1989, 2012 software was used for statistical analysis purpose. $P<0.05$ was considered as significant.

Results

The diabetic control rats showed progressive hyperglycemia and the standard drug showed persistent decrease in the blood glucose level from 1st to 28th day, while the test drug, Olmesartan did not show appreciable decrease in blood glucose levels from 1st to 3rd day but thereafter produced consistent decrease in blood glucose levels upto 28th day. There was reduction in CBG level of Standard and Olmesartan group which was very minimal on 1st day but started producing consistently progressive fall in CBG level from day 3-day 28. Week wise comparison in blood glucose level showed that the Olmesartan group showed lesser reduction in the first week compared to the standard. At the end of second week standard continued similarly but there was continued fall in the test group. At the end of 4th week both Olmesartan and standard almost performed same activity.

There was a gross increase in total cholesterol, triglyceride and LDL level in diabetic control but the level of total cholesterol was very minimal with respect to standard, whereas there was moderate increase in total cholesterol with respect to Olmesartan. There was gross decrease in HDL level in diabetic control group. The fall in HDL was very minimal with standard. The decrease in HDL was very minimal with Olmesartan (better than standard).

In diabetic control group, there was reduction of 20% body weight and in standard group 7% increase in body weight. In the Olmesartan group there was 3.53% increase in the body weight.

Discussion

Angiotensin II exerts several cytokine like actions via the AT1 receptor and can stimulate multiple signalling pathways, activate several growth factor receptors, and promote the formation of reactive oxygen species (ROS) and other proinflammatory responses (12).

Angiotensin II, have a potential role in endothelial cell dysfunction, insulin resistance, inammation, and proliferative effects (13). Insulin resistance of Angiotensin II is by interfering with the insulin-stimulated increase in insulin receptor substrate 1-associated PI3K activity (14). Angiotensin II also stimulates the production of superoxide radicals, TGF-β, endothelin, and plasminogen activator inhibitor (PAI-1), which ultimately interferes in NO action (15).

The main insulin-sensitizing action of adiponectin results from decrease in hepatic gluconeogenesis and increase in muscle glucose transport and, secondly from enhancement of energy consumption and fatty acid oxidation in peripheral tissues with the aim of increasing ATP production. Accumulating evidence from clinical, experimental animal and genetic studies support a close association between hypoadiponectinemia and insulin resistance/ type 2 diabetes (16).

The primary effects of adiponectin on muscle are to

augment uptake and combustion of free fatty acids (FFAs), whereas decreased liver triglyceride content results from secondary reductions in serum FFA and triglyceridelevels (17).

The induction of adiponectin in fact might be caused by secondary effects involving other PPAR- inducible genes and not by specific activation of the PPAR response elements (18).

The strong inverse correlation between serum adiponectin levels and intra-abdominal fat mass may in part underlie the link between visceral fat and insulin resistance (19).

Olmesartan has been applied most frequently because of having partial peroxisome proliferator-activated receptor gamma (PPARγ) agonist activity (20).

The possible mechanisms by which ARBs may improve the insulin resistance are hemodynamic effects, increase of glucose transport and improvement of the intracellular signal transduction of insulin, through the blockade of Ang II and inhibition of oxidative stress. (21, 22, 23).

Angiotensin receptor blockers have a partial agonist action of PPARγ and are expected to have beneficial effects on insulin resistance by increasing adiponectin levels.

In the present study, the standard group treated with glibenclamide (0.5 mg/kg) showed a steady decrease in blood glucose levels from 3588 mg/L on Day 0 before administration of drug to 1736.6 mg/L on day 28 thus indicating that the standard drug has a good immediate and also prolonged hypoglycemic action. Diabetic Olmesartan group decreased blood glucose level from 3553.3 mg/L on day 0 to 2008.3 mg/L on day 28. The difference in the blood glucose readings between D0 to D28 for Olmesartan group is 1545 mg/L. The control group treated with gum acacia increased the blood glucose level from 3511 mg/L on day 0 to 4388 mg/L on day 28.

The progressive consistent hypoglycemic effect with respect to duration of administration and maximum effectiveness of the test drug was seen after 1st week, but persistent continued hypoglycemic activity was continued up to end of 4 weeks. At the end of study the percent reduction of blood glucose level in Olmesartan group was 54.23% when compared to diabetic control, while, 16.57% decrease in blood glucose level when compared to Standard (Glibenclamide) group and has shown similar reduction in mean percent blood glucose level, and it was statistically significant ($p<0.005$) compared to diabetic control group. This indicate that Olmesartan has significant and sustained hypoglycemic activity persisting till last day (28th day) compared to standard in their respective experimental dosages. The above data conclude that Olmesartan has the capacity to improve the glycemic status in experimentally induced diabetes in animals and the glycemic status of test drug is almost equal to that of standard at all-time intervals (Table I).

In diabetic control there was a gross increase in total cholesterol (93.17 mg/L), triglyceride (1088.3 mg/L) and LDL level (858.3 mg/L), whereas there was gross decrease in HDL level (101.7 mg/L) compared to both standard and test group from 0-28 day. While, the test drug Olmesartan also showed moderate increase in total cholesterol (313.4 mg/L), triglyceride (540 mg/L) and LDL levels (386.7 mg/L)

Table I: Blood glucose levels in different groups.

	Groups	*D0*	*D1*	*D3*	*D7*	*D14*	*D21*	*D28*
1	Diabetic control	3511.6±118.0	3703.3±158.5	3821.6±20.15	3893.3±238.2	4055±227.9	4206.6±237.6	4388.3±257.6
2	Standard	3588.0±159.4	3516.6±212.7	3273.3±17.52	3013.3±190.7	2655±227.5	2001.6±247.0	1736.6±244.8
3	Olmesartan	3553.3±186.9*	3518.3±205.3*	3251.6±23.04*	3025.0±183.9*	2713.3±100.1*	2308.3±241.6*	2008.3±220.6*

Data expressed in Mean mg/L ± SD values. *P<0.05 compared with control.
D0 = before giving the drug.
D1, D3, D7, D14, D21, D28 = 1st, 3rd, 7th, 14th, 21st, 28th days of administration of the drugs respectively.

from 0-28 day when compared to control. The decrease in HDL levels of Olmesartan was 66.6 mg/L from 0-28 day. Olmesartan was inferior to standard (60 mg/L) in reducing the total cholesterol from 0-28 days. Olmesartan was inferior to standard (76.7 mg/L) in reducing the triglyceride levels from 0-28 days. Olmesartan was inferior to standard (26.7 mg/L) in reducing the LDL levels from 0-28 days. Olmesartan was inferior to standard (23.3 mg/L) in increasing the HDL levels from 0-28 days. Thus to conclude the test drug Olmesartan is better in improving the lipid profile compared to control and is comparable with that of standard (Table II, III, IV and V).

As a consequence of induction of diabetes by Streptozotocin there was significant reduction in body weight in the control group of rats between 0-28 days. In the standard group of rats there was no reduction in the body weight rather there was slight improvement in weight from 0-28 days but in the Olmesartan group there was no much change in the body weight between 0-28 day of experimentation (Table VI). Improved body weight of the treated animals indicates the efficacy of Olmesartan in controlling the glucose excretion and blood glucose level of diabetic rats. The activity and behavior of diabetic control was less and gradually decreased from 0-28th day but the activity and behavior was almost normal throughout the study in standard and Olmesartan group.

Conclusion

Thus, the hypothesis put forth in the beginning, the glucose lowering effect of Olmesartan was through the mechanism like inducing adiponectin protein expression, via PPAR γ activation, AMP kinases activation, decreasing the inflammatory response of IL1, IL6, TNF-α and reduction in catecholamine levels (noradrenaline). Lipid lowering effect of Olmesartan was through PPAR α expression, induction of hepatic ACSL1 (acyl coA synthetase long chain), CPT1A (carnitine palmitoyl transferase).

Hence the present study establishes the hypoglycemic activity of Olmesartan group when

TABLE II : Statistical Analysis showing comparison of Total cholesterol levels between different groups on day 1 and day 28.

Groups	*Mean±SD on 1st day*	*Mean±SD on 28th day*	*Difference in TC levels*
Diabetic control	1193.3±107.8	2125±81.1	931.7±26.7
Standard	1066±77.6	1006±78.9	60±1.3
Olmesartan	1141.6±76.0*	1455±58.2*	313.4±17.8*

Data expressed in Mean mg/L±SD values. *P<0.05 compared with control.

TABLE III : Statistical Analysis showing comparison of Triglyceride levels between different Groups on day 1 and day 28.

Groups	*Mean±SD on 1st day*	*Mean± SD on 28th day*	*Difference in TG levels*
Diabetic control	1013.3±143.4	2101.6±144.6	1088.3±1.2
Standard	1095±145.5	1018.3±53.4	76.7±92.1

TABLE IV : Statistical Analysis showing comparison of LDL levels between different Groups on day 1 and day 28.

Groups	*Mean±SD on 1st day*	*Mean± SD on 28th day*	*Difference in LDL levels*
Diabetic control	1278.3±101.4	2136.6±91.1	858.3±10.3
Standard	1113.3±98.9	1140±107.5	26.7±8.6
Olmesartan	1203.3±35.5*	1590±50.1*	386.7±14.6*

Data expressed in Mean mg/L±SD values. *P<0.05 compared with control.

TABLE V : Statistical Analysis showing comparison of HDL levels between different Groups on day 1 and day 28.

Groups	*Mean±SD on 1st day*	*Mean± SD on 28th day*	*Difference in HDL levels*
Diabetic control	373.3±52	271.6±42.6	101.7±9.4
Standard	423.3±30.7	446.6±36.1	23.3±5.4
Olmesartan	401.6±38.6*	335.±39.3*	66.6±0.7*

Data expressed in Mean mg/L±SD values. *P<0.05 compared with control.

TABLE VI : Table showing mean values of body weight of rats in different groups on different days.

Groups	*Before STZ*	*D0*	*D1*	*D3*	*D7*	*D14*	*D21*	*D28*
Diabetic control	215	169	172	153	161	159	171	173
Standard	200	182	173	181	192	189	201	214
Olmesartan	198	170	160	155	178	188	192	205

Values in grams.

compared to control, which is statistically significant ($p<0.05$) and comparable to standard drug Glibenclamide in Streptozotocin induced diabetic rats. Also, Olmesartan shows less improved lipid profile action when compared to control, which is statistically significant ($p<0.05$) and comparable to standard drug Glibenclamide in Streptozotocin induced diabetic rats.

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Nil

Conflict of interest

Nil

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Original Article

Cognitive Status in Hypothyroid Patients Before & After Attainment of Euthyroid State

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Abstract

Thyroid hormones play a significant role in adult brain function. Cognition affects everyday activities and has substantial importance to the population. In this case control study we evaluated cognition in hypothyroid patients compared to euthyroid controls and their response after treatment. Various neuropsychological tests: Mini Mental State Examination, Digit Symbol Substitution Test, Letter Cancellation Task and Trail Making Test, were used to assess cognitive status of 30 newly diagnosed hypothyroid patients and compared with euthyroid controls. Tests were repeated in hypothyroid patients after the attainment of euthyroid state and in euthyroid controls at an interval of three months. We also measured the correlation between cognitive status and serum TSH levels of hypothyroid patients. Cognitive measures for attention/concentration, information processing and executive function were impaired in hypothyroid patients in pretreatment state and significant improvement was found after the attainment of euthyroid state suggesting that thyroxine therapy restores cognition.

Introduction

Cognition is the mental activities involved in the acquisition, storage, retrieval and use of information (1). Integration of a variety of processes and activities such as perception, imagery, memory, reasoning, problem solving, decision-making and language plays an important role in cognition. Cognition affects everyday activities and has substantial importance to the population. Changes in cognition do impact efficiency of multiple operations such as working memory, attention, information processing etc (2). Attention is a basic cognitive mechanism by which a person can focus on relevant objects and ignore the irrelevant ones. It is of various types like selective attention, vigilance or divided attention (3). Inability to focus attention on the task at hand results in inability to cope with the environment.

Amongst the other important biological processes, thyroid hormone is an important neuroregulator in fetal development of the central nervous system, and

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plays an important role after development as well. Hypothyroidism is a well-known reversible factor causing cognitive impairment, including dementia (4). Some theories suggest that the neuropsychiatric symptoms are related to changes in the brain secondary to multiple factors, including the direct effects of thyroid disease, as well as hormone deprivation in brain tissue. Hypothyroidism impacts aspects of cognitive functioning and mood. The degree by which hypothyroidism does correlates to the symptoms remains controversial (4). There remains a debate about which parameters will be predictive of cognitive decline. There have been conflicting reports about the correlation of cognition with TSH (5, 6). Improvement or complete resolution of associated neuropsychological symptoms by adequate treatment of the thyroid disorder has been reported (7-9).

Various tests are used to measure the speed of information processing and specific cognitive domains like attention/concentration, executive functions, spatial working memory, visual attention, task switching etc. Among the psycho-physiological assessment of vigilance, paper pencil tests like the Mini Mental State Examination (MMSE), Digit Symbol Substitution Test (DSST), Letter Cancellation Task (LCT) and Trail making test (TMT) are well established (10, 11). As they are paper-pencil based the advantage of these tests is that they can be administered bedside and persons with minimal literacy level can be tested easily with these tests. As most of our subjects were of lower literacy status we preferred to use these tests.

Most of the earlier studies are done in western population with fewer numbers of subjects. In the present study, we evaluated the effect of hypothyroidism on cognitive functions in Indian population. Various neuropsychological tests are used and results compared after attainment of euthyroid state. Serum TSH levels were correlated with the performance of these tests.

Materials and Methods

Thirty newly diagnosed hypothyroid cases in the age group of 18-50 years were taken from thyroid clinic, GTB Hospital, Delhi and thirty euthyroid controls were recruited for the study after taking written informed consent. The cases and controls were age and sex matched.

The subjects who were suffering from neuropsychiatric illness like depression using DSM IV criteria, or were on medications, especially anti-allergic etc., with a history of alcoholism or any other drug addiction, had a history of myocardial infarction, hypertension or diabetes mellitus, or who were illiterate or had less than 5 years of schooling were excluded from the study.

The estimation of serum levels of free T_3 (fT3), free T_4 (fT_4) and Thyroid stimulating hormone (TSH) of the patients and controls were done in the Endocrine and Metabolic laboratory of Guru Teg Bahadur Hospital, Delhi by using Radioimmunoassay (RIA) kit from Immunotech Bechmancoulter. Ethical clearance was taken from the Institutional Ethical committee for the study. The patients were then started on treatment and were asked for a follow up when they had attained euthyroid status after three months of treatment as evident by changes in the levels of hormones (Table I).

The patients and controls were made to abstain from nicotine and caffeine for at least 12 hours before testing. Testing was done following a restful overnight sleep.

The following tests were done

Mini Mental State Examination (MMSE):

It is an eleven-question measure that tests cognitive functions: orientation, registration, attention, calculation, recall and language. Subjects with a score of <24 were not recruited for the study. The mean scores of the controls and cases were recorded and analyzed (12).

Digit symbol substitution test:

This is a test of visuomotor coordination, motor persistence, sustained attention and response speed. The task requires rapid information processing in order to substitute the symbols accurately and

quickly. The test consists of numbers (1 to 9) arranged randomly in 4 rows of 25 squares each. The subjects were asked to substitute each number with a symbol using a number-symbol key given on each page. The time taken to complete the test along with the errors was noted (13).

Letter cancellation task:

Letter Cancellation Task is measure of sustained attention, concentration, visual scanning, and rapid response activation and inhibition (14). One and Three Letter Cancellation Tasks were used to assess the effect of increasing complexity of task. In the One Letter Cancellation Task the subjects were asked to cancel out letter 'A'. The time taken to complete the task along with the error score was noted. In the Three Letter Cancellation Task, the subjects were asked to cancel out letter 'A', 'Q' and 'T'. The time for completion with the number of errors was noted.

Trail making test parts A & B:

Trail Making is a timed test that measures complex visual scanning, motor speed, and cognitive flexibility (15). Trail Making Test consists of two parts each having 25 circles distributed over a sheet of paper. In part A the circles are numbered 1-25, and the patient was asked to draw lines to connect numbers in ascending order. In part B, the circles include both numbers (1-13) and letters (A-L); the patient draw lines to connect the circles in ascending pattern as in part Abut with the added task of alternating between the numbers and letters. Time taken to complete the task including the time for correction of errors was noted.

Testing of the patients was done before initiating treatment and after attainment of euthyroid state. The controls were tested twice at an interval of three months. Cognitive status of cases and controls were compared in both states. Serum TSH levels of hypothyroid patients were correlated with cognitive status.

Statistical analysis

The data obtained was analyzed by SPSS version 20.0. Results were analyzed by Two way repeated measure ANOVA followed by Tukey's test. p value <0.05 was considered significant.

Results

The mean age of the cases (31.67±8.40 years) and controls (31.00±8.004 years) had no statistical difference. We found significant difference in the serum fT3, fT4 and TSH levels of the patients at diagnosis when compared to the levels post treatment after 3 months ($p<0.01$) (Table I). All patients were found to be euthyroid after 3 months of treatment with thyroxine.

As expected, the MMSE (Table II) showed significant impairment in scores of cases ($P<0.001$) in

TABLE I: Thyroid profile of cases before and after treatment.

	Before (Mean±SD)	*After (Mean±SD)*	*p value (F test)*	*Significance (Tukey's test)*
TSH levels (µIu/mL)	26.43±10.244	3.4863±0.1963	<0.001	Significant
fT_3 (pg/ml)	0.2980±.09408	1.93±0.5690	<0.001	Significant
fT_4(ng/dl)	0.2736±0.0973	1.8986±0.2853	<0.001	Significant

(p value <0.05 is significant)

TABLE II: MMSE score of cases and controls.

	Before (Mean±SD)		*After (Mean±SD)*	*p value (F test)*	*Significance (Tukey's test)*
Cases	29.53±0.681		29.73±0.450	0.012	Significant
Controls	29.97±0.183		29.97±0.183		Not Significant
p value (F test)		0.012			
Significance (Tukey's test)	Significant		Significant		

(p value <0.05 is significant)

comparison to controls at the start of treatment. But there was significant improvement (P<0.001) after treatment on attainment of euthyroid state. Though the difference in reaction time of cases and controls for Digit Symbol Substitution Test (Table III) was not statistically significant (P=0.249). However, the attainment of euthyroid state led to a significant improvement (P=0.015) in comparison to the pre-treatment state. The error hits (Table IV) on DSST were higher in cases before treatment in comparison to controls (P<0.001) and the difference was significant. Significant improvement was found in error hits after treatment (P<0.001).

The cases showed a significant improvement in reaction time (P<0.001) and error hits (P<0.001) on One Letter Cancellation Task (Table III) after treatment in comparison to pretreatment state. But the difference for reaction time between cases and controls was not significant (P=0.234) and for error hits (Table IV) was significant (P<0.001) in both states. The reaction time on Three Letter Cancellation Task (Table III) was also more in cases in comparison to controls but the difference was not significant (P=0.597). The cases showed a significant improvement (P<0.001) after treatment. The error hits (Table IV) were higher in cases (before and after treatment) in comparison to controls (P<0.001). Significant improvement was found in error hits of cases (P<0.001) after treatment.

Cases showed a significant improvement in response time of Trail Making Test A (P<0.001) and Trail Making Test B (P<0.001) after treatment in comparison to pretreatment state.

