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MODULATION OF THE RESPIRATORY RHYTHM BY GAP-JUNCTION BLOCKERS 18-α-GLYCYRRHETINIC ACID AND 18-β-GLYCYRRHETINIC ACID

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Abstract : Gap junction proteins are expressed in the pre-Bötzinger complex of the respiratory network but it remains under discussion how they modulate the respiratory rhythm generation. In the present study we tested whether the gap junction blockers 18-β-glycyrrhetinic acid (18-β-GA) and 18-α-glycyrrhetinic acid (18-α-GA) change either the phrenic nerve (PN) discharge frequency or amplitude. The PN discharge was recorded using the working heart-brainstem preparation of adult wistar rats (P22-P28) exposed to increasing concentrations of the gap junction blockers (0.1; 1; 10; 20 μM). With the two lower concentrations (0.1; 1 μM) of 18-β-GA, PN discharge frequency decreased to 46±15% (p=0.007) of the control value while it increased to 173±57% (p=0.16) with the two higher concentrations (10; 20 μM). Surprisingly, with 18-α-GA the PN discharge frequency was not significantly changed with any of the concentrations used. Enhancing the respiratory drive with 12% CO_2, the PN discharge frequency increased tendencially with rising concentrations of 18-β-GA, but again no significant change was observed with 18-α-GA. PN-amplitudes were slightly reduced over the course of the experiments, while the frequency of the heartbeat was not significantly changed at any time with any concentration.

Key words : gap junctions
gap junction blockers
respiratory rhythm generation
working heart brainstem preparation
18-α-glycyrrhetinic acid
18-α-glycyrrhetinic acid

INTRODUCTION

Breathing in mammals is controlled by a neuronal network of medullary neurons, which generates rhythmic activity and leads to periodic contractions of abdominal, diaphragm and thoracic muscles. The neurons involved are connected through

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a respiratory network, which is located in the pre-Bötzinger complex (PBC), a morphologically defined region within the ventral brainstem (1). Respiration is divided into three distinct phases: inspiration, post-inspiration and expiration. Depending on their discharge pattern, the neurons of this network can be distinguished by their activity in relation to the respiratory cycle. Accordingly, they are classified as early-inspiratory (early-I), augmenting-inspiratory (aug-I), late-inspiratory (late-I), post-inspiratory (post-I), augmenting-expiratory (aug-E) and pre-inspiratory (pre-I) neurons (2). Each subtype of the inspiratory neurons has its specific function for rhythm generation and pattern formation (3, 4).

Gap junction channel-proteins consist of connexins that establish a direct connection between neighbouring cells, and therefore provide a direct exchange path for small molecules and ions. They also couple neurons electrically (5, 6). Electric communication via gap junctions is compulsory for pacemaker neuron synchronization in mammals, as it was demonstrated for the heart and neocortical pattern generator (7, 8). Gap junctions have also been detected in the brainstem, and recently, it has been demonstrated that gap junctions are also important for rhythmogenic inspiratory neurons in the pre-Bötzinger complex of both neonatal and adult rats (9, 10). Using immunohistochemical methods Solomon and co-workers (9, 11) detected connexin 26 (Cx26) as well as connexin 32 (Cx32), although their relative amounts and distribution varied with age. Connexin (Cx26) was present in high amounts in neonatal rats (<7d) but declined with age, while connexin 32 (Cx32) was sparse in the juvenile tissue but increased with age.

The influence of gap junction coupling within the respiratory network was demonstrated by Bou-Flores and co-workers (12) by pharmacological blockade, which resulted in a reduced inspiratory burst frequency. These experiments were done using the brain stem-spinal cord (en bloc) or medullary slice preparation of neonatal (P1-P5) Swiss Webster mice (12). On the other hand, Solomon and co-workers (13) found an increase of the respiratory frequency when blocking gap junctions. They performed their experiments with adult rats (5-6 weeks old) utilizing an *in situ* "working heart-brainstem preparation" (WHBP) (14). It is unknown whether differences in these results are due to the way the tissue was prepared, or are simply a reflection of the different ages or species of the animals used.

Here we test if the gap junction blockers 18-β-glycyrrhetinic acid (18-β-GA) and 18-α-glycyrrhetinic acid (18-α-GA) change PN discharges using the *in situ* WHBP with adult wistar rats (P22-P28). In light of the results presented above, our objective was to study a concentration response relationship for the gap-junctions blocker 18-β-GA and 18-α-GA. Our study provides data that might help to illustrate the functional role of gap junctional communication in the respiratory network.