MMSE score showed a negative correlation (correlation coefficient = –0.755) with serum TSH levels. Reaction time of DSST, One Letter Cancellation Task, Three Letter Cancellation Task, Trail Making Test A and Trail Making Test B showed a positive correlation with serum TSH levels (Table V).

TABLE III: Reaction time in cases and controls (in seconds).

		Before (Mean±SD)	*After (Mean±SD)*	*P value (F test)*	*Significance (Tukey's test)*
DSST	Cases	239.07±63.425	218.70±62.625	0.015	Significant
	Controls	223.43±36.938	222.13±34.483		Not Significant
One Letter Cancellation Task	Cases	261.17±29.813	242.93±30.908	<0.001	Significant
	Controls	250.33±34.004	251.47±36.630		Not Significant
Three Letter Cancellation Task	Cases	291.1±29.463	279.30±29.573	<0.001	Significant
	Controls	275.70±53.607	277.87±52.433		Not Significant
Trail Making Test part A	Cases	86.5±13.985	81.20±12.380	<0.001	Significant
	Controls	83.13±14.897	82.43±12.353		Not Significant
Trail Making Test part B	Cases	102.53±23.948	95.67±17.373	<0.001	Significant
	Controls	97.57±10.150	97.60±12.025		Not Significant

No significant difference in the reaction time of DSST, LCT, TMT A & TMT B between cases and controls was found.

TABLE IV: Error hits in cases and controls.

		Before (Mean±SD)	*After (Mean±SD)*	*P value (F test)*	*Significance (Tukey's test)*
DSST	Cases	1.73±1.048	0.90±.803	<0.001	Significant
	Controls	0.30±.535	0.30±.484		Not Significant
One Letter Cancellation Task	Cases	1.77±.935	0.90±.803	<0.001	Significant
	Controls	0.20±.484	0.20±.407		Not Significant
Three Letter Cancellation Task	Cases	1.77±1.073	0.73±.640	<0.001	Significant
	Controls	0.23±.504	0.20±.407		Not Significant

The error hits of DSST & LCT were significantly higher (P<0.001) in cases in comparison to controls in both states (before & after).

TABLE V : Correlation between Cognitive measures and serum TSH levels of patients.

Cognitive measures	*TSH*	
	Coefficient	*P value*
Mini Mental State Examination	–0.755*	0.000
Digit Symbol Substitution Test	0.559*	0.001
One letter cancellation Task	0.699*	0.000
Three letter cancellation Task	0.697*	0.000
Trail Making Test A	0.794*	0.000
Trail Making Test B	0.923*	0.000

Pearson correlation is significant at the 0.01 level.

Discussion

In the present Case Control study we compared cognitive status of hypothyroid patients with age and sex matched controls and also assessed cognitive status of hypothyroid patients before and after treatment. Mini Mental State Examination (MMSE), Digit Symbol Substitution Test (DSST), Letter Cancellation Task (LCT) and Trail making test (TMT) were used for assessment of cognition.

We found statistically significant impaired MMSE score in overt hypothyroid patients. Studies by Baldini et al (16) and Bono et al (17) on subjects with subclinical hypothyroidism also found neuropsychological changes in global cognitive functioning by using MMSE score while de Jongh et al (18) & Formiga et al (19) did not found impaired MMSE score in subclinical hypothyroidism.

In a follow up study by Gussekloo et al (20), an association was found between low fT_3 levels and decreased global functioning by using MMSE score in an unselected general population of 558 individuals aged 85 years. However there was no correlation of MMSE found with serum levels of TSH or fT4 in their study**.** Our study as well found a negative correlation of MMSE score with serum TSH levels in hypothyroid patients.

Studies that used MMSE as a general-purpose cognitive screening test have reported clinically significant impairments that were refractory to treatment in patients with hypothyroidism (21, 22) Correia et al (23) studied the cognitive functions in normal, hypothyroid and subclinical hypothyroid patients and reported specific cognitive deficits rather than a general decrease in cognitive performance. They found deficits in visuospatial, verbal, and associative memory before levothyroxine treatment. After 6 months of therapy, patients with overt hypothyroidism showed improvement unlike subclinical hypothyroid patients. Our findings are also consistent with their findings.

Jorde et al (24) used a battery of tests in a population study to assess attention, visual and verbal memory, intelligence and executive functions. Our findings of no significant difference in reaction time in DSST between cases and controls are in line with findings of Jorde et al (24).

The case group in their study was subclinical hypothyroid patients, defined as patients with elevated serum TSH level with serum fT_4 and fT_3 levels within the normal range and no overt symptoms of hypothyroidism, in contrast to overt hypothyroid patients in our study.

We found statistically significant post treatment improvement in reaction time & error hits of DSST in comparison to pretreatment state.

In hypothyroid patients we found increased reaction time in Three Letter Cancellation Tasks in comparison to that in One Letter Cancellation Tasks suggesting that processing of attention load was hampered during hypothyroidism. Restoration of euthyroid state led to functional normalcy as revealed by significant decrease in reaction time and error hits in One and Three Letter Cancellation Tasks. In a recent study by Samuel et al on women receiving TSH suppressive or replacement LT-4 doses did not show affect on memory & executive functions (Letter Cancellation Task & Trail Making Test) compared to healthy controls (25).

We found positive correlation in TMT A & TMT B with serum TSH levels in our study. Baldini et al (16) and Bono et al (17) also reported an association between subclinical hypothyroidism and neuropsychological changes with regard to logical

memory, attention, global cognitive functioning and some executive functions by using MMSE and TMT A & TMT B.

For cognitive flexibility/executive function, Jorde et al (24) also used the Trail Making Test part A and part B but they did not found any difference between patient with subclinical hypothyroidism and euthyroid individuals.

Our study shows that time taken to complete Trail Making test- B was more than in Trail Making Test-A, suggests that processing of additional information is affected more in hypothyroidism since Trail Making Test-B involves an additional attention load.

Our finding of post treatment significant improvement in performance of Trail Making Test in hypothyroid patients are in line with Osterweil et al (21) who reported an improvement using TMT part A and Symbol Digit Modalities Test (SDMT) after therapy for eight months in hypothyroid patients. Similar improvement in attention using TMT parts A have been also reported by Capet et al (26).

Contrary to our finding, Miller et al (27) and Schraml et al (28) found no improvement in attention and executive function in overt hypothyroid patients even after treatment by using TMT part A and TMT part B as a cognitive test. Similarly Whybrow et al (6) found no change in attention by using TMT part A and part B after 10.5 months of treatment in hypothyroid patients. Their results demonstrated that when the hypothyroidism was long standing, organic brain impairment persisted after thyroid replacement therapy. In a recent study by Parsaik et al (29) they found no significant association between MCI & clinical or subclinical hypothyroidism using TMT B & DSST in elderly persons.

Former studies (6, 24, 27) reveal that severity and duration of hypothyroidism are also determining factors in cognitive assessment in hypothyroid patients.

In the present study, we assessed the cognitive status in newly diagnosed overt hypothyroid patients and euthyroid controls. We found an overall improved performance in hypothyroid patients after treatment. The improvement was in various domains of cognition including global cognition, attention, concentration, visuospatial organization, executive function and psychomotor speed. Our findings are consistent with previous reports (21, 23, 26) that thyroxine therapy appears to restore cognitive functions in hypothyroid patients.

Conflicting findings in different studies potentially reflect differences in the age of the populations studied, differing degrees of severity of subclinical hypothyroidism, differing degree of response to therapy with irreversible changes in cognitive functions in chronic versus newly diagnosed cases of hypothyroidism and a lack of uniformity in administered cognitive tests.

The current study concludes that there is a significant reduction in cognitive skills with hypothyroidism but treatment does have significant effect on restoration of cognitive functions. This reduction in cognition is best correlated with TSH values in comparison to T3 and T4.

Conflict of Interest

None

Acknowledgements

We would like to thank all the patients participating in the study.

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Original Article

Protective Role of Eugenol on Acute Promyelocyte Leukemia Drug Arsenic Trioxide Induced Renal Injury in Wistar Rats

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Abstract

Background: Arsenic trioxide (As_2O_3) has been shown to have substantial efficacy in treating refractory or relapsed acute promyelocytic leukemia (APL). However, its therapeutic potential was limited due to its toxic side effects in vital organs. The kidneys are a major elimination pathway for many antineoplastic drugs and their metabolites. Renal impairment can result in delayed drug excretion and metabolism of chemotherapeutic agents, resulting in increased systemic toxicity.

Objective: This study was designed to evaluate modulation of anti leukemic drug As_2O_3 induced renal toxicity by eugenol, a natural monoterpene found in clove oil.

Methods: Arsenic trioxide (4 mg/kg body weight) was given orally to Wistar rats for a period of 30 days. Renal function parameters (Urea, creatinine, creatinine clearance), enzymatic (Glutathione-S- transferase, glutathione peroxidase, superoxide dismutase, catalase) and non-enzymatic antioxidant (reduced glutathione), lipid peroxidation marker were analyzed. The kidneys were examined histopathologically for the confirmation of oxidative stress based injury in renal tissue.

Results: Oral administration of arsenic trioxide significantly increased renal function markers, lipid peroxidation byproduct level, and altered antioxidant system. Rats treated with arsenic trioxide had significantly higher oxidative stress and kidney arsenic accumulation. The co-treatment with eugenol (5 mg/kg body weight) significantly reduced the oxidative damage compare with arsenic treated group.

Conclusion: Our observations indicate that supplementation with monoterpenoid eugenol alleviated nephrotoxicity by improving antioxidant capacity in renal tissue. These findings suggest that eugenol act as a potential agent in combating arsenic trioxide induced renal toxicity.

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Introduction

Arsenic trioxide (As_2O_3) was discovered more than 2000 years ago for treating diseases such as syphilis, tuberculosis, cancer and in 2000, US Food and Drug

Administration approved As_2O_3 as a drug for the treatment of relapsed or refractory acute promyelocytic leukemia (APL) (Antman, 2001). But it has been associated with profound toxicity in the heart (Raghu and Cherian, 2009; Mathews et al., 2013; Mathews et al., 2016), liver (Mathews et al., 2012) and kidney (Zhang et al., 2001). Das et al (2005) reported that generation of reactive oxygen species (ROS) is very common in arsenic toxicity. Arsenic induces oxidative stress, which causes antioxidant defense system dysfunction and lead to oxidative damage to cellular macromolecules by increasing the generation of free radicals (Emadi and Gore 2010; Wang et al., 2012). The kidney is highly vulnerable to damage caused by ROS which was observed by Rodrigo et al (2002). Pharmacological and dietary strategies have targeted to control oxidative stress produced by ROS. Research findings have suggested that the administration of various antioxidants can prevent or subdue side effects of various chemotherapeutic agents. Although many naturally occurring molecules with excellent antioxidant properties are good candidates for prevention or control oxidative stress based tissue damage. Eugenol (1-allyl-4-hydroxy-3-methoxybenzene) has emerged as a potential food constituent with antioxidant potential (Kamatou et al., 2012). It is a naturally occurring phenolic compound from clove oil. Eugenol belongs to the class of essential oils that are generally recognized as safe (GRAS) by the Food and Drug Administration. On this background, the present study was under taken to explore whether eugenol can reduce the antileukemia drug As_2O_3 induces renal toxicity.

Materials and Methods

Chemicals and Reagents

Arsenic trioxide, Sodium pyruvate, Reduced glutathione (GSH), Oxidized glutathione (GSSG), Phenazine methosulphate (PMS), Nitroblue tetrazolium (NBT) were obtained from Sigma-Aldrich, Banglore, India. Eugenol was purchased from Hi Media Laboratories Pvt. Ltd, Mumbai. 2,4-dinitro bis Nitro benzoic acid (DTMB), Nicotinamide adenine dinucleotide (NADH), Thiobarbituric acid (TBA), Nicotinamide adenine dinucleotide phosphate (NADPH), 1-chlor, 2,4 dinitro benzene (CDNB), Potassium chloride (KCl), Ethylene diamine tetra acetic acid (EDTA), Hydrogen peroxide (H_2O_2), Trichloroacetic acid (TCA), Magnesium sulfate (MgSO4), Adenosine triphosphate (ATP), Ammonium molybdate, 8-anilino-1-naphthalesulfonic acid (ANSA), sodium nitrite, Orthophosphoric acid, Naphthyl ethylene diamine dichloride were purchased from Merk Specialities Pvt. Ltd, Mumbai, India. Other chemicals and solvents of analytical grade were purchased from the local retailer.

Animals and Treatment

Rats weighing 180-250 g were purchased from Pharmacology Unit, Nagarjuna Herbal Concentrates Ltd, Thodupuzha, Idukki, Kerala, India and acclimatized for six days. All the animals were maintained under standard laboratory conditions of temperature (25°C) and 12 hour light and dark cycles throughout the experiment period. The rats were provided with laboratory chow (Hindustan Lever Ltd. India) and tap water ad libitum. Experiments were conducted as per the guidelines of Institutional Animal Ethical committee, School of Biosciences, Mahatma Gandhi University, Kottayam, Kerala, India (B29122014/3).

After an acclimatization period of one week, a total of 24 male rats were randomly allocated into four equally sized groups. Each group contains six rats; includes a normal control group, a eugenol control which received 5 mg/kg body weight of eugenol, a group administered with 4 mg/kg body weight of arsenic trioxide and a combination group treated with 4 mg/kg body weight of arsenic trioxide and 5 mg/kg body weight of eugenol.

At the end of the experimental period, all rats were sacrificed, blood was collected and serum was separated by centrifugation (3000 × g for 10 min). The kidneys were dissected out and washed in ice cold saline. The tissue was minced and homogenized accordingly with the experimental procedures.

Detection of Arsenic deposition in kidney

Kidney tissue was digested by thermal acid microwave digestion and diluted with double distilled

water. Total arsenic deposition in heart tissue was analyzed (Mathews et al., 2013) by standard inductively coupled plasma-optical emission spectroscopy (Optima 2000 DV ICP-OES, Perkin Elmer, Inc., Waltham, Massachusetts, USA).

Assay of tissue GSH

GSH was measured in tissue homogenate according to the method described by Ellman (1959). In the assay mixture contained 0.1 mL of sample, 0.85 mL of PBS (0.3 M, pH 7.4), and 0.05 mL of DTNB (10 mM). The reaction was read at 412 nm, and results were expressed as µmoles of GSH/g protein.

Assay of tissue GST

GST level was assayed by the method of Habig et al (1974). Tissue was washed in 1.15% KCl and homogenized in phosphate buffer (pH=7.4), centrifuged at 9000 rpm for 20 minutes. After centrifugation, the supernatant was mixed with 3 ml of the reaction mixture (1.7 mL Phosphate buffer + 0.1 mL of CDNB + 1.2 mL GSH) and it was measured spectrophotometrically at 340 nm.

Assay of Glutathione Peroxidase (GPx)

The activity of GPx was determined by the method of Rotruck et al. (1973). Briefly, the reaction mixture contained 0.2 mL of 0.4 M of Tris-HCl buffer (pH 7.0), 0.1 mL of 10 mM of sodium azide, 0.2 mL of homogenate (homogenized in 0.4 M of Tris-HCl buffer; pH 7.0), 0.2 mL of glutathione, and 0.1 mL of 0.2 mM of H_2O_2. The tubes were incubated at 37°C for 3 minutes, and the reaction was terminated by the addition of 0.5 mL of 10% trichloroacetic acid (TCA). To determine the residual glutathione content, the supernatant was removed after centrifugation and to this 1 mL of DTNB reagent was added. The color that developed was read at 412 nm against a reagent blank, and results were expressed as µg of GSH consumed/mg protein.

Assay of Superoxide dismutase (SOD)

SOD activity was determined by the method of Kakkar et al. (1984). The assay mixture contained 0.1 mL of sample, 1.2 mL of sodium pyrophosphate buffer (pH 8.3, 0.052 M), 0.1 mL of PMS (186 µM), 0.3 mL of NBT (300 µM), and 0.2 mL of NADH (750 µM). The reaction was started by the addition of NADH. After incubation at 30°C for 90 seconds, the reaction was stopped by the addition of 0.1 mL of glacial acetic acid. The reaction mixture was stirred vigorously with 4.0 mL of n-butanol. The mixture was allowed to stand for 10 minutes, centrifuged, and the butanol layer was separated. The color intensity of the chromogen in butanol layer was measured at 560 nm against n-butanol, and the concentration of SOD was expressed as units/g of renal tissue. One unit was taken as the amount of enzyme that gave 50% inhibition of NBT reduction/mg protein.

Assay of Catalase (CAT)

Catalase activity in the sample was measured according to the method of Aebi (1974) by measuring the decrease in absorbance of H_2O_2 at 240 nm.

Assay of tissue TBARS

Thiobarbituric acid reactive substances (TBARS) activity was estimated by the method of Beuge and Aust (1978). That was measured after the addition of 2 mL of TBARS (15% w/v trichloroacetic acid and 0.25 N of HCl) to l mL of heart tissue homogenate, then heated in a boiling water bath for 15 minutes. After cooling, the suspension was centrifuged at 1000 rpm for 10 minutes. The supernatant was then separated, and absorbance was measured at 535 nm. The results were expressed as nM of TBARS formed/mg protein.

Blood urea, serum creatinine, serum γ – glutamyl transferase (GGT), creatinine clearance test were detected (Agappe Diagnostic Ltd., Ernakulam, Kerala, India) using UV-Visible Spectrophotometer (U-5100, Hitachi High Technologies, America, Inc.).

Histopathology

Small sections of kidney were fixed in 10% buffered formalin and processed for embedding in paraffin. Sections of 5-6 µm were stained with hematoxylin and eosin and examined for histopathological changes

under the microscope (Motic AE 21, Wetzlar, Germany). The microphotographs were taken using Moticam-1000 camera at the original magnification of 100X.

Statistical Analysis

The experimental results were expressed as mean±SD. Statistical analysis was evaluated by one-way analysis of variance (ANOVA) using SPSS software (version 20.0, SPSS Inc, Chicago, IL, USA).Values were considered significantly different if P<0.05.

Results

Effect of eugenol on arsenic accumulation in the renal tissue is shown in Fig. 1. Co-treatment with eugenol attenuated arsenic accumulation in the kidney compared with arsenic trioxide treated group. A significant (p<0.05) increase in the level of blood urea nitrogen, urea and creatinine were observed in arsenic treated rats when compared with normal rats. Administration of eugenol (5 mg/kg body weight) along with arsenic trioxide (4 mg/ kg body weight) significantly (p<0.05) restored the levels of urea, uric acid and creatinine near to normal levels when compared with arsenic alone treated rats. Eugenol did not alter the level of BUN, uric acid, and creatinine. Rats treated with arsenic showed a high level of γ-glutamyl transferase activity with a decrease (p<0.05) rate of creatinine clearance compared to control. Eugenol treatment provided marked protective effect and improvement on these parameters (Table I).

TABLE I: Effect of eugenol on renal function diagnostic markers in rats.

Parameters	*Normal control*	*Arsenic trioxide*	*Eugenol*	*Arsenic trioxide+Eugenol*
Blood Urea Nitrogen (BUN) (mg/dl)	20.02±1.21	78.94±1.49a	18.49±1.02	40.95 ±0.94b
Serum Urea (mg/dl)	32.45±0.21	102.69±0.28a	31.09±0.13	64.85 ±0.37b
Serum Creatinine (mg/dl)	0.98±0.04	4.16±0.06a	0.85±0.02	1.29 ±0.04b
Creatinine Clearance (ml/min)	0.69±0.03	0.17±0.02a	0.61±0.05	0.48 ±0.01b
γ-glutamyl transferase activity(U/L)	100.02±1.48	523.11±1.93a	94.27±1.86	248.08 ±2.14b

Normal control, Arsenic trioxide (4 mg/kg body weight), Eugenol (5 mg/kg body weight), Arsenic trioxide (4 mg/kg body weight) + Eugenol (5 mg/kg body weight). Data represented as mean ± SD, n=6. 'a' (significant difference between Normal control and Arsenic trioxide), 'b' (significant difference between Arsenic trioxide and Arsenic trioxide +Eugenol). P< 0.05.

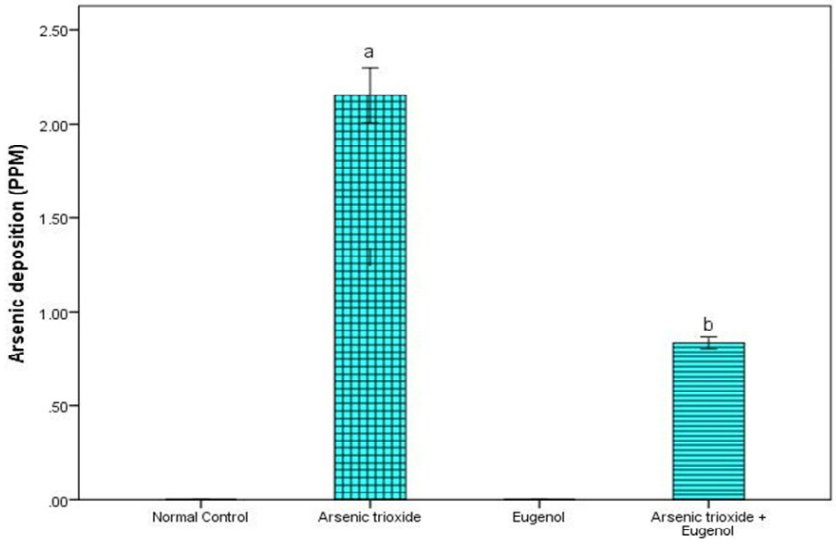

Fig. 1: Effect of eugenol on arsenic deposition pattern in the kidney. Experiment consist of Normal control, Arsenic trioxide – 4 mg/ kg body weight, Eugenol – 5 mg/kg body weight, and Arsenic trioxide – 4 mg/kg body weight) + Eugenol – 5 mg/ kg body weight. Data represented as mean±SD, n = 6. 'a' (significant difference between Normal control and Arsenic trioxide), 'b' (significant difference between Arsenic trioxide and Arsenic trioxide + Eugenol). P<0.05.

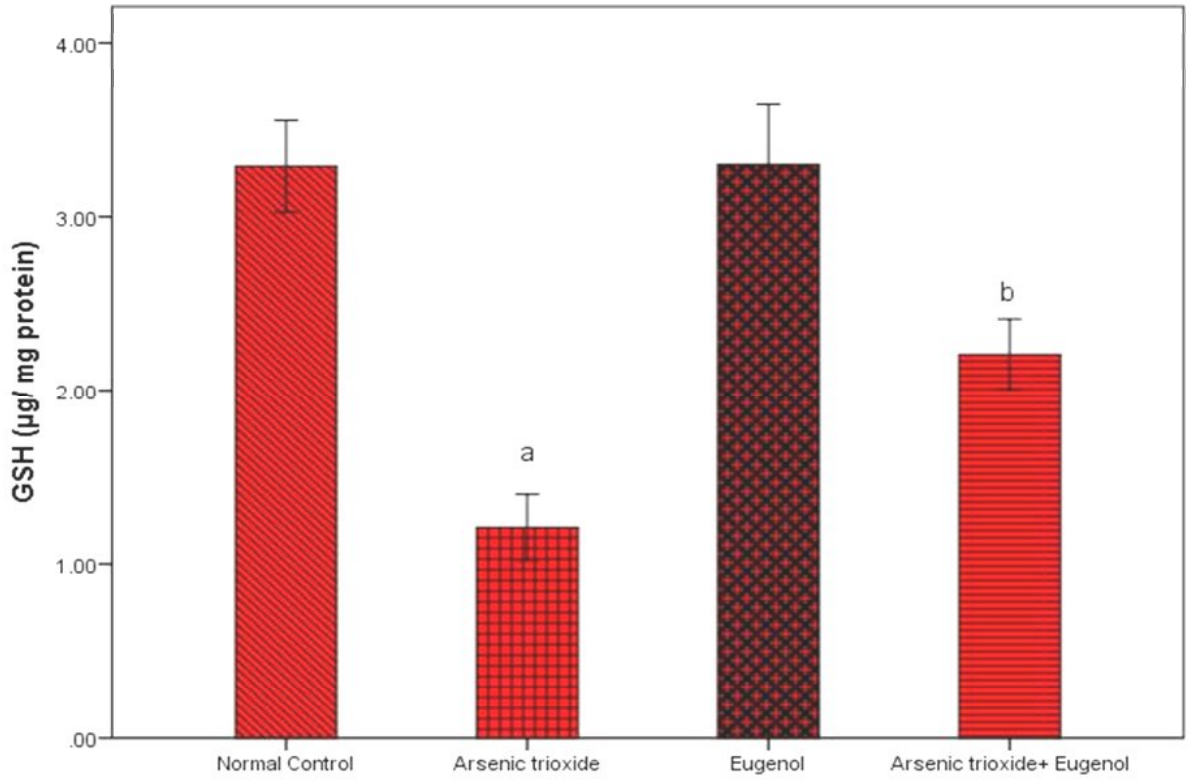

Fig. 2 : Effect of eugenol GSH in kidney. Experiment consist of Normal control, Arsenic trioxide – 4 mg/kg body weight, Eugenol- 5 mg/kg body weight, and Arsenic trioxide – 4 mg/ kg body weight) + Eugenol – 5 mg/kg body weight. Data represented as mean±SD, n=6. 'a' (significant difference between Normal control and Arsenic trioxide), 'b' (significant difference between Arsenic trioxide and Arsenic trioxide + Eugenol). P<0.05.

Treatment of normal rats with eugenol produced significant changes in kidney GSH level (Fig. 2) compared to normal controls. Treatment of rats with arsenic significantly ($p<0.05$) decreased the activity of GSH. Administration of eugenol (5 mg/kg body weight) along with arsenic trioxide significantly ($p<0.05$) increased the level of non-enzymatic antioxidant reduced glutathione in renal tissue. The changes in the activities of enzymatic antioxidants GST, GPx, SOD and CAT in control and experimental rats were shown in Fig. 3, 4, 5 and Fig. 6 respectively. Significant decreases ($p<0.05$) in the activities of these enzymes were observed in arsenic treated rats. However, treatment with eugenol caused a significant ($p<0.05$) increase in GST, GPx, SOD & CAT activity compared with arsenic treated group. These findings suggest that eugenol exerted its protective effects on arsenic induced renal toxicity may via the antioxidant pathway. The arsenic treated rats showed significant ($p<0.05$) elevated renal tissue TBARS level compared to controls (Fig. 7). Co-treatment of rats with eugenol attenuated the arsenic trioxide increases in MDA level in kidney.

Histopathological change is a direct indication of renal injury. The H&E-stained renal tissues appeared to have normal kidney tubules in the control (Fig. 8A) and eugenol treated group (Fig. 8C). In contrast, it was demonstrated that arsenic trioxide induced histopathological changes in the renal tissues, such as the destruction of tubular structures, necrosis, and disorganization, interstitial fibrosis (Fig. 8B). However, co-treatment with eugenol significantly diminished arsenic induced histological alterations (Fig. 8D).

Discussion

An outstanding benefit of As_2O_3 for APL is due to its ability to the degradation of PML/RAR alpha in the oncoprotein of acute promyelocytic leukemia (Zhang et al., 2010). However, the therapeutic use of As_2O_3 is limited by its toxic side effects in various organs.

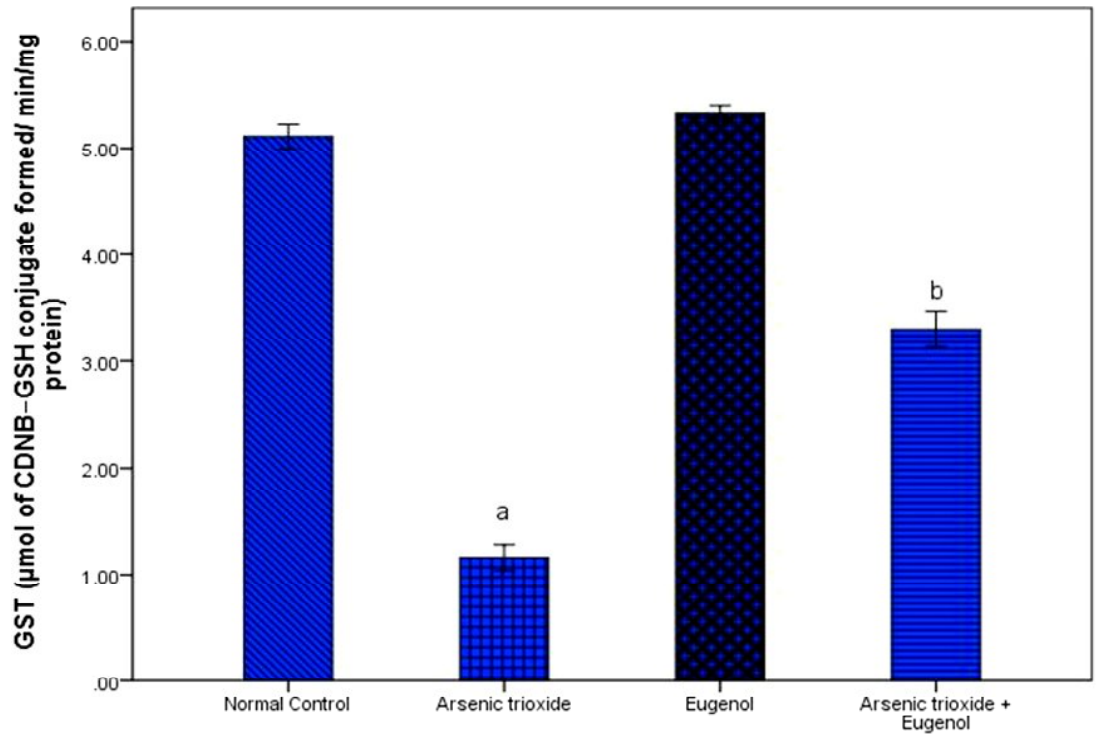

Fig. 3 : Effect of eugenol on glutathione –s-transferase level in renal tissue. Experiment consist of Normal control, Arsenic trioxide – 4 mg/ kg body weight, Eugenol – 5 mg/kg body weight, and Arsenic trioxide – 4 mg/kg body weight) + Eugenol – 5 mg/ kg body weight. Data represented as mean±SD, n=6. 'a' (significant difference between Normal control and Arsenic trioxide), 'b' (significant difference between Arsenic trioxide and Arsenic trioxide + Eugenol). P<0.05.

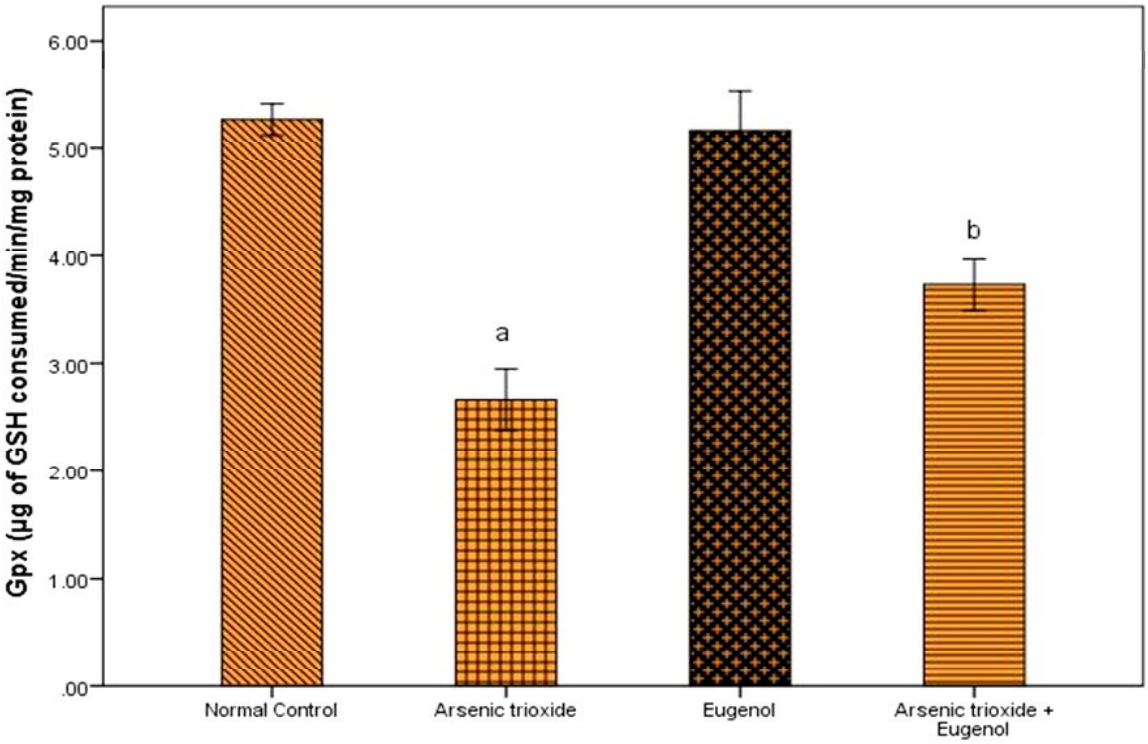

Fig. 4 : Effect of eugenol on renal tissue glutathione peroxidase activity. Experiment consist of Normal control, Arsenic trioxide – 4 mg/kg body weight, Eugenol – 5 mg/kg body weight, and Arsenic trioxide – 4 mg/kg body weight) + Eugenol – 5 mg/kg body weight. Data represented as mean±SD, n = 6. 'a' (significant difference between Normal control and Arsenic trioxide), 'b' (significant difference between Arsenic trioxide and Arsenic trioxide + Eugenol). P<0.05.

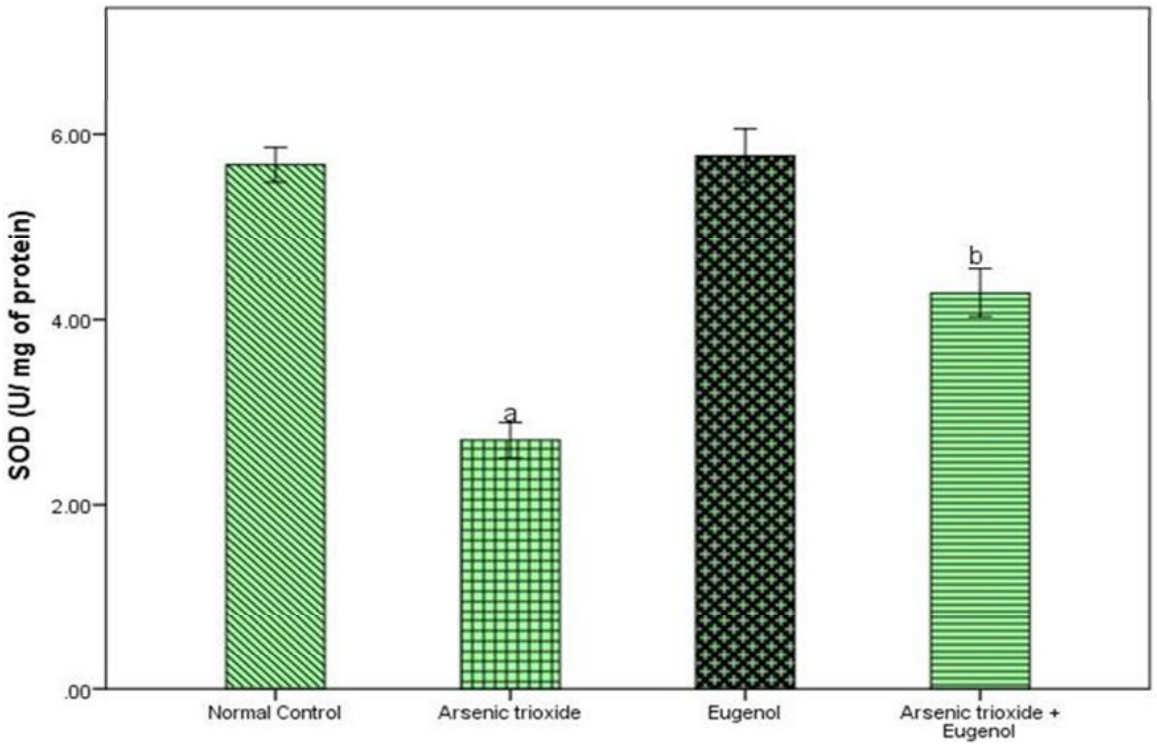

Fig. 5 : Effect of eugenol on superoxide dismutase activity in kidney. Experiment consist of Normal control, Arsenic trioxide – 4 mg/ kg body weight, Eugenol – 5 mg/kg body weight, and Arsenic trioxide – 4 mg/kg body weight) + Eugenol – 5 mg/kg body weight. Data represented as mean±SD, n = 6. 'a' represents the significant difference between Normal control and Arsenic trioxide), 'b' represents significant difference between Arsenic trioxide and Arsenic trioxide + Eugenol). P< 0.05.

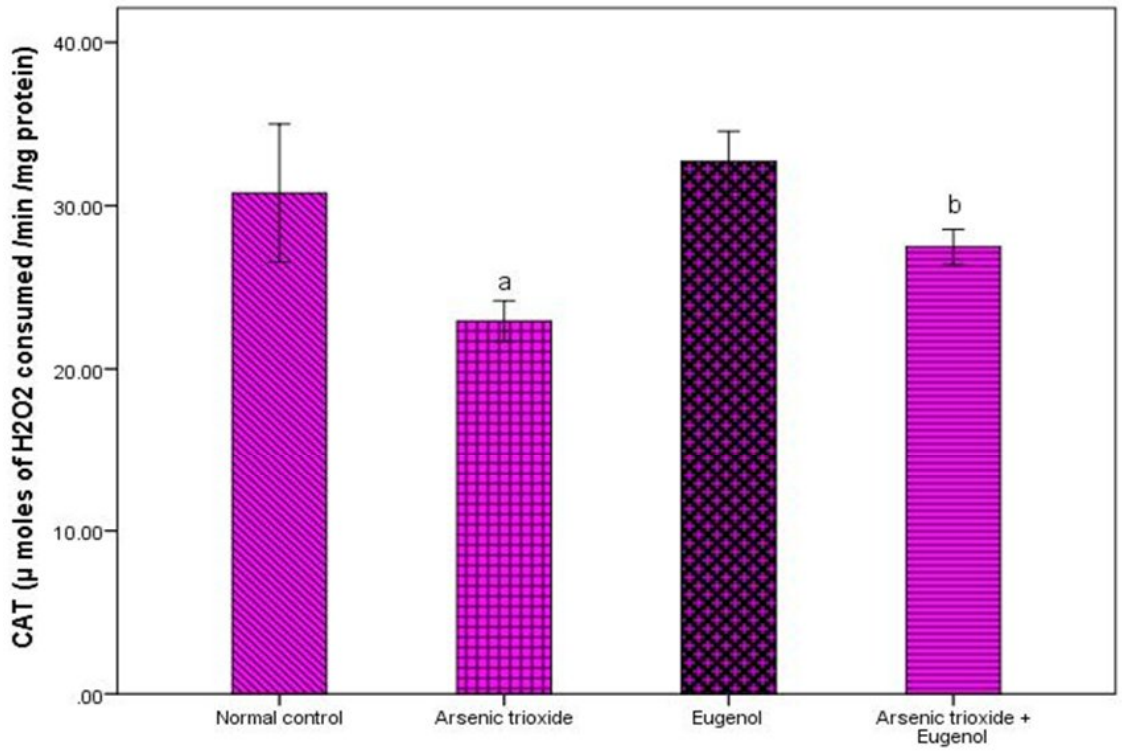

Fig. 6 : Effect of eugenol on enzymatic antioxidant catalase activity in renal tissue. Experiment consist of Normal control, Arsenic trioxide – 4 mg/kg body weight, Eugenol – 5 mg/kg body weight, and Arsenic trioxide – 4 mg/ kg body weight) + Eugenol – 5 mg/ kg body weight. Data represented as mean±SD, n = 6. 'a' represents the significant difference between Normal control and Arsenic trioxide), 'b' represents significant difference between Arsenic trioxide and Arsenic trioxide + Eugenol). P<0.05.