METHODS

Preparation

In order to test the effects of gap junction blockers 18-α-GA and 18-β-GA (both purchased from Sigma-Aldrich Chemie GmbH, Germany), we used the *in situ* perfused brainstem preparation (14) with adult

Wistar rats P22-P28 (*Rattus norvegicus familiaris*). The animals were bred in the animal facility of the University-Clinic of Essen. Our procedures have been extensively described (15, 16) and ensured a maximum reduction of discomfort and pain to the animals. The following are the main steps of the preparation: animals were deeply anaesthetized with isoflurane, and the absence of the tail flick reflex was taken as an indicator that the animals were pain free. The skull was opened with a scalpel and the cortex was removed at the pre-collicular level. The animals were then transected right above the diaphragm and the lower part of the body was removed. These steps were performed as quickly as possible to avoid ischemia time. The preparation was transferred into ice-chilled artificial cerebrospinal fluid (ACSF) of the following composition: 125 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 2.5 mM $CaCl_2$,1.25 mM $MgSO_4$, 1.25 mM KH_2PO_4, 1.1 mM glucose. The ACSF had an osmolarity of 290 mosmol and was gassed with carbogen (95% O_2/5% CO_2) for at least 15 minutes. The pH was adjusted with HCl or NaOH and maintained at 7.3 to assure *in vivo* like conditions. In addition, 3.5 g Ficoll (Sigma-Aldrich Chemie GmbH, Germany) was applied, and the total osmolarity was increased to 300-330 mosmol. The animals were transferred to the recording chamber and were retrograde perfused with ACSF via the abdominal aorta. To assure the respiratory drive, we gassed the perfusion with oxygen which contained either 5% CO_2 (ACSF pH 7.35) or 12% CO_2 (ACSF pH 7.2) to achieve a higher chemo-stimulation. The temperature of the perfusate was maintained at 31°C. We used a peristaltic pump (Watson Marlow 205 U) to circulate the ACSF through the preparation. Bubble traps and filters in the flow system minimized the risk of embolism by air bubbles in the animal. The pump was running at 60-80 rotations per minute, which provided a pressure between 50-80 mmHg at the level of the abdominal aorta. To assure muscular paralysis we applied Vecuronium bromide 0.04 µg/ml (Norcuron, Inresa Arzneimittel GmbH, Germany). The PN was isolated, cut and the end was taken into the tip of a glass electrode to record the nerve discharges.

Drug solutions

The gap junction blockers 18-α-GA and 18-β-GA were dissolved in ethanol (stock solution 10 mM). Final concentrations (0.1-20 µM) were made immediately before the experiments, diluting the stock solution with ACSF.

Electrophysical recordings

PN discharges were recorded by suction electrodes in order to monitor central respiratory activity. The electrodes were made from soda glass without filament, with an outer diameter (Ø) of 1.4 mm (Hilgenberg, Malsfeld/Germany). The glass electrodes were produced with a micropipette puller Model Flaming/Brown P-87 (Sutter Instrument Co. USA). The electrode was filled with the ACSF, which was connected by a silver wire to an amplifier (custom-made at the electronics workshop of the University of Mainz/Germany). PN activities were amplified (2,000–10,000x), band pass filtered (100-5000 Hz) and monitored on a strip chart recorder. This set-up allowed recording of both PN electrical discharge and heartbeat frequency simultaneously.

Experimental protocol

The major focus of this study was to observe PN discharges before and during pharmacological blockade (i.e., uncoupling) of gap junctional proteins. To monitor the PN discharges under control conditions (CTR), they were recorded for 15-20 minutes during perfusion with normal ACSF gassed with either 5% CO_2, or 12% CO_2. The preparation was then perfused with the gap junction uncoupling agents. During the experiment the concentrations increased in steps from 0.1, to 1 and 10 µM and finally to 20 µM. The concentrations were changed after 30 minutes of application. PN discharges were recorded continuously throughout the entire experiment.

Data analysis

The data were recorded on hard disc for off-line analysis. PN discharges were also recorded on paper charts throughout the entire length of the experiment. To standardize our experiments, we analyzed the PN discharges after 30 minutes exposure of gap junction uncoupling agents. Mean values (±S.D.) were calculated with Microsoft Excel. Frequencies of both the PN discharges and the recorded heartbeats were standardized by expressing all values as a percentage of the control value, which was set as 100%. To evaluate the statistical significance of the experiments we used the student's t-test and presented them as P-values. The statistical significance was set as (*) $P<0.05$, (**) $P<0.01$ and (***) $P<0.001$.

RESULTS

Results were obtained from a total of 37 experiments (22 experiments using 5% CO_2 and 15 experiments with 12% CO_2). 18-α-GA and 18-β-GA were applied in 19 experiments each.

Phrenic nerve discharge characteristics under control conditions

Eupnoenic discharges are characterized by a ramp like discharge pattern (Figs. 1A, B, C, D). PN discharge frequency under control conditions (5% of CO_2) was 10±2.43 discharges/min and with 12% of CO_2 the frequency was 11.6±3.58 discharges/min. The effects of the uncoupling agents on PN discharge were mostly reversible in experiments using the ACSF without gap junction uncoupling agents after 45-60 min (data not shown; n=3).

Concentration dependence of phrenic nerve discharge frequency after blockade of gap junctions

When the solution was gassed with 5% CO_2, the PN discharge frequency varied depending on the different 18-β-GA concentrations used (Fig. 2A). At a concentration of 0.1 µM, PN discharge frequency was decreased to 46±15.4% ($p=0.008$, $n=5$) compared to control conditions. The PN frequency decreased slightly further when 1 µM was applied (49.3±26.9%; $p=0.007$, $n=5$). But, when the concentration of 18-β-GA was increased to 10 µM, PN discharge frequency was no longer reduced, but rather increased to 134.6±66.1% (not significantly different from the control value; $p=0.4$, $n=4$). Further increasing the 18-β-GA concentration to 20 µM made this observation more pronounced (173.4±57.2%; $p=0.2$, $n=3$).