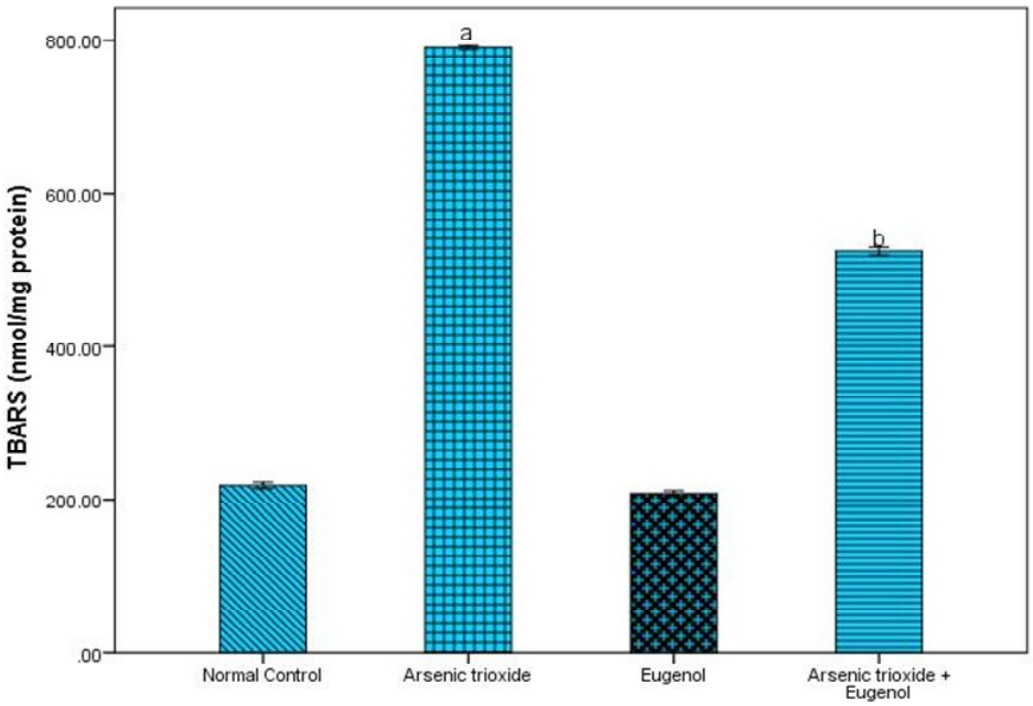

Fig. 7 : Effect of eugenol on lipid peroxidation in renal tissue. Experiment consist of Normal control, Arsenic trioxide – 4 mg/ kg body weight, Eugenol – 5 mg/kg body weight, and Arsenic trioxide – 4 mg/kg body weight) + Eugenol – 5 mg/kg body weight. Data represented as mean±SD, n = 6. 'a' represents the significant difference between Normal control and Arsenic trioxide), 'b' represents significant difference between Arsenic trioxide and Arsenic trioxide + Eugenol). P<0.05.

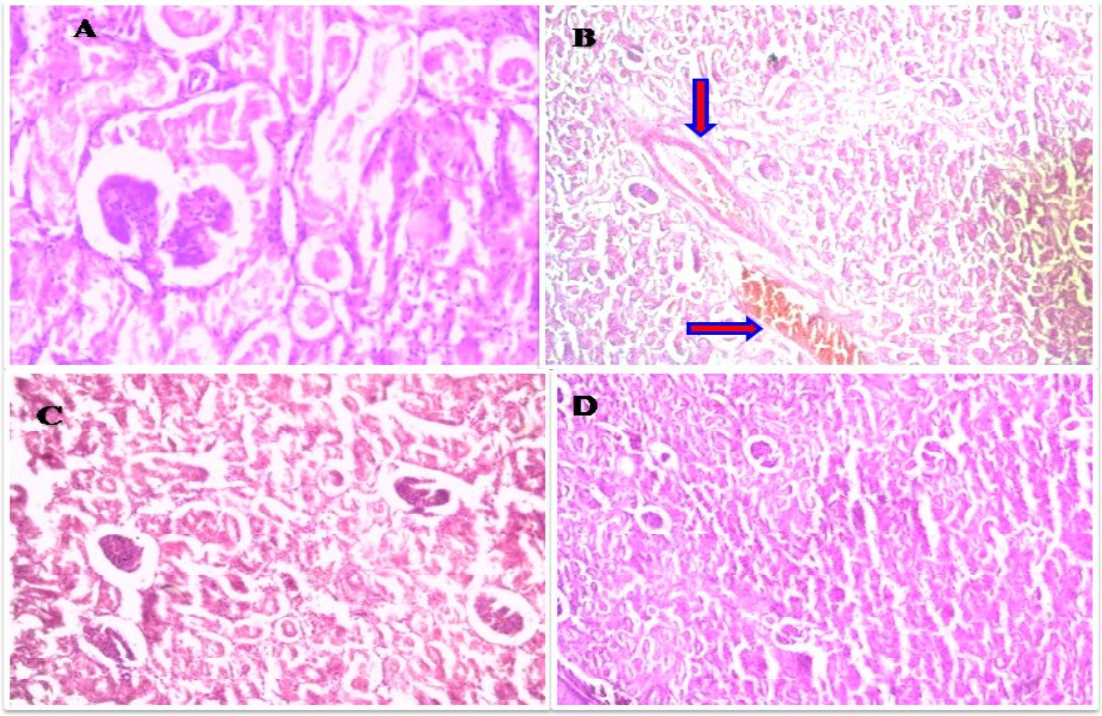

Fig. 8 : Effects of eugenol on arsenic trioxide induced oxidative stress in the histology of renal tissue. (A) – Normal rat kidney (H&E100X). Intact glomeruli in the cortical region with patent tubular epithelium, glomeruli and tubules were normal. (B) – Arsenic trioxide (4 mg/ kg body weight) treated rat kidney (H&E 100X). Showing proximal and distal tubular necrosis, tubular degeneration, tubular dilation, thickened basement membrane and marked atrophy of many glomeruli cells. (C) – Eugenol (5 mg/kg body weight) treated rat kidney (H&E 100X) showing normal renal histoarchitecture with glomeruli and renal tubules. (D) – Arsenic trioxide + Eugenol (4 mg/kg body weight +5 mg/kg body weight)-treated rat kidney (H&E 100X) Atrophy of glomerular tufts accompanied by moderate necrosis and congestion, degeneration and desquamation of tubular epithelium.

Therefore the development of strategies to reduce these toxic effects would allow us to explore the full therapeutic potential of As_2O_3 in cancer therapy. In this study, our goal was to investigate the renal protective effects of eugenol, a naturally occurring monoterpenoid in combination with APL drug arsenic trioxide in Wistar rats.

The tissue uptake of arsenic was analyzed by inductively coupled plasma emission spectroscopy (ICP-OES). Arsenic trioxide treated rats has shown deposition of arsenic in renal tissue. During chronic exposure, arsenic is known to accumulate in the liver, kidneys, heart, spleen, and muscles (Ratnaike et al., 2003). Emadi and Gore (2010) reported that arsenic compounds are cytotoxic and cause renal tissue damages. In addition to this kidney is sensitive to arsenic because it has an inefficient oxidative system (Prozialeck et al., 2007). We observed the administration of eugenol with arsenic trioxide prevent or reduce the accumulation of arsenic trioxide in the kidney.

Arsenic administration caused marked renal dysfunction as evidenced by the significant increase in blood urea, serum creatinine, and GGT levels. As_2O_3 has been reported to cause renal injury, elevated serum creatinine (CREA) and blood urea nitrogen (BUN) levels in clinical studies (Sakurai et al., 2006). Urea is the major nitrogen containing the metabolic product of protein metabolism. An elevated level of blood urea is known to be correlated with increased protein catabolism. The elevation in the levels of BUN and CREA in the serum of As_2O_3 treated rats is considered to be an important marker of renal dysfunction (Augusti et al., 2008). Vaezi et al (2017) have reported arsenic trioxide related renal toxicity in mice. Combination therapy with eugenol protected the kidney function from arsenic intoxication as indicated by significant restoration of serum and blood urea, creatinine and GGT as well as creatinine clearance rate.

The intra cellular antioxidant system comprises of different free radical scavenging antioxidant enzymes along with some non-enzymatic antioxidant like GSH and thiols (Fatma et al. 2009). Thus to eliminate free radicals, these antioxidant play a key role and maintain an equilibrium with oxidants and reductants. In the present study, arsenic treatment resulted in a dramatic decline in the level of GSH, GST, GPx, SOD, and CAT. In a study by Sener et al (2005) observed ROS generated in tissues are normally scavenged by enzymatic and non-enzymatic antioxidants. The non-enzymatic antioxidant GSH has a crucial role in cellular defense mechanisms against toxicity and oxidative stress resulting from exposure to arsenic (Chen et al., 2012). The reduced level of GSH in our study also agree with the observations of Akhand et al (2004) that arsenic is known to be a potent sulfhydryl reactive chemical capable of binding with proteins. Jones (2002) reported that GSH with its sulfhydryl group functions in scavenging of free radicals as well as xenobiotics. Our results substantiated with the observations of Srivastava and Shivanandappa (2010) that GSH depletion leads to lowered cellular defense mechanism. While GST activity appears to be linked to the activity of GSH (Hayes et al., 2005) and the declined level of GST activity may be due to the deficiency in GSH level. In the present study arsenic intoxication also significantly reduced the activity of GPx. GPx is an enzymatic antioxidant play an important role in the elimination of H_2O_2 and lipid hydroperoxide using GSH as a hydrogen donor (Lubos et al., 2011). Ekor et al (2006) observed that intracellular SOD and CAT enzymes are prominent in the antioxidant system that catalyzes the dismutation of superoxide radicals ($.O^{2-}$) to hydrogen peroxide, and decomposition of H_2O_2 to H_2O respectively. Furthermore, Yamanaka et al (1991) demonstrate that in arsenic metabolism one electron reduction of molecular oxygen by dimethyl arsenic produces superoxide anion radicals. SOD is a primary defensive enzyme and prevents the further generation of free radicals (Yamanaka et al., 2004). Decreased SOD activity in organs suggests that the accumulation of superoxide anion radical, which would leads to a raised level of hydroperoxides along with the lowered activity of CAT and GPx (Mahza and Shivanandappa, 2013). The recent study report from Nasiry et al (2017) well established renal toxicity related antioxidant depletion by arsenic trioxide in rats. Our findings indicate combination treatment with eugenol, a naturally occurring antioxidant would scavenge the free radical produced by arsenic and this might be

the reason of enhanced antioxidant status. It has been reported that phenolic antioxidants, including eugenol, has powerful free radical scavenging effect (Binu and Harikumaran Nair, 2015). The allyl group in the phenolic rings of eugenol is responsible for radical scavenging effect through the mechanism of electron spin resonance (Kuhn and Winston, 2007).

Eugenol interferes with initiation as well as propagation of lipid peroxidation and it is attributed to the free radical scavenging effect of eugenol (Nagababu and Lakshmaiah, 1994). The experimental rats administered with arsenic trioxide showed a rise in membrane peroxidation rates compared to normal control rats. Earlier studies proved that the compound arsenic increases lipid peroxidation and suppresses antioxidants in the kidney (Mittal and Flora, 2006; Nandi et al., 2006). Manimaran et al (2010) have reported that kidney is relatively more susceptible to oxidative stress induced by arsenic. Malonyldialdehyde (MDA) levels, an indicator of free radical generation and it formed as a breakdown product of the major chain reactions leading to oxidation of polyunsaturated fatty acids (Draper et al., 1990). Wang et al (2006) observed the role of MDA in assessing oxidative stress and different types of biological damages. Co-treatment with eugenol reduces the lipid-peroxidation which could be agreed with the report by Nagababu and Lakshmaiah (1994) that eugenol has significantly decreased the rate of lipid-peroxidation. Murakami et al (2005) also reported the effect of eugenol on lipid peroxidation and oxidation of low-density lipoproteins.

Our results showed increased lipid peroxidation in the kidney of arsenic treated rats which are associated with renal damage. Histopathological examination of renal tissue of arsenic trioxide treated rats showed the destruction of the tubular structure, necrosis, disorganization and interstitial fibrosis. These findings are in agreement with previous reports of Emadi and Gore (2010). Arsenic trioxide produces ROS, which may act as a signaling molecule in the reduction of oxidative stress and tissue injury (Yu et al., 2013). However, co-treatment with eugenol could prevent the changes and could also maintain the ultra structure almost similar to that of normal controls. The nephroprotective effects of eugenol can be partially attributed to the properties that scavenge free radical activity and enhance the antioxidant defense system.

In this study, eugenol has shown a protective action against antileukemic drug arsenic trioxide induced oxidative stress to the renal tissue as evidenced by the lowered urea, uric acid, creatinine and GGT enzyme activities and elevated levels of the enzymic and non-enzymic antioxidants along with creatinine clearance rate. In addition, lowered rate of lipid peroxidation decreased arsenic accumulation and renal tissue damage in the kidney demonstrated that eugenol offers protection against arsenic. The facts that eugenol is an antioxidant, and that it prevents arsenic induced toxicity *in vivo* make the present conclusions and provide an impetus for further studies.

Conflict of Interest

The authors declare that there is no conflict of interest involved in this study.

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Original Article

Assessment of Heart Rate Recovery and Chronotropic incompetence in Subclinical Hypothyroid Adults

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Abstract

Overt hypothyroidism leads to altered cardiac functions due to hemodynamic and autonomic dysfunction, however, role of subclinical hypothyroidism (SCH) remains doubtful. A cross-sectional study was conducted on 30 adult subclinical hypothyroid subjects and equal number of euthyroid controls. Submaximal exercise was used for assessing the Heart Rate Recovery (HRR) in upright position at 1 min & 2 min and Chronotropic response (CR) was calculated. SCH subjects had decreased HRR2 (P=.012) and CR(P=.002) as compared to controls. Chronotropic incompetence (CI) was seen to be associated with SCH (P=.007). Stepwise regression analysis of Serum TSH with variables which are significantly different between cases & control, observed a negative relationship with peak heart rate (p=0.012). Slow HRR2 and decreased CR in SCH, indicates insidious subtle changes in cardiac responses in SCH before progression to overt hypothyroidism. HRR2 can be used as a preliminary test to make decisions regarding treatment at an early stage to prevent the further escalation of the derangement.

Introduction

Subclinical hypothyroidism (SCH) is usually an asymptomatic condition with a prevalence ranging from 9.4% to 21.5% across adult Indian population (1, 2) and is characterized by elevated level of serum thyrotropin (TSH) but normal free T3 and free T4 concentrations (3, 4).

Thyroid physiology plays an important role in regulating many organ systems of the body and metabolism (5). Parallel increase in levels of total lipids & cholesterol with increasing Serum TSH levels has been observed conferring to abnormal Lipid metabolism especially in SCH (6, 7). Changes in lipid profile leading to atherosclerosis along with alteration in cardiac functions due to hemodynamic & autonomic dysfunction (8) together has increased the risk of CAD and MI in SCH (9, 10). SCH persons more often presents with symptoms of fatigability, weakness and low exercise tolerance which is justified - among other factors - by the decrease in myocardial contractile force due to structural changes in the ATPase activity and down regulation of epinephrine activity. Impairment of cardiac autonomic activity in SCH is also evidenced by a hypo-functional parasympathetic system and an increase

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sympathetic tone as evaluated using heart rate variability (11, 12).

Exercise repeated at certain intervals causes pituitary thyroid reaction possibly elevating the requirement of thyroid hormone to fulfill exercise induced need of high oxidation fuel like fatty acids (13). With the cardiac tissue deriving most of the energy from fatty acids at rest exercise in hypothyroid cases may compromise the energy fuel and together with hypo-functional parasympathetic activity may cause intolerance to exercise. More over a recent study have observed a decrease in baseline heart rate with unchanged baseline systolic blood pressure (SBP) and diastolic blood pressure (DBP) in subclinical hypothyroid patients on levothyroxine treatment following sub minimal exercise indicating improvement in cardio pulmonary exercise performance (14).

Decrease in HR after exercise is considered to be due to decrease in sympathetic activity (15) & to reactivation of parasympathetic activity (16). Recently attenuated (HRR) has been demonstrated as a predictor of all – cause mortality (17, 18, 19). Asymptomatic SCH subjects may has a normal heart rate and cardiac structure & function at rest but exercise may bring out the subtle cardiovascular abnormality in them (11). Although impairment of cardiac autonomic activity in subclinical hypothyroidism has been studied (20) there is paucity of work onpost exercise HRR and chronotropic response (CR) in the SCH which is simple to administer and can help bring out latent abnormalities and may help identify apparently asymptomatic SCH cases who are at risk of developing coronary artery disease.

Materials & Methods

A cross sectional analytical study on adult SCH subjects was conducted in the department of physiology, Himalayan Institute of Medical Sciences (HIMS), SRH university, Dehradun after approval from the Institute ethical committee. Written informed consent was taken from the subjects for inclusion in the study.

Sample size and method

The study volunteers were selected by method of probability sampling. With the mean difference in HRR in 1 min of 8 (21) and pooled SD as 8.5 formula for difference in mean of two equal groups = $(r+1)(Z\alpha/2 + Z1-\beta)^2 / r(\Delta mean)^2$ a sample of 24 was calculated for each group which was increased to 30 in each group. An equal number of euthyroid control group with comparable age were also recruited (n=30).

Selection of subjects

Clinically healthy adults with history of weakness and lethargy reporting at medical OPD were followed for their thyroid status and 30 clinically healthy adults diagnosed with subclinical hypothyroidism in the age group of 20-40 years of both the sexes were recruited. The clinical diagnosis of SCH was established by a normal values of Serum FT3 and FT4 and a Serum TSH value of >5 micro IU/ml to 15 micro IU/ml (22). As per the hospital laboratory reference range the normal range of values of Serum FT3 and FT4 were 2-4.2 pg/ml & 0.6–1.7 ng/dl respectively. Equal number of clinically healthy euthyroid adults (normal FT3 & FT4 and TSH 0.34-5 micro IU/ml) were recruited from attendants of patients and residents in and around SRHU campus.

Common exclusion criteria were used to recruit the participants in both euthyroid and SCN group. The exclusion criteria assessed by detailed history, systemic examination & eye examination included, diabetes mellitus, hypertension, bipolar disorder, obesity (BMI >/=30 Kg/m2), recent delivery (</=9 months) tuberculosis, anemia, multiple endocrine syndromes, neuromuscular disorder, severe myopia, cataract, glaucoma and maculopathy, CNS dysfunction, smokers, alcoholics and those taking drugs affecting the thyroid status (Litium, NSAIDs) and acting on CNS. Basic investigations of fasting blood sugar, hemoglobin estimation, ECG and X ray chest were done along with other investigation as per specific exclusion criteria.

Study tools

Structured case reporting forms was used to generate

demographic, relevant history and anthropometric data. Treadmill (Company: JKEXERSno. A0047762) was used for submaximal exercise for assessing the Heart Rate Recovery after exercise. Bio Impedance Machine (Omeron KARADA SCAN Model HBF-375) was used to measure percentage of body fat and visceral fat. Fixed metered scale and weighing machine (Company: Krups) were used to measure height and weight. Non-invasive techniques were used to minimize the discomfort to the recruited subjects

Study protocol

Following written informed consent volunteers were asked to report to the Physiology department in morning hours on all working days. A standard case reporting form was administered by the investigator at the point of entry to collect information on demographic, anthropometric characteristics, personal medical history of past and present illness, and family history with detailed history of chronic medication, addiction and smoking. Volunteers were familiarized with the procedures to follow.

a) Demographic characteristics like age, gender and occupation were recorded. They were measure for standing height nearest to 1 cm, weight nearest to 100 grams using standard protocol and for body fat (% Body fat, Visceral fat) using the bio impedance method Blood was drawn to assess the serum levels of TSH, free T3 and T4. They were then subjected to sub-maximal exercise design using Ellestad Protocol using age predicted heart rate and Borg's scale for perceived excretion to assess heart rate recovery at 1 min & 2 min and chronotropic incompetence following exercise.

b) Experimental procedure: All subjects underwent "symptom-limited" treadmill exercise testing using the Ellestad protocol (23) for sub-maximal exercise designed to bring the subject up to a plateau at approximately 60-85% of age predicted Maximum HR. Age predicted maximum HR was calculated as 220—age. Prior to testing; all subjects were instructed not to eat, drink any beverages, or smoke for 3 hours before the test. After subjects were cleared for testing, they were fitted with a heart rate monitor and instructed to lie supine in a resting position for 5 minutes. At the end of this period, the subject's resting heart rate and blood pressure was recorded. Heart rate was recorded at the end of each stage. The subject was given a 5 minute warm-up on the treadmill at 4 miles per hour, as well as light stretching prior to beginning of sub maximal exercise. All subjects held on to the front rail of during the treadmill test and the polar ElctroInc HR monitor was tied around the chest. Subjects commenced walking at their comfortable pace on the treadmill and with a speed of 1.7 mph for 3 minute. Then the speed was increased to 3 mph for next 2 minutes and subsequently by 1 mph every 2 minutes for next 10 minutes until the criteria for test termination are achieved [60-85% age predicted maximum HR] but were instructed to stop exercise if they experienced any symptoms related to angina, light headedness, confusion, fatigue or subject had grade III level of exertion on Borg scale of exertion. When 60-85% of age predicted maximum HR was achieved on HR monitor the speed was held constant for 2 minutes and after recording of peak HR achieved during the exercises the procedure was stopped. Subject was made to immediately sitting on a chair and arm with arm rest. HR recovery was monitored following stoppage of the treadmill after 1 min and after 2 min into recovery. Chronotropic response was assessed by (peak HR–resting HR) / ((220–age)–resting HR); and a value of $\geq$0.80 was considered as Chronotrophic incompetence (CI) (24). HRR was defined as the difference between peak HR at sub-maximal exercise and 1 minute and 2 min into recovery in an upright position (25).

Data management & Statistical analysis

SPSS (Statistical Package for the Social Sciences, 17.0 version) was used to analyze the collected data. Mean and SD were used to represent the demographic, anthropometric and other measured variables in the two groups. χ^2 analysis was used to assess the differences between dichotomous variables. Differences in means of quantitative variables (eg HRR1, HRR2, CR, Relative decrease in

HR) in the groups were tested using unpaired "t" test. Correlation of the S.TSH with the measured variables for continuous variable eq BF%, HRR1, HRR2, CR, and relative decrease in HR was assessed by Pearson correlation. Level of significance was set at p<0.05.

Results

The study analyzed 30 cases of adults (20-40) clinically diagnosed subclinical hypothyroidism for cardio vascular variables, HRR and chronotropic response. An equal number of controls were taken for comparison.

The number and proportion of females were considerably more than males in SCH cases i.e., 26 (86.6%). As expected the difference of serum TSH levels among SCH cases and euthyroid controls in this study was statically significant (p=<0.001). No significant difference of FT3 levels and FT4 levels was observed between SCH case and control (Table I).

No significant difference was found in all the three parameters including PR, SBP and DBP of euthyroid control & SCH case (Table II).

A lower peak HR during exercise was observed in SCH cases and was statistically different from that achieved by euthyroid controls (P<0.001). HRR was less in SCH cases than in euthyroid controls but the difference was significant only at 2 second into recovery post exercise (p=0.002). Chronotropic response was also significantly lower in SCH than euthyroid controls following moderate exercise. Chi square test for association of CI (<0.08) with SCH established a significant positive association (p=0.005) (Table III & Fig. 1).

Serum TSH showed a negative relation to both Heart

TABLE I: Comparison of Anthropometric and Biochemical parameters among Euthyroid Controls and SCH Cases.

S. No.	*Parameter*	*Control (n=30)*	*Case (n=30)*	*P-value*
1	Age (years)	33.1±7.5	35.5±5.9	0.170
2	Height (cm)	159.3±9.7	155.9±6.9	0.116
3	Weight (kg)	65.8±12.9	64.1±9.8	0.568
4	BMI (kg/m²)	25.7±3.5	26.4±3.0	0.464
5	Body Fat (%)	28.6±7.5	32.4±4.1	0.015*
6	Visceral Fat (%)	9.3±3.6	10.5±5.0	0.257
7	Serum FT3 (pg/ml)	3.0±0.7	2.7±0.7	0.120
8	Serum FT4 (ng/dl)	1.1±0.6	1.1±1.0	0.875
9	Serum TSH (µIU/ml)	2.3±1.0	7.2±2.5	0.000***

Values in Mean±SD; Un-paired "t" test; P<0.05 is significant; FT3: Free T3; FT4: Free T4.

TABLE II: Cardiovascular parameters among Euthyroid control (n=30) & SCH cases (n=30).

S. No.	*Parameter*	*Control (n=30)*	*Case (n=30)*	*P-value*
1	PR (beats/min)	86.9±7.6	84.6±7.2	0.234
2	SBP (mmHg)	110.3±11.7	110.8±13.1	0.885
3	DBP (mmHg)	72.8±6.8	72.1±8.2	0.720

Values in mean±SD; Un-paired "t" test; P<0.05 is significant; PR: pulse rate; SBP: systolic blood pressure; DBP: diastolic blood pressure.

TABLE III: Exercise stress parameters among SCH case and Euthyroid control.

S.No.	*Parameter (mean±SD)*	*Euthyroid Control (n=30)*		*SCH Case (30)*		*p-value*
1	Resting HR(bpm)	88.0±9.3		85.3±7.9		0.231
2	Maximum HR(bpm)	186.9±7.5		184.5±5.9		0.170
3	Peak HR(bpm)	153.7±20.5		134.8±16.3		0.000***
4	HRR1(bmp)	30.3±11.8		27.0±10.6		0.269
5	HRR2(bpm)	47.1±0.4		38.6±11.9		0.012*
6	CR	0.6±0.2		0.5±0.2		0.002**
7	Cut off value=0.8 Chronotropic Incompetence	<0.8	≥0.8	<0.8	≥0.8	Yates: 7.12; p=0.007
	No. of subjects	20(66.6%)	10(33.3%)	29(96.6%)	1(3.3%)	Fischer p=0.005

Values in Mean±SD; HR: Heart Rate; HRR1: Heart Rate Recovery 1 min; HRR2: Heart Rate Recovery 2 min; CR: Chronotropic Response; CI: Chronotropic Incompetence. Values in mean±SD; Un-paired "t" test; P<0.05 is significant.

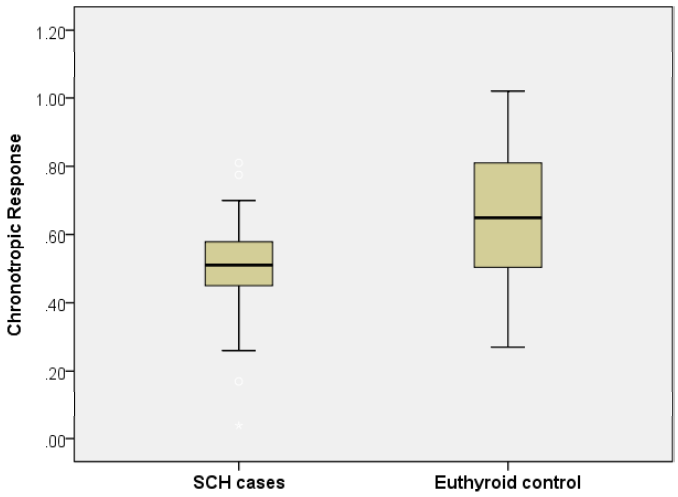

Fig. 1 : Boxplot showing chronotropic response among SCH cases (n=30) and Euthyroid controls (n=30).

rate recovery at 2 min and chronotropic response but was not statistically significant due to small sample size. Stepwise regression analysis of Serum TSH with variables like RHR, Peak MHR, %BF, %VF, HRR2 & CR which were significantly different among SCH & controls showed that peak MHR was the only factor showing a significant negative relationship to levels of Serum TSH ($\beta = -0.32$; p=0.012).

Discussion

The present study was carried out on 30 Subclinical hypothyroid case to assess the chronotropic response and HRR in comparison to euthyroid control.

Although asymptomatic SCH subjects having high serum TSH concentration in the setting of normal levels of serum T4 and T3 they are at risk for many cardiovascular manifestations due to altered lipid metabolism, structural and autonomic derangements (10). HRR following sub-minimal exercise is well correlated to vagal reactivation primarily in 1st minute after exercise (26) and have been suggested as an independent marker of cardiovascular risk factor (27, 28). The study investigated the evidence of impaired cardiac activity in SCH similar to that in overt hypothyroid (29). Chronotropic response and HRR2 were found to be significantly different between SCH and euthyroid control in our study. Study by Galetta et al observed an impaired cardiac autonomic function by reduced Heart rate variability in SCH patients (12). Study by Akcakoyun et al have also reported a reduced HRR and CR in SCH subjects (P<0.003; P<0.03, respectively) against similar gender and BMI controls (21). How ever they did not find any difference in RHR, Systolic and diastolic blood pressure. Our study observed a reduced HRR in both 1st & 2nd second but was statistically significant in only 2 min into recovery (p=0.012). HRR per se depends on the parasympathetic reactivity which may is attenuated in subclinical hypothyroid (30) however HRR in 2 min represents decrease in sympathetic activity along with reactivation of parasympathetic (31). A significantly lower HRR in two minute observed in the study may be related to altered activity of both sympathetic & parasympathetic nervous system. On the contrary Sunita et al found similar HRR (peak to 1-5 min of recovery) and percentage change in HRR (1 min recovery to 2-5 min of recovery) in SCH cases and euthyroid control (15).

The peak HR in SCH cases was significantly lower compared to the control (P<0.01) which may be due to decreased sympathetic tone and increased parasympathetic activity often a feature associated with increased TSH levels (32). Lower response to sympathetic activity during exercise could be the reason for lower peak HR reached during exercise in SCH cases.

An attenuated HR response to exercise called as CI is predictive of mortality and CAD even after adjustment for age, physical fitness and standard Cardio vascular risk factors (33, 34). Also it is unaffected by exercise protocol, and stage of exercise used for measurement (35). We observed a significantly decreased CR in SCH (p=0.002) as compared controls and definite association of Serum TSH levels with CI(<0.8) (p=0.005) in the SCH population. Akcakoyun M et al found similar impaired chronotropic response in their study on 25 patients of SCH indicating impaired cardiovascular autonomic function in SCH (21). However, Sunita et al found that CR was similar in both SCH cases and Euthyroid controls (15). The lower chronotropic responses in SCH cases in our study may be explained by deceased sympathetic reactivity (30), depressed systolic function at rest, left ventricular

diastolic dysfunction at rest and exercise (36). Although studies on effects of SCH on heart show conflicting results but SCH seems to be associated with increased cardiovascular risks of coronary heart disease and total mortality.

Conclusion

Slow HRR2 and decreased CR indicates insidious subtle changes in cardiac responses to exercise in SCH which in due course may cause alteration in cardiac contractility, myocardial oxygen consumption, cardiac output, blood pressure, and systemic vascular resistance. Negative association of HRR2 & CR with S TSH in SCH may suggest that abnormal TSH concentrations may be a novel cardiac risk factor not only for overt hypothyroid (37) but for SCH as well. Also HRR2 can be used as a preliminary test to make decisions regarding pharmacological treatment at an early stage to prevent progression to overt hypothyroidism but also to improve cardiac function & competence.

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Original Article

Nephroprotective Potential of *Lens Culinaris* Against Cisplatin-induced Nephrotoxicity

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Abstract

Aim: The current study was aimed to investigate the nephroprotective potential of hydroalcoholic extract of seeds of *Lens culinaris* against cisplatin-induced nephrotoxicity in male wistar rats. **Materials and methods:** The renal injury was modeled with cisplatin (5 mg/kg b wt, i.p.). Nephroprotection was investigated by administration of extract at two different dose levels (200 and 400 mg/kg b. w.) in both curative and prophylactic regimens. Nephrotoxicity was assessed by determining serum and urinary parameters, renal oxidative stress markers, histological studies. **Results:** Induction of cisplatin neprotoxicity indicated by raise in the levels of Serum markers, Urinary Total protein and Lipid peroxidation and decreased Urinary creatinine and antioxidant enzymes. Treatment with extract reversed the effects induced by cisplatin in dose dependent manner. Histological and immunohistochemical studies substantiated the biochemical parameters. **Conclusion:** Thus the present study promises beneficial use of *Lens culinaris* in nephrotoxicity.

Introduction

From past several years people suffering from kidney diseases is increasing. Increased use of drugs such as gentamicin, doxorubicin, NSAIDS, cisplatin is one of the cause of acute kidney injury. Cisplatin (*cis*-diamminedichloroplatinum (II)) is a platinum based chemotherapeutic drug used to treat a variety of malignancies, such as bladder, cervical, ovarian, and testicular cancers (1). However its use in chemotherapy has been limited largely due to its diverse dose related side effects, including kidney, hematological and testicular toxicity. Previous reports suggested that Oxidative stress has been associated with cisplatin-induced kidney damage (2).

Researchers have evaluated different approaches in allopathic medicine but the treatment for nephrotoxicity is still empirical. Owing to the limitations of the agents of modern medicine, researchers are exploring the traditional system of medicine. Many medicinal plants have been claimed and proved as good nephroprotective agents (3). *Lens culinaris* (F:Fabaceae) commonly called as lentils. Seeds of *Lens culinaris* are being considered as one of the most beneficial legumes for health. In

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traditional and folklore medicine lentils are used as blood purifier, diuretic, anti fungal and to treat various kidney and gastric ailments (4), and till date there were no reports on nephroprotective activity of this plant. Hence the present study is designed to evaluate the nephroprotective potential of hydroalcoholic extract of seeds of *Lens culinaris* (HALC) against cisplatin-induced nephrotoxicity in rats.

Materials and Methods

Collection of Seeds of *Lens culinaris*:

Lens culinaris seeds were purchased from local market and authenticated by botanist Dr. K. Madhava Chetty, Asst. Professor, Dept. of Botany, S.V.University, Tirupati, Andhra Pradesh and a voucher specimen was deposited in Dept. of Botany, S.V.University, Tirupati.

Preparation of hydroalcoholic extract:

Seeds of *Lens culinaris* were powdered in a Wiley mill. The powdered seeds were macerated with ethanol and water (70:30) for 24 h. Macerated material was refluxed for 3h and then filtered. The procedure was repeated twice and obtained filtrate was combined and subjected to distillation under reduced pressure. Obtained semisolid was stored in desicator urine further use.

Preliminary phytochemical screening:

Preliminary phytochemical screening was carried out for hydroalcoholic extract of *Lens culinaris* (HALC) for the presence of active phytochemical constituents like alkaloids, carbohydrates, steroids, proteins, tannins, flavonoids, gums mucilage glycosides, saponins and terpenes (5).

Pharmacological studies:

The experimental protocol was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India and approved by the Institutional Animal Ethical Committee (IAEC) (Registration No.: 1677/PO/a/12/CPCSEA/9).

Animals:

Healthy Wistar strain albino rats between 2-3 months of age and weighing about 150-200g had been used in the present study. They were maintained in a 12 h light/dark cycle at a constant temperature 25°C with free access to standard rat pellet diet and water *ad libitum*.

Acute toxicity studies:

Acute toxicity studies were carried out according to OECD 423 guidelines. Animals were observed for gross behavioral studies like changes in skin, fur, eyes, and mucous membranes, respiratory, circulatory, motor and behavioral pattern. The animals were also observed keenly for the presence of any signs of tremors, excessive salivation, diarrhea, lethargy, sleep and coma for 14 days. (6).

Evaluation of nephroprotective effect:

Albino strain wistar rats were divided into nine groups of six each. Nephroprotective activity of extract was tested at two different dose levels *i. e.,* 200 and 400 mg/kg b. w.

TABLE I: Treatment schedule for evaluation of nephroprotective activity.

	Group
I	Normal control- vehicle for 5 days
II	Cisplatin on day 1 + vehicle from day 5 to day 9
III	Cisplatin on day 1 + HALC (200 mg/kg b. w.) from day 5 to day 9
IV	Cisplatin on day 1 + HALC (400 mg/kg b. w.) from day 5 to day 9
V	Vehicle from day 1 to day 5 + cisplatin (5 mg/kg, i.p.) on day 5
VI	HALC (200 mg/kg b. w.) from day 1 to day 5 + cisplatin on day 5.
VII	HALC (400 mg/kg b. w.) from day 1to day 5 + cisplatin on day 5.
VIII	Cisplatin on day 1 + cystone from day 5 to day 9
IX	Only higher dose of HALC (400 mg/kg b. w.) from day 1to day 5

Assessment of nephroprotective activity:

On day 5 from animals of group-I & IX and on day

9 from remaining groups urine was collected keeping rats individually in metabolic cages; the urine samples were subjected for estimation of urinary functional parameters. The animals were sacrificed on the respective following days by cervical decapitation and blood samples were collected by cardiac puncture and were used for estimation of serum markers. Biochemical parameters such as Blood Urea Nitrogen ((BUN) by DAM method), Serum creatinine ((SC) by Jaffe's Alkaline picrate method), Urinary total protein ((U_{TP}) by Turbidity method) and Urinary creatinine ((U_{Cr}) by Alkaline picrate method) were estimated by using commercial kits (7). Kidneys were isolated and used for anti-oxidant and histological studies.