18-α-GA did not change the PN discharge

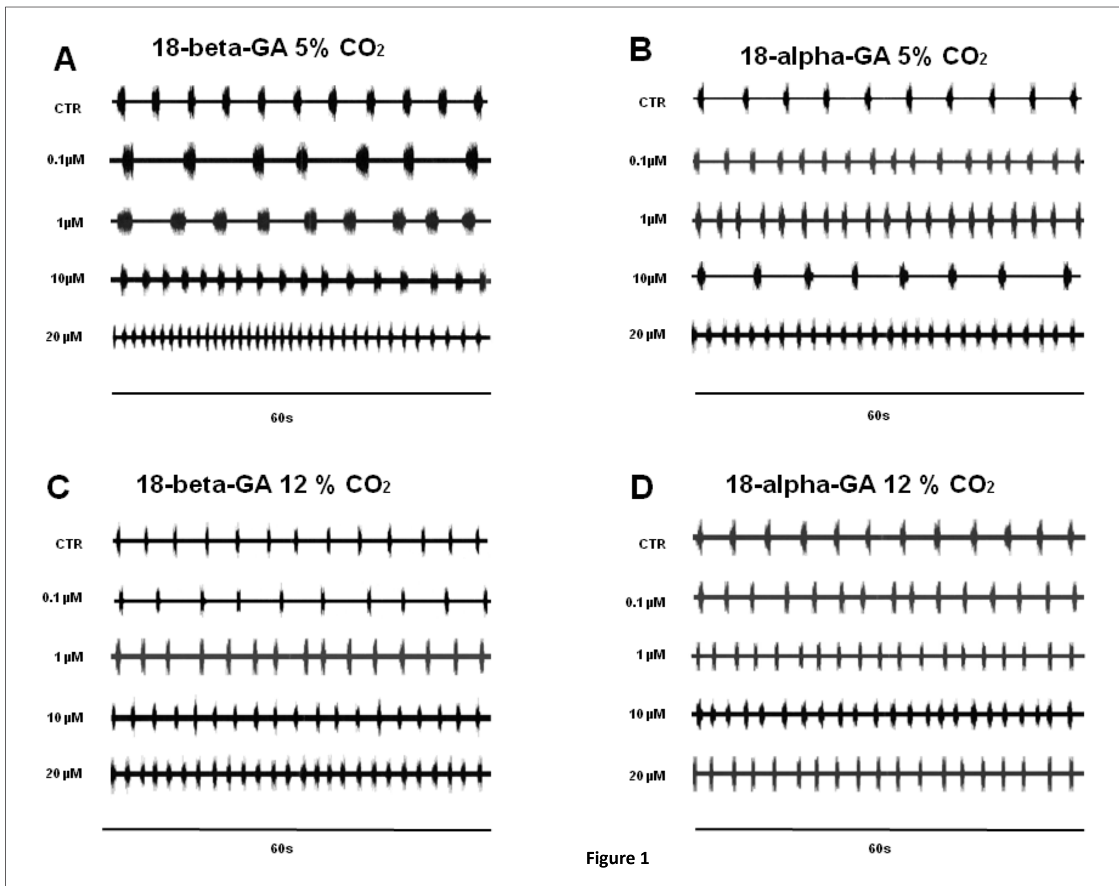

Fig. 1 : Characteristic changes of burst discharges in the PN of adult wistar rats (P22–P28) under control conditions (CTR) and after blockade of gap junction proteins with 18-α-glycerrhetinic acid and 18-β-glycerrhetinic acid with rising concentrations: A) Changes of PN frequency after application of 18-β-GA (0.1-20 µM; 5% of CO_2). B) Changes in PN discharge frequency after different applications of 18-α-GA (0.1-20µ M; 5% CO_2). C) Changes of PN frequency after application of 18-β-GA (0.1-20 µM; 12% of CO_2. D) Changes in PN discharge frequency after increasing applications of 18-α-GA (0.1-20 µM; 12% CO_2).

frequency significantly at any concentration using 5% CO_2 (Fig. 2B; 0.1 µM: 107.4±50.6%, p=0.9, n=5; 1 µM: 132.8±36.8%, p=0.2, n=3; 10 µM: 107.4±58.1%, p=0.7, n=3; 20 µM: 145.6±25%, p=0.09, n=3).

When the respiratory drive was boosted by the use of 12% CO_2 in the ASCF, neither of the two gap junction blockers altered the frequency of the PN discharges significantly at any concentration used. With 18-β-GA (0.1-20 µM) the observed PN discharge frequency was: 0.1 µM: 97±43%, p=0.7, n=2; 1 µM: 116.8±34%, p=0.09, n=8; 10 µM: 142±64%, p=0.18, n=7; 20 µM: 146±75%, p=0.54, n=2). Applying 18-α-GA, the frequencies were as follows: 0.1 µM: 91±31%, p=0.5 n=4; 1 µM: 131±15%, p=0.05, n=3; 10 µM: 123±40%, p=0.3, n=4; 20 µM: 96±19%, p=0.7, n=4.