Anti-oxidant studies:

Kidneys were homogenized in ice cold 0.05 M phosphate buffer p^H 7.8 to obtain a 20% (w/v) homogenate. The homogenate was centrifuged at 10,000 rpm for 15 min and the clear supernatant obtained was immediately used for the analysis of antioxidant enzymes. Anti-oxidant studies were carried out by the estimation of levels of reduced glutathione, catalase, superoxide dismutase, and lipid peroxidation (8-11).

Histological studies:

Kidneys of two animals from each group were used for histological studies. The isolated kidneys were fixed in 10% neutral buffer formalin and processed to paraffin wax. Sections (5 microns) were stained with haematoxylin and eosin and are examined under light microscope (Magnification 10X).

Immuno-histochemical studies:

Sections of formalin-fixed, paraffin-embedded kidneys were obtained on poly-L- lysine coated slides. Sections were deparaffinized in xylene, then rehydrated through a graded alcohol series. Antigen retrieval was performed by incubating slides in citrate buffer (pH 6.0) (10 mM) at 95°C for 20 min. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 30 min. To detect active kidney injury molecule-1 (Kim-1/Havcr1) sections were incubated under humid conditions overnight at 4°C with the anti-Kim-1/Havcr1 antibody (1:200; R&D systems, Inc., USA). Next day, the slides were washed three times in Tris buffers (pH 6.0) and were incubated with a biotinylated Goat Anti-Polyvalent Plus (Thermo Fisher Scientific, USA) for 30 min at room temperature. This step was followed by further wash in Tris buffer and incubation of slides at room temperature with a Streptavidin Peroxidase Plus (Thermo Fisher Scientific, USA) that binds to the biotin present on the secondary antibody. After washing in Tris buffer, the immunostaining reaction product was developed using 3,3-diaminobenzidine (DAB Plus substrate, Thermo Fisher Scientific, USA). After immunoreactivity, slides were dipped in distilled water, counterstained with Harris haematoxylin and finally the sections were dehydrated in xylene, mounted with DPX and cover slipped. Slides prepared were examined by light microscopy (Magnification 40X) (12).

Statistical analysis:

The data was expressed as mean±standard error. Mean values between the groups was considered statistically significant $p<0.05$ after analyzing by one way ANOVA and was compared using Tukey-Kramer multiple comparison tests.

Results

Preliminary phytochemical studies:

Phytochemical screening of the hydroalcoholic extract of seeds of *Lens culinaris* revealed the presence of carbohydrates, proteins, saponins, flavonoids, tannins and phenolic compounds.

Acute toxicity studies:

Animals which received extract at 2000 mg/kg b. w observed for 14 days had not shown any clinical signs of toxicity and mortality. Hence the extract is safe at 2000 mg/kg b.w.

Assessment of nephroprotective activity:

Based on acute toxicity studies 1/10th and 1/5th doses were selected as lower and higher doses for screening

of nephroprotective activity. Effect of seeds of *Lens culinaris* on cisplatin-induced nephrotoxicity was represented in Table-II and Table-III. Administration of high dose of hydroalcoholic extract for 5 days does not show any deteriorative effects on kidney.

Effect of seeds of *Lens culinaris* on blood urea nitrogen:

The levels of blood urea nitrogen increased significantly ($p<0.05$) in group-II (43.63±0.32) and group-V animals (44.25±1.15) when compared with normal control group-I animals (12.61±0.91). While the elevated levels of blood urea nitrogen were significantly ($p<0.05$) decreased by the administration of the ethanolic extract of seeds of *Lens culinaris* at 200 and 400mg/kg b.w. p.o. to 24.67±1.52 (group III) and 20.01±1.39 (Group IV) in curative regimen and to 33.14±0.37 (group-VI) and 26.60±3.37 (group-VII) in prophylactic regimen in dose dependent manner. (Table II)

Effect of seeds of *Lens culinaris* on serum creatinine:

The levels of serum creatinine increased significantly ($p<0.05$) in group-II animals (2.18±0.07) and group-V animals (2.40±0.13) when compared with normal control group-I animals (0.71±0.04). While serum creatinine were significantly decreased ($p<0.05$) by the administration of the ethanolic extract of seeds of *Lens culinaris* at 200 and 400 mg/kg b.w. p.o. to 1.60±0.12 (group III) and 1.25±0.04 (Group IV) in curative regimen and to 2.36±0.23 (group-VI) and 1.78±0.07 (group-VII) in prophylactic regimen in dose dependent manner against cisplatin-induced elevation of serum creatinine (Table II).

Effect of seeds of *Lens culinaris* on urinary total protein:

The levels of urinary total protein increased significantly ($p<0.05$) in group-II (4.70±0.29) and group-V animals (5.48±0.29) when compared with normal control group-I animals (1.15±0.11). While the raised levels of urinary total protein were significantly ($p<0.05$) decreased in animals treated with ethanolic extract of seeds of *Lens culinaris* at 200 and 400mg/kg b.w. p.o. to 3.64±0.26 -Group III and 2.32±0.14 -Group IV of curative regimen and to 4.25±0.18 -Group-VI and 2.75±0.16 -Group-VII in prophylactic regimens in dose dependent manner (Table II).

Effect of seeds of *Lens culinaris* on urinary creatinine:

The levels of urinary creatinine decreased significantly ($p<0.05$) in group-II (7.91±0.32) and group-V animals (6.38±0.39) when compared with normal control group-I animals (18.57±0.49). While the levels of urinary creatinine were significantly ($p<0.05$) increased by the administration of the ethanolic extract of seeds of *Lens culinaris* at 200 and 400mg/kg b.w. p.o. to 10.84±0.52 (group III) and 15.33±0.51 (Group IV) in curative regimen and to 7.70±0.63 (group-VI) and 12.30±0.58 (group-VII) in prophylactic regimen in dose dependent manner (Table II).

Effect of seeds of *Lens culinaris* on antioxidant levels:

Animals which received Cisplatin alone *i.e.*, group II and group-V significantly ($p<0.05$) increased the levels of LPO and decreased the levels of GSH, CAT, SOD when compared to normal control group I animals. Animals treated with hydroalcoholic extract at 200 and 400 mg/kg b.w. reversed the effects induced by

TABLE II: Effect of HALC on serum and urinary parameters on Cisplatin-induced nephrotoxicity.

Group	*BUN (mg/dl)*	*SC (mg/dl)*	*U_{TP} (mg/24 hrs)*	*U_{Cr} (mg/24 hr)*
I	12.61±0.91	0.71±0.04	1.15±0.11	18.57±0.49
II	43.63±0.32[a]	2.18±0.07[a]	4.70±0.29[a]	7.91±0.32[a]
III	24.67±1.52[b]	1.60±0.12[b]	3.64±0.26*[b]	10.84±0.52[b]
IV	20.01±1.39[b]	1.25±0.04[b]	2.32±0.14[b]	15.33±0.51[b]
V	44.25±1.15[a]	2.40±0.13[a]	5.48±0.29[a]	6.38±0.39[a]
VI	33.14±0.37[c]	2.36±0.23[ns;c]	4.25±0.18[c]	7.70±0.63[ns;c]
VII	26.60±3.37[c]	1.78±0.07[c]	2.75±0.16[c]	12.30±0.58[c]
VIII	21.04±1.82[b]	1.33±0.06[b]	1.56±0.07[b]	15.93±0.49[b]
IX	11.96±0.66[ns;a]	0.81±0.07[ns;a]	1.09±0.07[ns;a]	18.83±0.41[ns;a]

Each value represents the Mean±S.E.M from 6 animals in each group. $p<0.05$; ns: not significant. a: Group-II, V and IX compared with Group-I; b: Group-III, IV and VIII compared with Group-II; c: Group-VI and VII compared with Group-V.

TABLE III : Effect of HALC on anti-oxidant levels on Cisplatin induced nephrotoxicity.

Group	*LPO (nmol/g of wet tissue)*	*GSH (µmol/gof wet tissue)*	*SOD (units/mg of wet tissue)*	*CAT (units/mg of protein)*
I	1.14±0.09	81.83±1.12	35.22±0.55	42.37±0.75
II	7.32±0.25[a]	21.83±0.42[a]	11.87±0.46[a]	15.68±0.92[a]
III	4.83±0.43[b]	42.98±0.30[b]	17.18±0.66[b]	26.05±0.43[b]
IV	2.82±0.14[b]	46.22±0.57[b]	23.71±2.14[b]	37.01±1.19[b]
V	6.76±0.29[a]	24.17±0.19[a]	13.08±0.35[a]	13.78±0.66[a]
VI	6.50±0.18[ns;c]	31.82±0.57[c]	14.84±1.18[c]	20.08±0.28[c]
VII	4.19±0.04[c]	39.70±0.57[c]	27.90±1.07[c]	28.49±1.09[c]
VIII	1.73±.15[b]	57.75±0.84[b]	29.95±0.12[b]	39.97±0.60[b]
IX	1.27±0.15[ns;a]	76.49±0.93[ns;a]	37.83±0.82[ns;a]	41.75±0.88[ns;a]

Each value represents the Mean±S.E.M from 6 animals in each groupp<0.05; ns: not significant. a: Group-II, V and IX compared with Group-I; b: Group-III, IV and VIII compared with Group-II; c: Group-VI and VII compared with Group-V.

the cisplatin on renal oxidative stress markers in dose dependent manner in both curative and prophylactic regimens (Table III).

Histological studies:

Kidney sections of rat which received cisplatin caused a marked necrosis in proximal tubules, degeneration of the tubular epithelial cells and glomeruli. The treatment with HALC in both curative and prophylactic regimens caused regenerative changes and reduced the renal damage. (Fig. 1 to Fig. 9)

Immuno-histochemical studies:

Section of rat kidneys which received cisplatin alone showed marked expression of Kim-1 in damaged renal tubular cells. While the kidney sections of rats treated with HALC showed reduced expression of KIM-1 (Fig: 10-17).

Discussion

Nephrotoxicity of the drugs is usually associated with their accumulation in renal cortex, dependent upon their affinity to kidneys and on kinetics of drug trapping process. Cisplatin is one such drug which causes nephrotoxicity. A minimum dose of cisplatin (5 mg/kg body weight) was sufficient to induce nephrotoxicity in rats (13). Earlier studies have demonstrated that several mechanisms including oxidative stress, inflammation and apoptosis are closely associated with cisplatin-induced nephrotoxicity (14). Clinical use of cisplatin is limited because of its nephrotoxicity (15). Currently in the market only ketosteril (synthetic drug) is available to treat nephrotoxicity but it is associated with severe

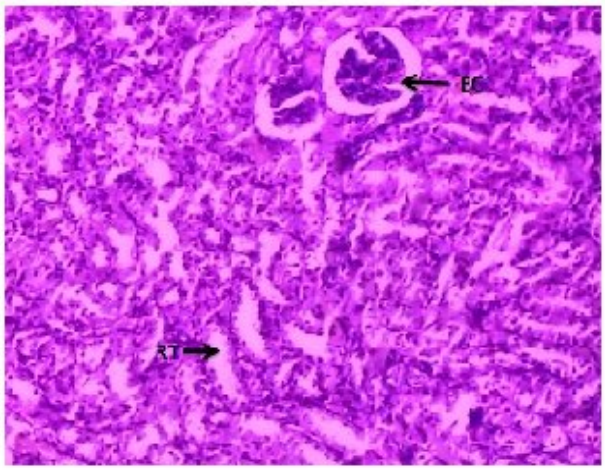

Fig. 1 : Group-I: Section of normal rat kidney showing normal organization of tubular epithelial cells and glomeruli.

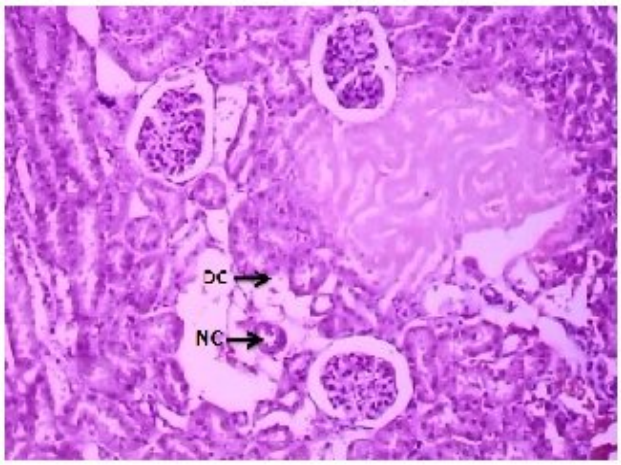

Fig. 2 : Group-II: Section of rat kidney showing necrotic changes in kidney tissues and congestion (Curative control)

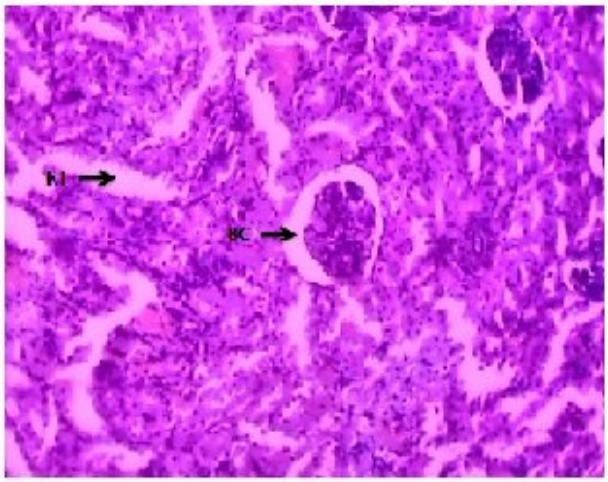

Fig. 3 : Group-III: Section of rat kidney showing moderate regenerative changes in kidney tissue (curative lower dose)

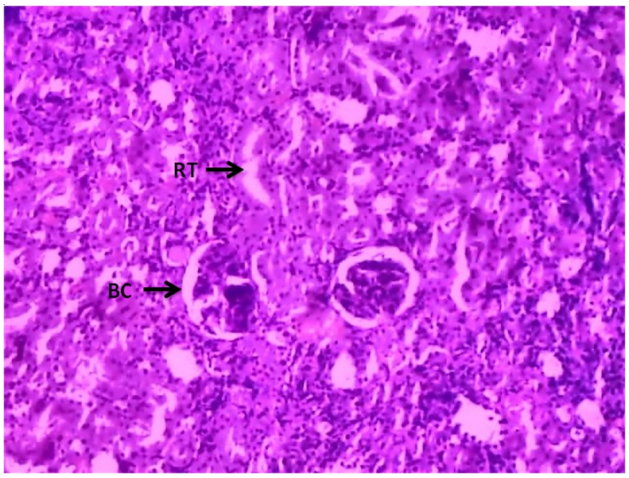

Fig. 4 : Group-IV: Section of rat kidney showing marked regenerative changes in renal tubule and Bowman's capsule (curative higher dose)

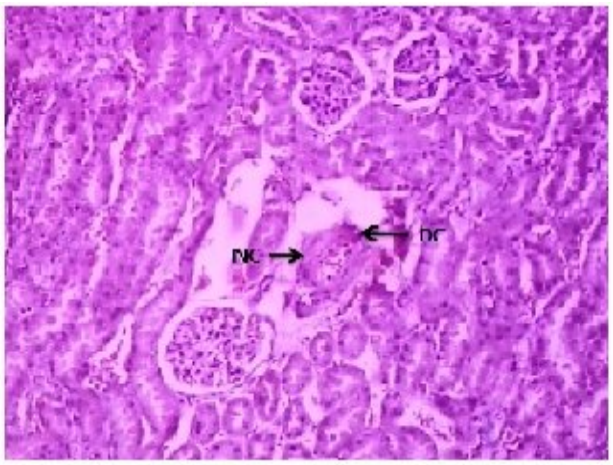

Fig. 5 : Group-V: Section of rat kidney showing congestion in glomeruli, vacuolization, and necrotic changes (Prophylactic control)

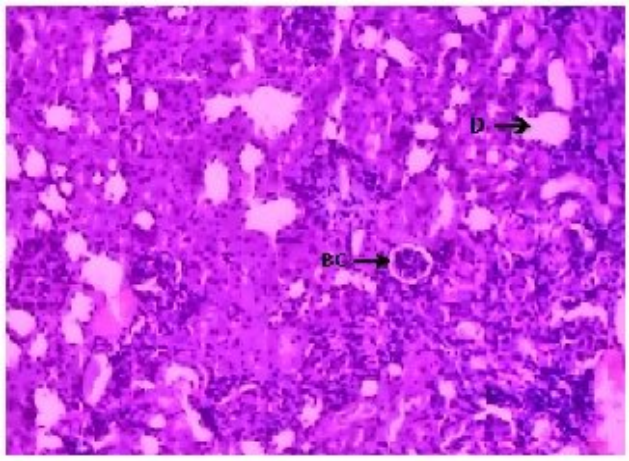

Fig. 6 : Group-VI: Section of rat kidney showing mild regeneration of kidney tissue (prophylactic lower dose)

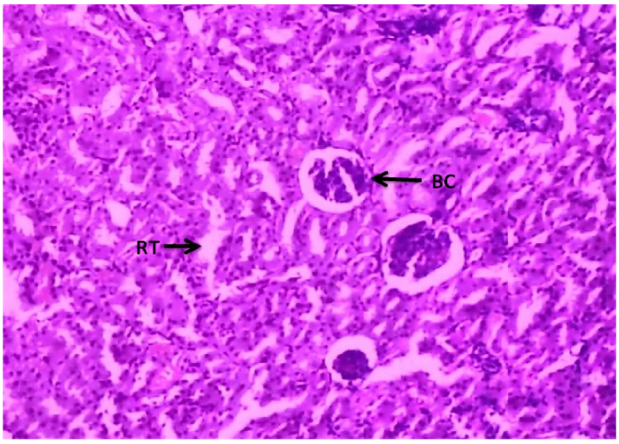

Fig. 7 : Group-VII: Section of rat kidney showing regenerative changes in kidney tissue and similar to normal architecture (prophylactic higher dose)

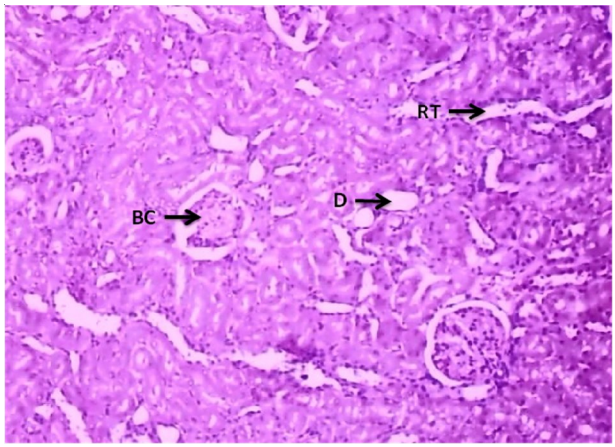

Fig. 8 : Section of rat kidney showing almost normal organization of Bowman's capsule and distal convoluted tubule (standard treated)

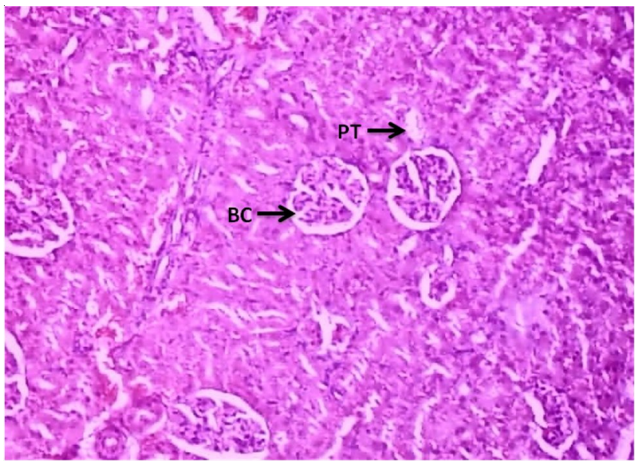

Fig. 9 : Section of rat kidney showing almost normal organization of Bowman's capsule and distal convoluted tubule (Only higher dose of HALC)

RT-Renal tubule BC- Bowman's capsule (Magnification 10X)

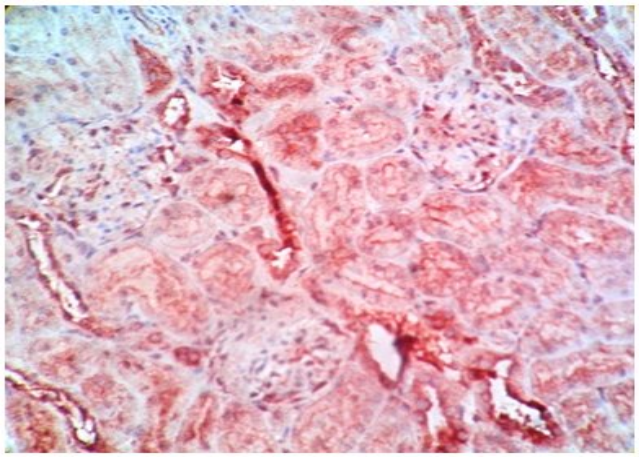

Fig. 10 : Normal Kidney architecture showing absence of KIM-1 expression (Group-I)

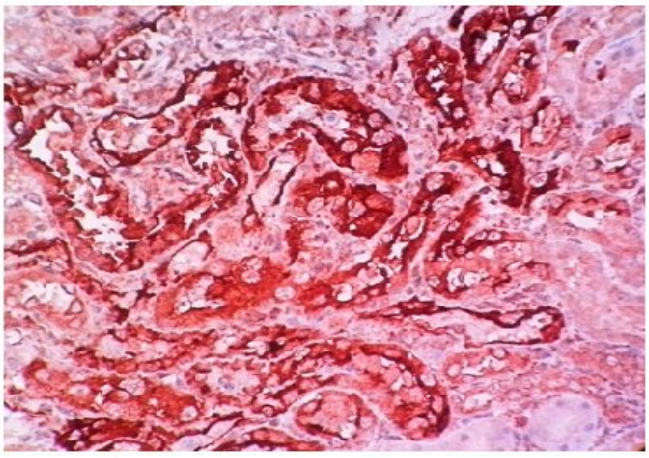

Fig. 11 : Kidney section showing marked expression of KIM-1 (Group-II)

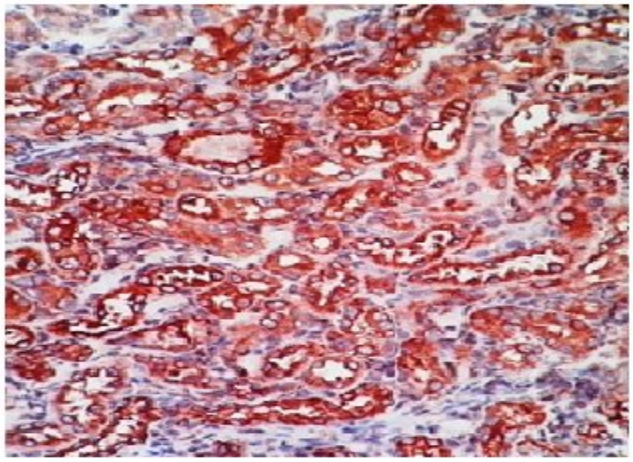

Fig. 12 : Kidney section showing moderate expression of KIM-1 (Group-III)

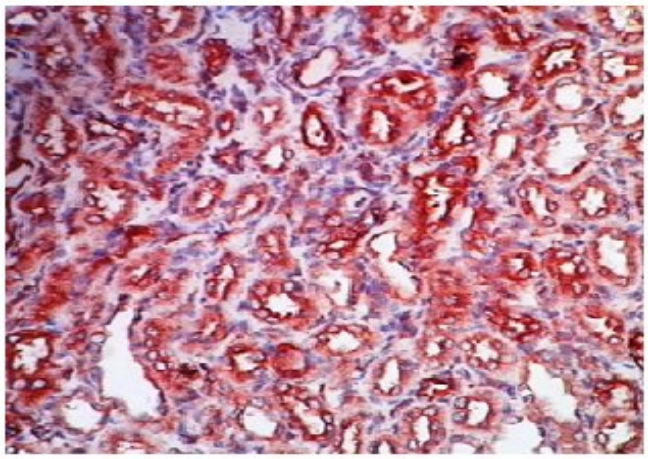

Fig. 13 : Kidney section showing mild expression of KIM-1 (Group-IV)

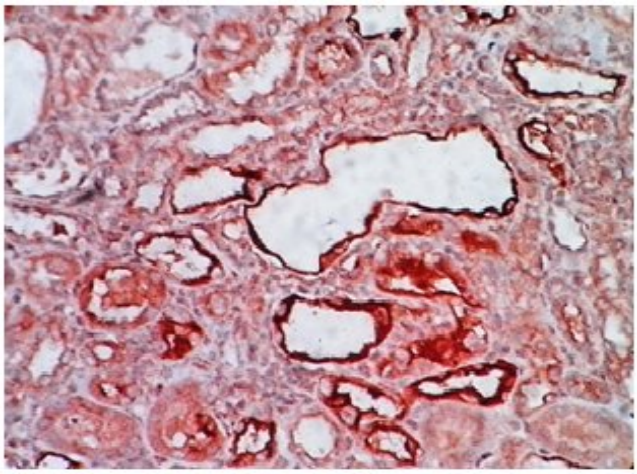

Fig. 14 : Kidney section showing profound expression of KIM-1 (Group-V)

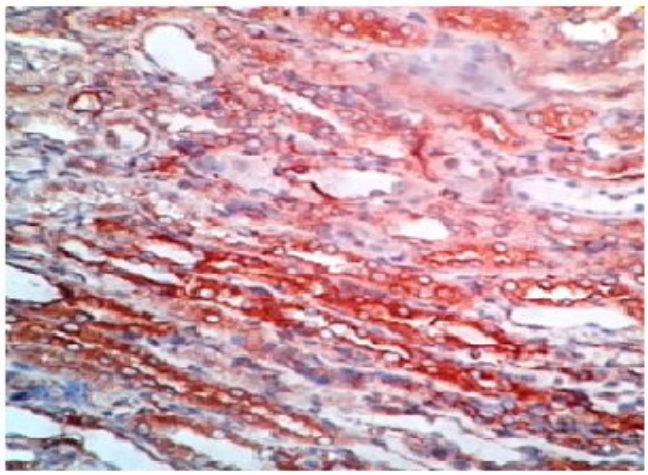

Fig. 15 : Kidney section showing moderate expression of KIM-1 (Group-VI)

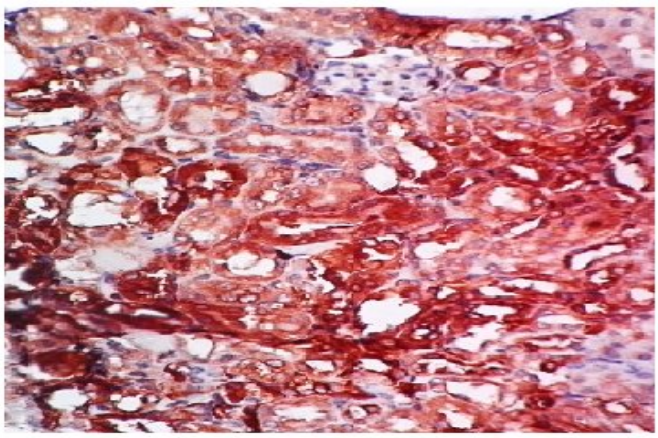

Fig. 16 : Kidney section showing reduced expression of KIM-1 (Group-VII)

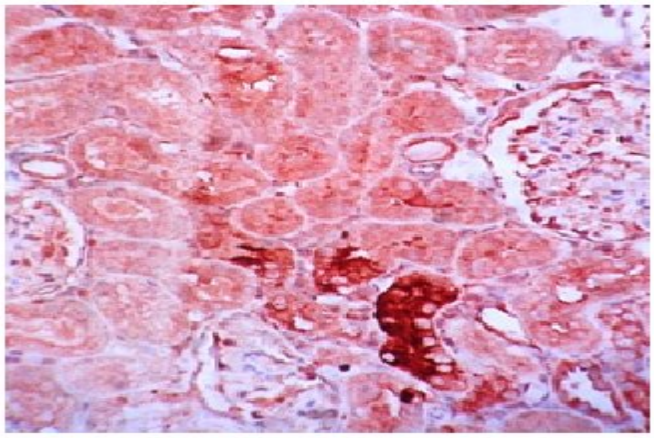

Fig. 17 : Kidney section showing mild expression of KIM-1 (Group-VIII)

side effects (16). Hence there is dire need to invent effective drugs to treat nephrotoxicity. Previous reports evidenced that number of medicinal plants exhibited significant nephroprotection against renal toxicity induced by different nephrotoxicants (17). *Lens culinaris* is one such plant in traditional and folklore medicine seeds of this plant is used to treat various kidney ailments (4). Hence present study is planned to evaluate nephroprotective potential of hydroalcoholic extract of *Lens culinaris* in both curative and prophylactic regimens. Hydroalcoholic extract is selected since many previous reports states that hydroalcoholic extract will be rich in antioxidant principles like flavonoidal glycosides and phenolic compounds and it is the most preferred type in ayurvedic formulations. The nephrotoxicity is a rapid process due to the reaction with the proteins in renal tubules. The renal damage is produced within one hour after administration. Hence the presence of protective agent in the renal tissues may reduce the toxic effects of cisplatin. This is the rationale behind the prophylactic regimen (16).

In the current study, cisplatin at a dose of 5mg/kg.b. w. induced nephrotoxicity in albino rats which was characterized by signs of injury, such as increased creatinine and BUN levels in plasma, raise in urinary total protein, reduced creatinine clearance and these results are in accordance with previous findings (18). Like *Hygrophila spinosa, Bassia malabarica, Berberis aristata* treatment with seeds of *Lens culinaris* restored the nephrotoxic effects

induced by cisplatin in dose dependent manner in both curative and prophylactic regimens (19, 20, 3).

Reactive Oxygen Species (ROS) generated during normal cellular processes are immediately detoxified by endogenous antioxidants like GSH, catalase, SOD etc., but according to Kim et al., excessive ROS accumulation by cisplatin causes an antioxidant status imbalance leading to lipid peroxidation and GSH depletion. Also, the increased reactive oxygen species that attack the cell membrane lipids leads to increased tissue lipid peroxides as manifested by increased MDA level and over accumulation of lipid peroxides in tissue causes over consumption and depletion of GSH and inhibition of antioxidant enzymes (21). In accordance with earlier reports in present study also administration of cisplatin decreased the activity of antioxidant enzymes SOD and CAT, depletion of GSH and enhancement of MDA production in renal tissue leading to increased LPO levels (22). In the current study, administration of extract in both regimens exhibited a clear protective action against the deleterious effects resulting from the administration of cisplatin on the antioxidant status. Nephroprotective activity of HALC was also supported by the histological and Immunohistochemical studies. Administration of HALC alone for 5 days did not show any deteriorative effects on kidney revealing that the extract is safe.

In conclusion, present study promises the beneficial use of seeds of *Lens culinaris* as a nephroprotective agent against cisplatin-induced nephrotoxicity.

Acknowledgements

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Medical Education / Original Article

Student Feedback in Medical Teaching Evaluation: Designing the Perfect Mechanism

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Ongoing evaluation and audit of the effectiveness of a teaching program is essential to constantly upgrade and improve upon the teaching learning experience, and do course correction for appropriate learning outcomes. There are several sources of information about the effectiveness of teachers. Faculty rating by students can be a useful and constructive tool for formative and summative assessment of an educational system (1). In the present context in medical education in India, it remains controversial and poorly utilized. Student feedback mechanisms are not professionally organized or planned. The instruments for feedback are not thoughtfully designed, validated and implemented. There is apprehension in the faculty about negative ratings with a potential fallout in terms of “professional melancholia” or a witch hunt by the administration (2, 3). This study aims to address the reasons why student evaluation has not formally been established into the system in Indian medical education, discusses what positive outcomes could be achieved by regular feedback and attempts to suggest solutions and methods to develop a simple feasible method of evaluation which can be effectively applied.

Medical education scenario in India: issues and difficulties

Student feedback has been proven by research to be valid and reliable and can provide valuable information for faculty, students and administrators for improvement of various courses in higher education (4). However, in medical education in India it is still controversial, and its mechanism poorly understood. Even if data is collected it does not find useful application in improvement of the curriculum or its implementation.

Most research on student evaluation of higher education has been from non-medical curricula, where faculty ratings are well established as a part of the system. These studies cannot be applied to medical education as the Medical curriculum differs in the four important ways which reflect teaching quality, i.e the structure, the processes, the learning outcomes and the individual teachers (5). The scenario in which a feedback mechanism from the students has to be designed is complex and needs to be understood if the feedback generated is systematically organized and to be used to improve the system.

The medical curriculum offers fewer choice options, and a fairly rigid structure is followed. The course is not modular and is covered over several years by various teachers on varied topics rather than it being taught in discrete cohesive modules. There is limited integration of various disciplines and in many instances the significance of a particular course of

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teaching is only understood by the student at a much later date. Designing a pretest, posttest and feedback for a concise module is relatively simple and specific, and feedback gained can be effectively applied to constantly improve upon the module and teaching effectiveness. The traditional Indian medical curriculum is vast and disconnected, and does not lend itself easily to constructive useful feedback and has been fairly resistant to change.

Clinical teaching and problem based learning are a significant part of the curriculum, making the process very different from other higher education teaching learning experiences (6, 7). Vast theoretical knowledge, practical training and deductive reasoning are intricately interwoven into the final outcome. The student imbibes the skills and attitudes of a doctor as he progresses through the system and often the process is physically and psychologically grueling. The best teachers are often the most unpopular.

The outcome of the process ideally would be to have a good basic doctor, well trained to deal with the Indian medical challenges. The assessment system, that is to evaluate this ability, has become more and more theoretical and reliant on multiple choice questions which do not efficiently evaluate the proficiency of the student in the field, in a practical setting. There is a parallel system of coaching for post graduate entrance examination, which occupy the students time and focus, and often are more “popular” with the students. The final learning outcomes of the two processes are different, the medical curriculum focusing on developing a good basic doctor, with clinical skills and appropriate attitudes, and the coaching class increasing the knowledge base with expertise in solving multiple choice questions. The latter becomes more attractive to the students, ambitious to reach the next level. There are therefore inherent biases to student feedback on assessment process, which would come in the way of designing an appropriate feedback tool.

Finally, a medical teacher performs multiple roles in teaching, research and patient care. Resources to teach are often inadequate and most institutions are plagued by shortage of teaching staff. There is pressure to develop a research profile on the faculty which has over the last few years become essential for placement and job enhancement. The clinical load in most teaching hospital is substantial, leaving the medical teacher with little time to prepare for the teaching activity.

There is much scope for improvement in the present system, and keeping in mind that the fundamental structure and implementation of the curriculum is not likely to change in the near future, there is a need to identify those aspects which feedback would be useful and how it may be implemented and incorporated into the system.

With the above aspect in mind a short online survey was carried out among faculty members of different medical colleges to explore some questions related to student feedback. We received about 39 replies and on evaluation the following points could be highlighted.

In response to question of ‘should student evaluate teachers?’ we found majority (78%) of the teachers agreed that student should evaluate teachers while only 9.38% disagreed. The rest were not sure (Fig. 1).

Opinion to second question‘ what points should student evaluate the teacher’, we found majority (75%) opined that teaching quality should be evaluated. However, some also supported that curriculum and process of teaching syllabus and assessment methods should be evaluated (Fig. 2).

When asked about the advantages and disadvantages of obtaining feedback from student. Most teacher (84%) selected improvement of teaching methods as best advantage while identifying teaching shortfall and better preparation for lectures were other advantages (Fig. 3). Most (86%) suggested that the utilization of feedback by administration against faculty as most dreaded disadvantage while decreased confidence among teacher as other disadvantage (Fig. 4).

This points are further dwelled upon in details and an attempt is made to explore valuable ingredients

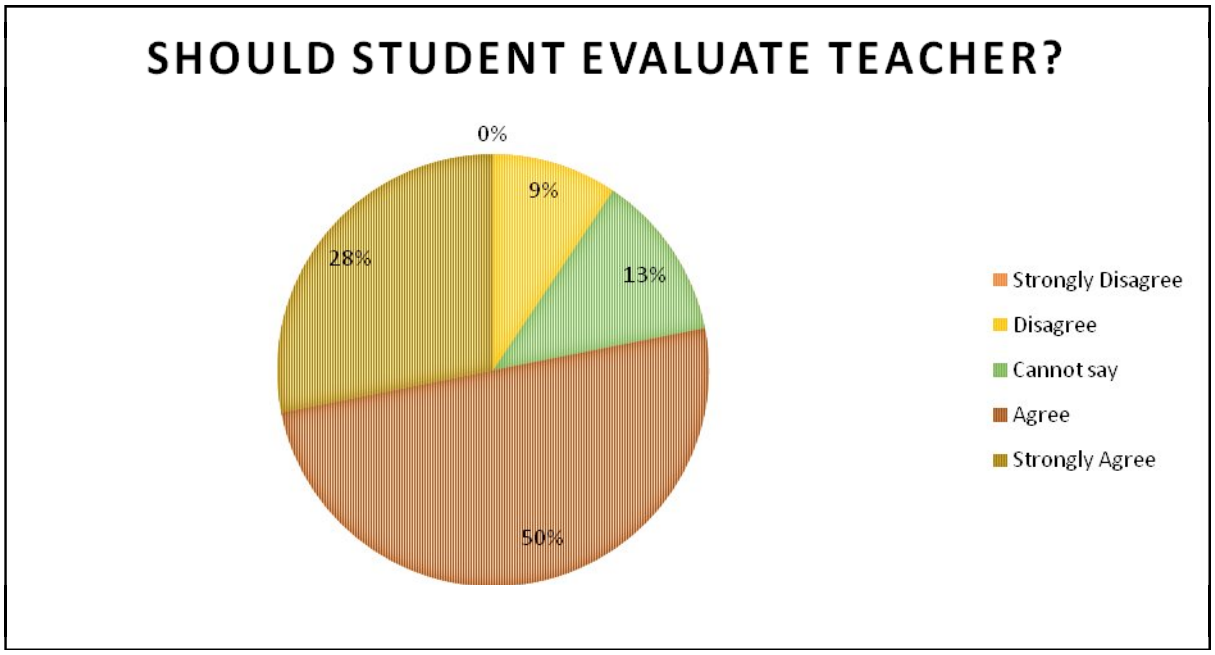

Fig. 1: Should student evaluate teacher? Responses as percentages.