Variations in the amplitudes of the PN discharges were measured as an indicator to

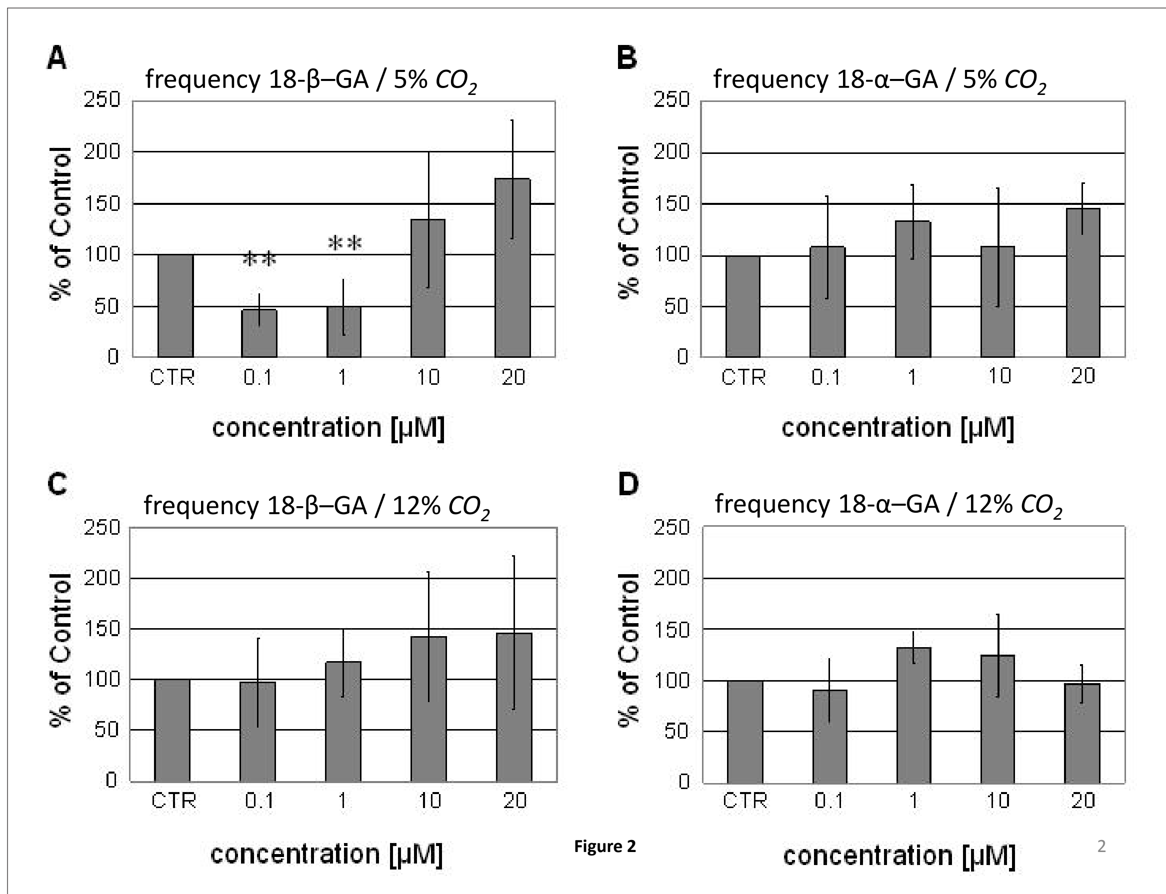

Fig. 2: PN discharge frequency under control conditions (CTR) and after pharmacological blockade of gap junction proteins with 18-α-glycerrhetinic acid or 18-β-glycerrhetinic acid. A) Changes in the phrenic nerve discharge frequency with increasing concentrations of 18-β-GA (0.1-20 µM; 5% of CO_2). B) Phrenic nerve burst frequency changes after application of 18-α-GA (0.1-20 µM) into the perfusate (5% CO_2). C) Changes in discharge frequency using 18-β-GA (0.1-20 µM; 12% CO_2). D) Changes in the phrenic nerve discharge frequency applying 18-α-GA (0.1-20 µM; 12% CO_2).

see whether the gap-junction blockers change the respiratory drive. In 5% CO_2, 18-β-GA and 18-α-GA changed the amplitude of the PN output inconsistently and insignificantly at the different concentrations (Figs. 3A, 3B). Using 18-β-GA (5% CO_2), changes in amplitude were: 0.1 µM: 72±20%, p=0.9 n=5; 1 µM: 67.7±14%, p=0.8, n=4; 10 µM: 71±12%, p=0.3, n=; 20 µM: 64±10%, p=0.03, n=3. For 18-α-GA (5% CO_2), the results were: 0.1 µM: 53±23%, p=0.003 n=6; 1 µM: 65±15%, p=0.03, n=4; 10 µM: 77±11%, p=0.05, n=4; 20 µM: 58±11%, p=0.05, n=3.

With 12% CO_2,the amplitude continuously decreased with increasing concentrations of the blockers using 18-β-GA: 0.1 µM: 94±23%, p=0.7 n=8; 1 µM: 80±19%, p=0.03, n=2; 10 µM: 62±21%, p=0.02, n=7; 20 µM: 76±4%, p=0.07, n=2. In the presence of 18-α-GA

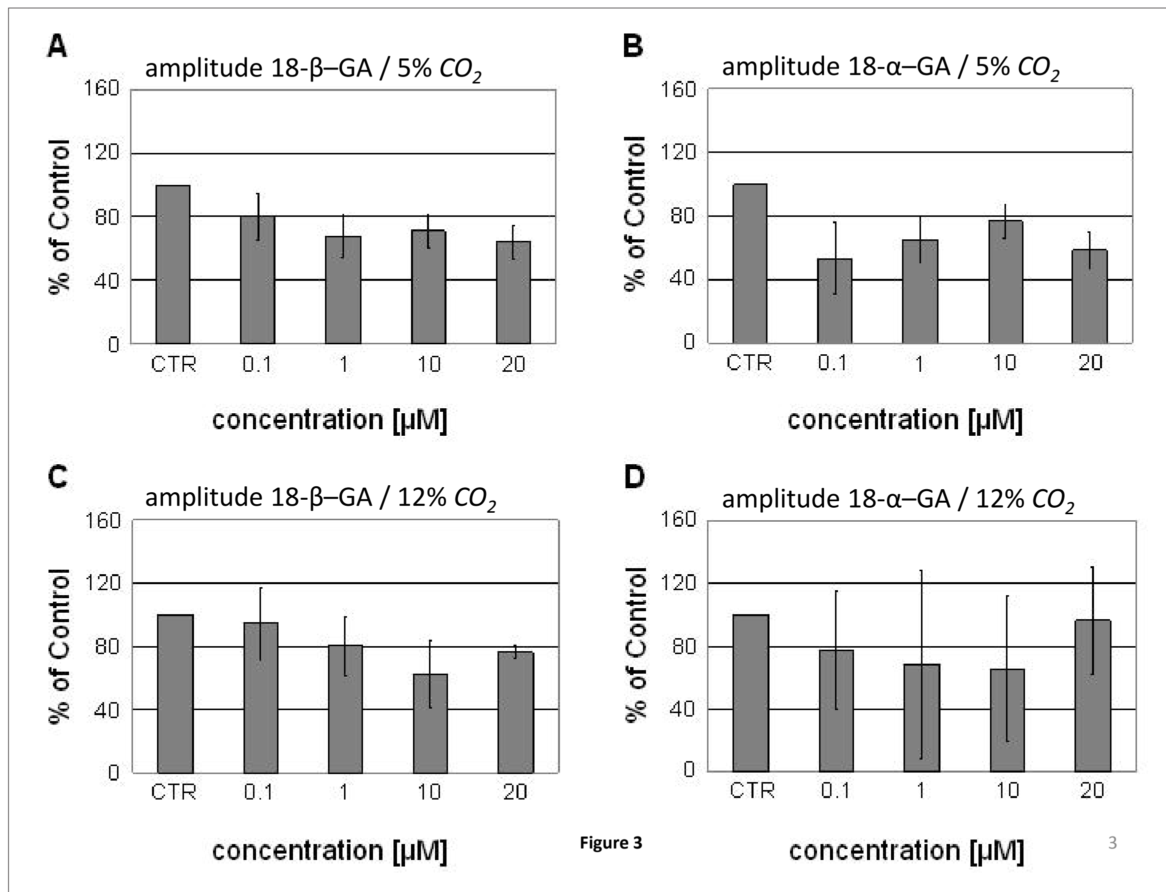

Fig. 3 : PN discharge peak amplitude under control conditions (CTR) and after pharmacological blockade of gap junction proteins with 18-α-glycerrhetinic-acid or 18-β-glycerrhetinic-acid. A) Changes of the phrenic nerve discharge peak amplitude with increasing concentrations of 18-β-GA (0.1-20 µM; 5% CO_2). B) Phrenic nerve discharge peak amplitude changes after application of 18-α-GA (0.1-20 µM; 5% CO_2). C) Changes in discharge peak amplitude using 18-β-GA (0.1-20 µM; 12% CO_2). D) Changes in the phrenic nerve discharge peak amplitude applying 18-α-GA (0.1-20 µM; 12% CO_2).

phrenic nerve amplitude changed as follows: 0.1 µM: 77±37%, p=0.38 n=4; 1 µM: 68±59.8%, p=0.8, n=3; 10 µM: 65±46.7%, p=0.4, n=4; 20 µM: 96±34%, p=0.83, n=4 (Figs. 2C, D, and 3C, D).

Since the myocardial cells are also connected via gap junctions (17), we expected a change in the heartbeat frequency; however the frequency did not change significantly from control conditions during the application of gap junction uncoupling agents, regardless of whether the perfusion was gassed with either 5% CO_2 or 12% CO_2 (Figs. 4A, B, C, D).