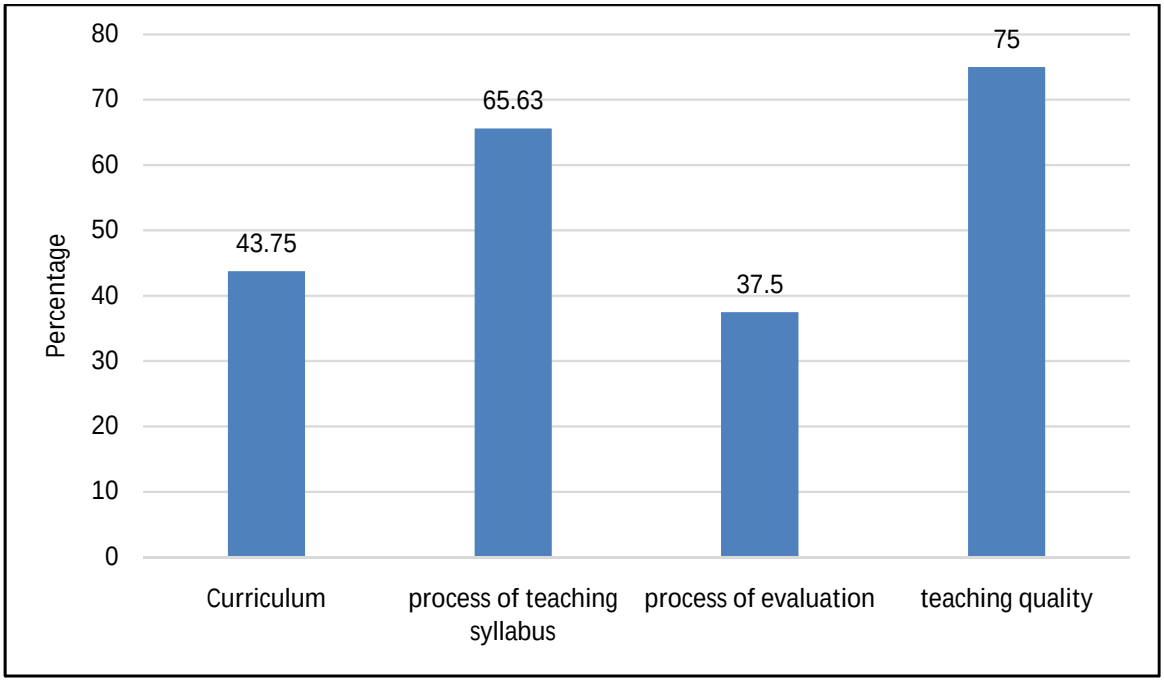

Fig. 2: What student should evaluate? Responses as percentages.

to be included in feedback to make a robust system.

Are students qualified to rate their teachers? What are the sources of bias

There are limitations to student ratings, and there are several aspects of the teaching learning experience that the students are not qualified to rate. The depth and breadth of the professor's knowledge, the content expertise are beyond the scope of student evaluation and should not be included in the

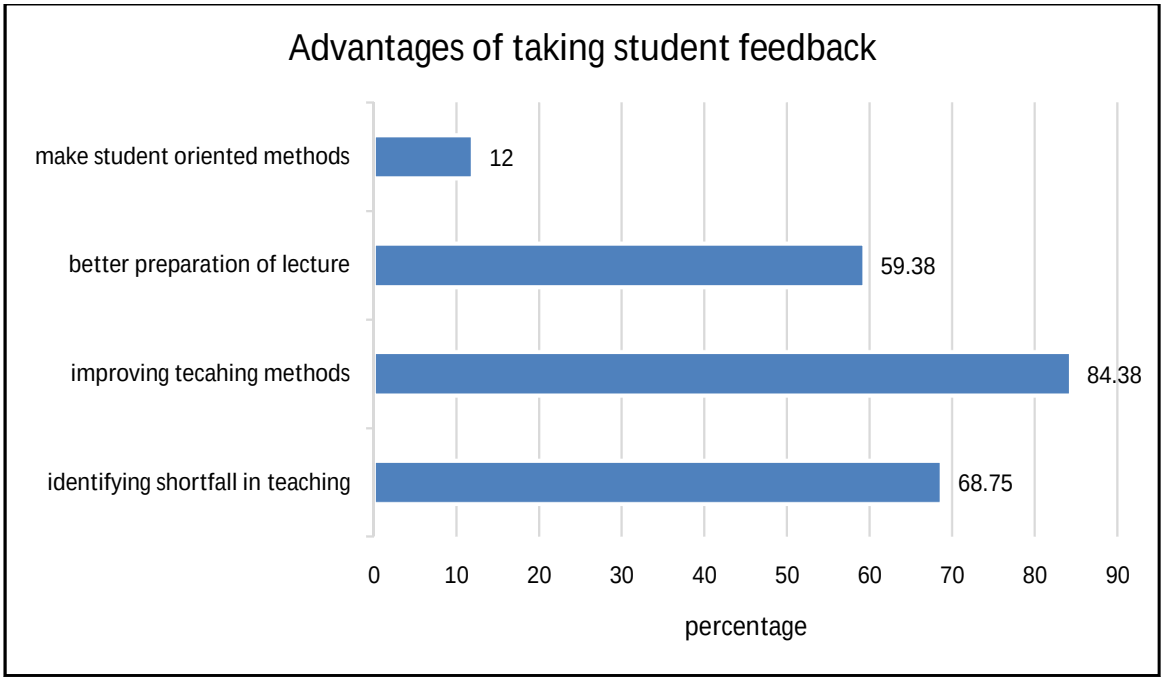

Fig. 3: Advantages of taking feedback from students-responses of teachers as percentages.

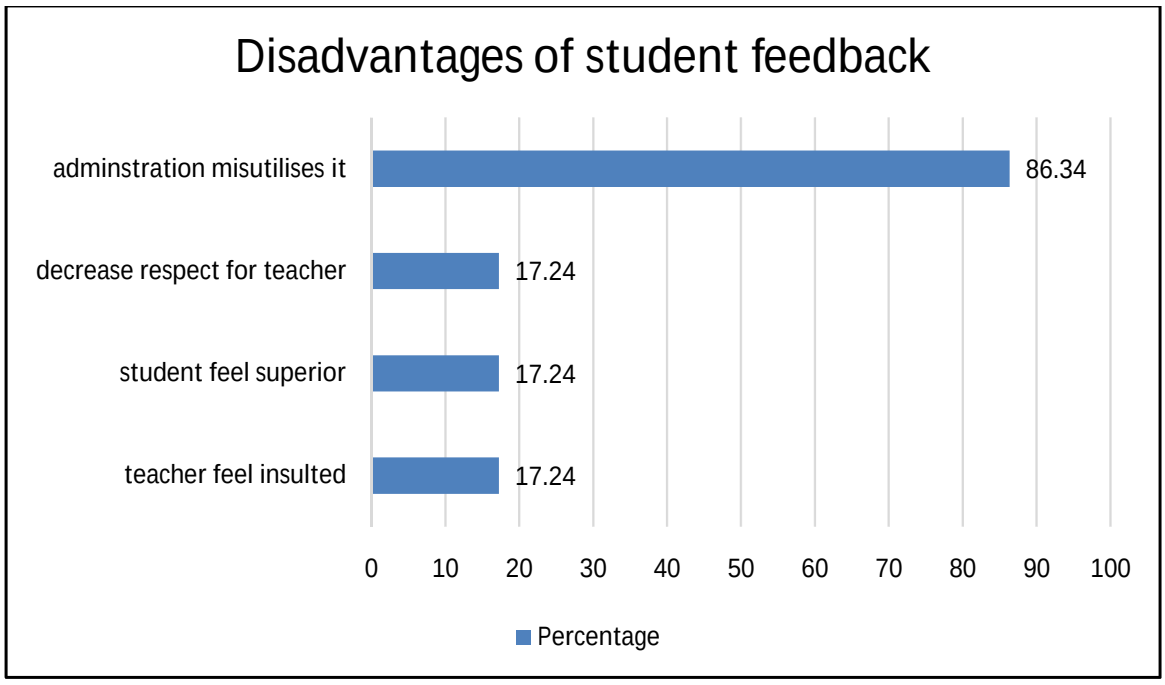

Fig. 4: Disdvantages of taking feedback from students-responses of teachers shown as percentages.

instrument designed for the purpose. Other aspects which contribute to teaching effectiveness such as teaching methods, use of technology, assessment methods students can give a certain degree of feedback, but it must be complemented with peer review or other professional methods of feedback (8). Students give only one facet of the information that is required to judge teaching effectiveness and if that alone is used it is incomplete and biased. There are several other sources of evidence for teaching

effectiveness. These include peer observation, peer review of course materials, external expert ratings, self ratings, videos, exit and alumni ratings and learning outcome measures to name a few.

Students are certainly qualified to express their satisfaction or dissatisfaction regarding the teaching learning experience, but there are several sources of bias which may be inherent, one being “Popularity” of the teacher. Good showmanship doesn’t necessarily mean a good teaching in terms of the final educational outcome. However, the students may express the classroom experience with a popular teacher in a very positive evaluation (3, 9).

Students grading and performance in examinations can be another source of faculty evaluation, but this too has its pitfalls. If other factors are controlled, research suggests that lenient inflated grading by a teacher in assessments can increase students ratings of a teacher, not necessarily reflecting better teaching quality (10).

The type of course taught, its relevance to the student’s final professional requirement may also have a bearing on the student evaluation or assessment, where highly theoretical courses which may be mundane or boring getting poorer ratings as compared to practical more clinically relevant courses which students enjoy. Both aspects may be essential to the final curriculum (11).

Finally, it is questionable whether students are qualified to rate the teaching quality while still in a course or not. Many believe that students understand the significance of a learning experience only several years later when working in the field. There have been studies on this which suggest that student ratings are fairly consistent over time and teachers or courses which were highly rated while in the course of instruction are still rated highly even after 13 years of joining the work force (3).

Research suggests the most consistently reliable outcome measure of a classroom experience is assessment of the learning outcome. Learning outcome may be assessed in an ongoing day to day formative manner, using both theoretical and practical tools (12).

What are the characteristics that students can rate and what is the purpose?

Purpose of collecting student ratings may be towards formative or summative assessment (3, 13). Formative assessment aims to use information collected to review and improve the teaching effectiveness. Information may also be collected to make decisions upon merit, promotion and tenure, and qualifies as summative assessment. Summative assessment should rely upon several sources of information aside from student feedback alone, and various other resources used as mentioned earlier in the text. Summative assessment that relies heavily upon student’s ratings alone is a main source of anxiety and disfavor amongst faculty. Centra (1993) suggested that all teacher evaluations by students be for formative purposes only initially and should help and encourage teachers to identify what is required of them (14, 15).

Once the purpose is defined, there are several domains of the teaching experience which the students can observe and these have been discussed in various reviews A Student evaluation tool designed in a management institution through a rigorous process involving students and faculty identified 10 key areas that the students can observe (16) :

Course Design

Instruction skills

Depth of knowledge

Facilitation skills

Student faculty interaction ability to motivate

Quality of assignments

Organization of assessment

Quality of feedback

A focused group discussion with medical faculty brought out the following elements :

Arousing interest in the subject that is taught

Organization of content in a sequential and logical manner

Quality of transference of information

Ability to contextualize the learning

Effective utilization of teaching aids

Ability in facilitating appropriate psychomotor skills

Role modelling appropriate attitude

The fundamental point is the ability of the teacher to engage the attention of the student, stimulate the learner to understand the topic being taught, and connect with previously known information. Clinical medical teaching also requires transference of psychomotor skills and attitude building of correct communication skills and empathy and compassion. These factors can be kept in mind while constructing an appropriate evaluation tool. It is important to keep in mind while designing the tool to ensure that one is actually objectively assessing teaching effectiveness and not “consumer satisfaction”.

Another aspect of discussion is that whatever the evaluation tool developed the students need to be sensitized as to the purpose of the tool and how to use it. The final purpose is to foster open communication and a climate of trust between the teacher and the learner. At no point should the teacher feel humiliated or the student be placed in a position that he/ she may be victimized (18, 19, 20).

Formal and informal Methods for taking student feedback after a single class or short course by a teacher :

The two ways to ensure that a system is followed are either to keep it very simple and informal, or to configure it into routine practice and into a time table. Faculty has traditionalized taking attendance at the end of a lecture. Formative feedback mechanisms have to be similarly structured into the system and reviewed in an ongoing manner, as either a self-evaluation by the concerned teacher or by peers or the administration.

Simple methods of self-evaluation feedback are informally practiced by almost all faculty in the form of eye contact, discussion, clarifications and similar activities. A quick and useful model at the end of a course of instruction is to pass around a shoe box, where written anonymous feedback can be collected. Once the chits are read, very often teachers “blind spots” or tricky areas come to the forefront and can be rectified. An oral vote at the end of a class regarding teaching effectiveness can be taken. Technology and smart phones are available with most students and faculty, and quick online surveys can be designed which can be filled up using social media.

To develop a validated tool which can be applied to give continuous aggregated quantitative assessment data, which can be applied across medical colleges across the country requires deeper research, using focused discussions between all stakeholders, students, administration and faculty with gradual sensitization of the whole community that the process of feedback is essential, its purpose not to victimize, and part and parcel of a healthy academic milieu. The students rating scale has to be tailored to the specific medical education scenario, where clinical teaching is akin to an apprenticeship, and developing psychomotor skills and appropriate attitude are also kept in mind.

After designing an appropriate tool, the student ratings data can be continuously collected using even a smart phone application. Daily classroom feedback data can be generated as can cumulative data collected over courses to give percentile feedback over several classes or even a complete course. In the present scenario, legal aspects would also be have to be considered, including confidentiality of data generated, its potential for misuse and these issues would have to be factored into the system (1, 3).

Conclusions

The use of student ratings for evaluation of teaching effectiveness in medical education in India is in the

present context is in its infancy. Most institutions have disorganized or limited systems for feedback, of cosmetic value rather than serving a systematic purpose for audit and improvement. The curriculum as it is being implemented at present does not take the student as a stakeholder in the education process. It is the need of the hour to develop a robust feedback system that can be applied for formative assessment and improvement of medical teaching across institutions in the country.

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Short Communication

No Effect of Long Distance Cycling on Physical Fitness of Medical Students Routinelycycling to College

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Abstract

Literature reports controversial findings about the effect of active commuting to and from school on the physical fitness. In the present study, we evaluated the effect of 6 months of cycling on the physical fitness and body composition in cycle commuting medical students. Twenty students of a premier medical college regularly cycling were randomly selected and divided into test and control groups. We compared the effect of 20 km long cycling 4 times /week apart from the normal routine with controls. We found that after 6 months of cycling there was no significant effect on the post-exercise heart rate after step test in the test group (132.8±17.2 vs 139.2±16.2, p>0.05) as well as control (143.2±11 vs 149.6±12.4, p>0.05) and VO2max values also did not change significantly (Test 55.6±7.2 vs 52.9±7.1, p>0.05 Control 51.2±4.6 vs 48.5±5.2, p>0.05). So we concluded, 6 months of long distance cycling in already cycle commuters does not improve the physical fitness.

Introduction

Physical fitness of an individual forms an important aspect of one's health. It is a powerful marker of health in young people which includes mainly cardiorespiratory fitness, muscular fitness, and motor fitness (1). Active commuting is defined as the phenomenon of using an active means of transportation in the form of either walking or bicycling, to and from school. Cycling is one of the commonly and easily available modality to the people. Being a category of sedentary people, medical students have the inherent tendency to gain weight and buy diseases in the bargain. Cycling as an intervention could be used to assess the change in physical performance. It is still controversial about the contribution of active commuting to physical fitness (2, 3) but some observational studies have shown that young people who actively commute to school tend to be more physically active (4, 5).

The study was done in a medical college where students used to go cycling to attend the lectures routinely. Apart from this, college had an active cycling club where students were going for cycling 20 km about 4 times a week. So in the present study we evaluated the effect of 6 months of active

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long distance cycling on the physical fitness and body composition of the medical students.

Materials and Methods

Twenty age, weight and height matched medical students of 18-21 years were included in the study. Student volunteers and those free from diseases were included in the study. While the exclusion criteria involved students suffering from major diseases which could interfere with the study. Detailed clinical examination of the subjects was done and ethical clearance got from the Institutional Review Board after obtaining the written informed consent. A prospective cohort study was carried out at the Department of sports medicine in a premier medical college by randomly sampling the subjects. Out of 20 students inducted, 10 underwent regular long distance cycling over a period of 6 months, were designated as the test group and other 10 were not given this exposure hence designated as control group.

Body composition analysis was done by BC-Analyzer machine, which is based on the principle of bioelectric impedance. The parameters measured included BMI, Fat%, Waist-Hip Ratio. We used a submaximal test Queens College step test to evaluate the cardio-respiratory fitness of the subjects. This test is performed on a wooden stool of the height of 16.25 inches (41.3 cm) for a duration of 3 min at the rate of 24 cycles per minute for male subjects and the rhythm is set by a metronome. After the completion of the exercise, immediately post-exercise recording of carotid pulse rate is done to get peak heart rate. Then estimated VO2 max is calculated = 111.33 - 0.42 x peak heart rate (bpm) (6).

Results

Data was analysed using Microsoft Office Excel, for within the group comparison paired 't' test was used. A significance value of $p<0.05$ was chosen for significant change.

Effect of cycling on body composition: No significant effect was seen.

Effect of cycling on the physiological parameters

Table I shows that there was significant increase in the resting heart rate of the test group after 6 months of cycling. No significant effect was seen in other parameters.

TABLE I: Pre & post intervention comparison of physiological parameters.

	Control		*Test*	
	Pre	*Post*	*Pre*	*Post*
Resting HR	72.8±9.6	70.9±8.7	68.3±8.5	75.3±9.9*
Peak HR	143.2±11	149.6±12.4	132.8±17.2	139.2±16.9
VO2 max	51.2±4.6	48.5±5.2	55.6±7.2	52.9±7.1

Values are mean±SD, * means $p<0.05$.

Discussion

The present study highlights that cycling for 6 months did not improve physical fitness of subjects who were already regularly cycling.

Active commuting is an inexpensive form of physical activity that can be integrated into individuals' routines, and if sufficient intensity is achieved, active commuting could lead to an increase in cardiovascular fitness (7). The present study did not find improvement in the physical fitness in the test group (Table I). We could find two studies from the literature which agree with the findings of our study. In one, authors did not find any significant association between active commuting to school and cardiorespiratory fitness (8). Other study done by Heelan K A et al also suggests that there is no association of active commuting and physical activity (9).

There are so many studies which do not agree with the findings of our study like controversial reports have been found about the contribution of active commuting to physical fitness (10, 11). Still other studies showed that active commuting by cycling is associated with a higher cardiorespiratory fitness level (12).

The reasons for the differing results in our study

from the previous ones may be due to the higher resting heart rate which we got in test group (sympathetic dominance) meaning that the training effect of 6 months of cycling was not sufficient to produce the full physiological effects to be visible in VO2 max values. The other reasons could be that most of the previous studies have evaluated the effect of active commuting to and from the school whereas in our study we have analysed the effect of 6 months of long distance cycling on the cardiorespiratory fitness. Still other reasons for the differing results in our study could be different modalities of testing physical fitness like some used cycle ergometer protocol (2), some used 20-m shuttle run test (12) and also 1-mile run time (9) and accelerometers. In the present study we used a very simple and handy objective method to evaluate the physical fitness by step test.

Limitations of the study

1) Strict adherence to training schedule of long distance cycling could not be followed religiously due to some college engagements like examinations and other unavoidable programmes. 2) Sample size in the study was a small due to limited number of cyclists in the college team. 3) There was no strict control over eating habits.

Conclusion

Six months of long distance cycling in cycle commuters does not improve the physical fitness in already regular cycle commuters.

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