DISCUSSION

Recent studies have shown the expression of connexin 26 (Cx26) and connexin 32 (Cx32)

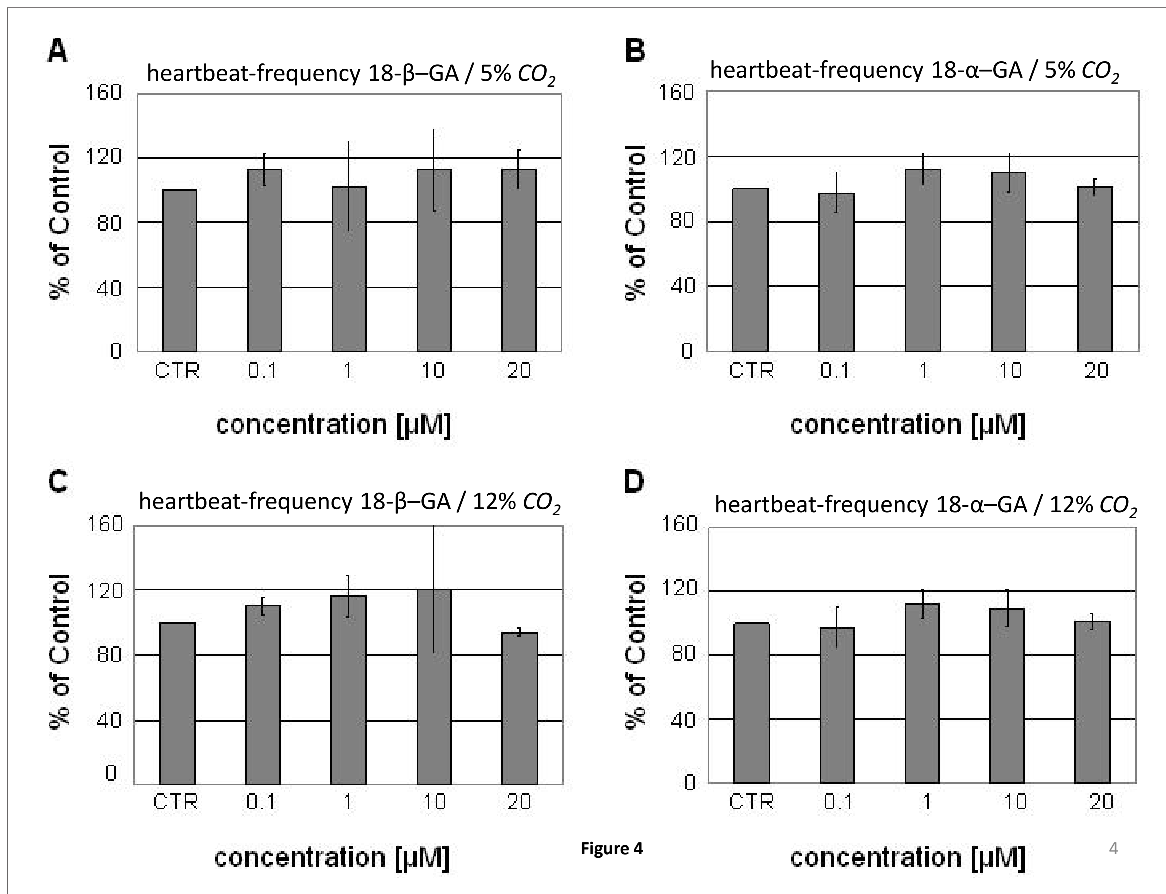

Fig. 4 : Changes in the heartbeat-frequency with varying concentrations of gap junction blockers 18-β-glycerrhetinic acid (A, C) 18-α-glycerrhetinic acid (B, D) gassed with either 5% CO_2 (A, B) or 12% CO_2 (C, D).

in the pre-Bötzinger complex (11), a CO_2-chemosensitive region of the brain stem (9), as well as in motor neurons (18).

While several gap junction blockers have been used to induce a change in the frequency of the PN discharge, we examined the effects of the most common gap junction blockers 18-α-GA and its isomer 18-β-GA to induce a pharmacological blockade of gap junction proteins. These two substances were tested in a variety of experiments, which have demonstrated a functional gap junction blockade (19-27). Clearly, when using the heart-brainstem preparation (14) all gap junction proteins in the brainstem and spinal cord will be blocked simultaneously, independent of whether they are involved in respiratory activity or not.

With rising concentrations of these gap junction blockers we observed a biphasic modification of the frequency of PN discharges while their amplitude decreased with rising concentrations; applying 18-β-GA in a concentration range from 0.1 μM to

1 μM reduced the discharge frequency, while concentrations from above 1 μM increased the PN discharge frequency.

Our results are comparable with the effects described by Solomon and co-workers (13) as well as with those presented by Bou Flores and Berger (12). Both also underline the importance of gap junctions for the maintenance of the respiratory rhythm. The findings presented here support both reported effects (decrease and increase of PN discharge) in a concentration-dependent manner. Like Solomon and co-workers (13), who used concentrations of the gap junction blockers in the range of 25-100 μM (5% CO_2), we observed an increase of PN discharge frequency using a concentration of 20 μM (18-β-GA; 18-α-GA). In contrast, Bou-Flores and Berger (12) described a reduction of PN discharge frequency over a concentration range between 50 and 100 μM. While there remain discrepancies in the results, the following differences in experimental design could be a possible explanation:

I. Type of preparation

Like Solomon and co-workers (13), we used the *in-situ* perfused heart-brainstem preparation and systemically applied gap junction blockers into the perfusion system; therefore, all gap junctional proteins were uncoupled simultaneously (as well as those in the entire preparation).

Gap junction proteins have been detected in the putative CO_2-chemosensitive brainstem areas (9) and in presumptive phrenic and hypoglossal moto-neurons (18). These areas could have contributed to the increase or decrease of PN discharge frequency due to the blockade of gap junction uncoupling agents, which block connexins throughout the whole brain and spinal cord. Nevertheless, it remains unanswered which cells are responsible for the effects. On the other hand, Bou-Flores and Berger (12) performed their experiments using the medullary slice preparation, which isolates the respiratory centre from inhibitory (or excitatory) input of other areas of the brain or the spinal cord, which might have influenced the results described in their presentation.

II. Age of animals

As in this study, Solomon and co-workers (13) used adult animals, whereas the animals used by Bou-Flores and Berger (12) were 1-5 days old. A different expression of gap junctions throughout the brainstem in relation to the age of the rats have been reported by Solomon and co-workers (9), which provides another possible explanation of the different respiratory effects which were reported.

III. Concentration of uncoupling agents

The biphasic effect we observed for 18-β-GA might be due to specific binding affinities at different receptors. Lower concentrations of gap junction blockers could specifically block connexins that contribute to the respiratory generation or synchronization of the signal within the respiratory network. Of the two gap junction blockers used in this study, 18-β-GA showed a bigger magnitude in its potency of modification than 18-α-GA. Using the same concentrations of both uncoupling agents, 18-β-GA had a more

pronounced effect on the frequency of phrenic nerve discharges. The gap junction blockers that we used in our experiments were not composed for specific Cx isoforms. Unfortunately, more specific agents are currently not available (25).

IV. Respiratory drive

In order to test the effect of the respiratory drive (28), we gassed the preparation with either 5% or 12% CO_2. With 12% of CO_2, any effects of gap junction blockade were not obvious because chemosensitive receptors and their modulation of the respiratory neurons might have masked the effects of the gap junction blockers.

The changes to the respiratory rhythm that we observed were definitely mediated by blockade of gap junction proteins, and not by nonspecific effects, because both glycyrrhetinic derivates performed similar modification to the respiratory rhythm. Furthermore, in our experiments, the effects of both gap junction uncoupling agents were reversible even after an exposure of more than 30 minutes. Additional evidence is that glycyrrhetinic derivates have been shown to functionally block ≤ 80% of electrical coupling between cells (21, 22, 25, 29, 30, 31). Nevertheless, it remains unclear where the precise site of gap junctional blockade is located on the connexin (25).

To determine the actual site of action of the gap junction blockade and to analyse whether a gap junction blockade at other parts of the nervous system might have influence the results were beyond the scope of this study but will be addressed in future studies. For example, we expected to see changes in heartbeat frequency after gap junction blockers were applied, but it did not change significantly in the concentration range we have used. Therefore, we assume that higher concentration will be needed to change the frequency of the heartbeat.

Conclusion

Electrical coupling between neurons in the respiratory network is compulsory for physiological respiratory rhythm generation. 18-β-GA as well as 18-α-GA changed PN discharge frequency (i.e., respiratory rhythm) due to the blockade of gap junctional proteins. Respiratory rhythm is modulated but not stopped; therefore, we assume that under this condition synaptic transmission is driving the respiratory network.

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EFFECTIVENESS OF PRANAYAMA ON THE LEVELS OF SERUM PROTEIN THIOLS AND GLUTATHIONE IN BREAST CANCER PATIENTS UNDERGOING RADIATION THERAPY : A RANDOMIZED CONTROLLED TRIAL

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Abstract : The effect of Pranayama on the levels of protein thiols and glutathione was studied among breast cancer patients receiving radiation therapy. 160 patients were randomised into experimental and control group using block randomisation. The experimental group received fractionated radiation for five days a week and performed Pranayama (Nadishodhana, Brahmari and Sheethali) for 30 minutes twice daily for five days a week. The control group received only radiation. Blood samples were collected from both the groups at the end of six weeks of radiation therapy and analysed for the levels of serum protein thiols and glutathione. An independent sample 't' test showed a significant difference in the level of serum protein thiols between the two groups (t = 4.43 p 0.001). A Mann-Whitney U test showed a significant difference (z = –3.07 p 0.002) in the

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level of glutathione as well. These Pranayama techniques improve the antioxidant status of breast cancer patients receiving radiation therapy.

Key words : protein thiols glutathione pranayama radiation therapy

INTRODUCTION

Breast cancer is a very commonly occurring cancer among women in the metropolitan cities of India. It is fast replacing cervical cancer as the leading cause of cancer among women (1). Breast cancer is treated with surgery, chemotherapy, radiation therapy and hormone therapy depending on the histological stage and other particulars of the patient. Radiation therapy is employed usually to prevent local recurrence of cancer after doing surgery and chemotherapy. Radiation therapy causes DNA strand breakdown to kill the cancer cells. In the process, normal cells are also affected by DNA damage (2).

Radiation therapy is known to reduce antioxidants in the body. Protein thiols are believed to have a role in the DNA repair thus reducing the side effects of these therapies (3, 4). Glutathione is a tripeptide, L-γ-glutamyl-L-cysteinylglycine, present in high concentrations in most cell types. Glutathione donates hydrogen ion and unpaired electron to neutralize peroxides and free radicals. In this process, glutathione gets oxidised to glutathione disulphide (5).

Pranayama literally means the control of breath. Pranayama has been an ancient technique practiced by the Yogis for the spiritual growth. Many scientific studies have proven that these breathing techniques improve human heath by maintaining a physiological balance and it affects many systems of the body. Pranayama has also been shown to improve the antioxidant level in healthy people (6, 7).

This study assessed the effectiveness of Pranayama (Yogic breathing techniques) on the levels of protein thiols and glutathione among breast cancer patients receiving radiation therapy. In this study, by Pranayama we mean three breathing techniques called Nadishodhana Pranayama, Sheethali Pranayama and Brahmari Pranayama.

MATERIALS AND METHODS

Patients

The study was conducted after obtaining institutional ethical committee clearance. The study group consisted of a total of 160 patients which included both the control and the experimental group. The patients were allocated into experimental and control group using block randomization procedure (sixteen blocks of ten patients) after obtaining informed consent. Patients with any psychiatric disorder, who have not undergone any surgical treatment for their breast cancer, those with extreme mobility issues (e.g., unable to get in and out of a chair unassisted), those who have practiced yoga or taken yoga classes prior to diagnosis, those diagnosed with lymphedema at baseline and those with recurrent breast cancer were

excluded to ensure a more homogenous sample. Patients were not receiving any vitamin supplements or any other antioxidant supplements during the study period.

Procedure of pranayama

The patients in the experimental group performed Pranayama (Sheethali, Brahmari and Nadisodhana Pranayama) along with radiotherapy whereas patients in the control group received radiotherapy only. Experimental group of patients performed Pranayama, morning and evening for 5 days a week for 6 weeks (from the day of starting radiotherapy till the last day of radiotherapy). Patients performed Nadisodhana for approximately 5 minutes (21–25 cycles), Sheethali for approximately 5 minutes (50–60 cycles) and Brahmari for approximately 8 minutes (10 cycles). The initial sessions on Pranayama were given in the Yoga department for one week. The patients performed Pranayama morning and evening for the next 5 weeks in a separate room in the hospital under the supervision of a co-investigator.

Patients who were having locally advanced breast cancer and who underwent Modified radical mastectomy or Breast conserving surgery, followed by 8 cycles of chemotherapy [Doxorubicin 60 mg/m^2 IV d1 (Cyclophosphamide 600 mg/m^2 d1) 3 weekly * 4 cycles Followed by Paclitaxel 175 mg/m2 IV 3 weekly * 4 cycles] were enrolled in this study. After chemotherapy, patients were given radiation of 50 Gy in divided doses. Patients performed pranayama on same days when they came for radiation therapy. Blood was collected in red coloured vacoutainers for serum sample and purple vacoutainers with EDTA for packed cell, from both the group at the completion of radiation therapy and analysed for serum protein thiols and glutathione. Pre-test for these enzymes were done in a sample of 80 patients before starting radiation therapy.

Chemicals

5, 5'dithio-bis (2-nitrobenzoic acid) (DTNB) was obtained from Sigma chemicals, St. Louis, MO, USA. All other reagents were of chemical grade. Spectrophotometer Genesys 10 uv was used for analysis.

Procedure for serum anti-oxidant enzymes

Preparation of serum

Blood samples were collected in plain vacoutainers without EDTA and the sample was allowed to stand at room temperature for half an hour and centrifuged at 3000 rpm for 10 minutes in refrigerator centrifuge and serum was separated.

Protein thiols

Thiols are compounds that contain carbon bonded sulfhydryl group. Protein thiols in the plasma include the protein sulfhydryl groups and protein mixed disulphides with homocysteine, cysteinylglycine, cysteine and glutathione. Most of the cytosolic thiol groups are maintained in their reduced state by a variety of pathways. During oxidative stress, these protein thiols get oxidised mainly by the formation of disulphide bonds in the plasma. Thus protein thiols play a major role in antioxidant defences (8).

Protein thiols can be measured with a spectrophotometric method using dithionitrobenzene. The spectrophotometric assay for protein thiols is based on the reaction of 5,52-Dithiobis (2-nitrobenzoic acid) (DTNB) or Ellman's reagent with protein thiols. DTNB combines with accessible thiol group in proteins and reduces to stable intermediate compound of mixed disulfide, protein S-S aromatic compound. The reduced product of DTNB is 5 mercapto -2-nitrobenzoate. There is formation of yellow colour due to liberated p-nitrothiophenol anion, whose absorbance is measured at 412 nm after 5 mins.

Procedure for protein thiol estimation

Sample blank

In a test tube 920 µl of 0.2 M disodium hydrogen phosphate containing 2 mM disodium EDTA is taken and 100 µl of serum is added. The contents are mixed and optical density was taken at 412 nm wavelength exactly after 5 mins.

Test

In a test tube, 900 µl of 0.2 M disodium hydrogen phosphate containing 2 mM disodium EDTA, 100 µl of serum and 20 µl of 10 mM DTNB are added. The contents are mixed and optical density was taken at 412 nm wavelength exactly after 5 mins.

Reagent blank

In a test tube, 1000 µl of 0.2 M disodium hydrogen phosphate containing 2 mM disodium EDTA and 20 µl of 10 mM DTNB are added. The contents are mixed and optical density was taken at 412 nm wavelength exactly after 5 mins.

Sample blank was measured because bilirubin, β-carotene, and other plasma constituents that absorb at 412 nm can interfere with protein thiol measurement. The absorbance for sample and reagent blanks was subtracted from serum absorbance values to obtain the corrected values (9).

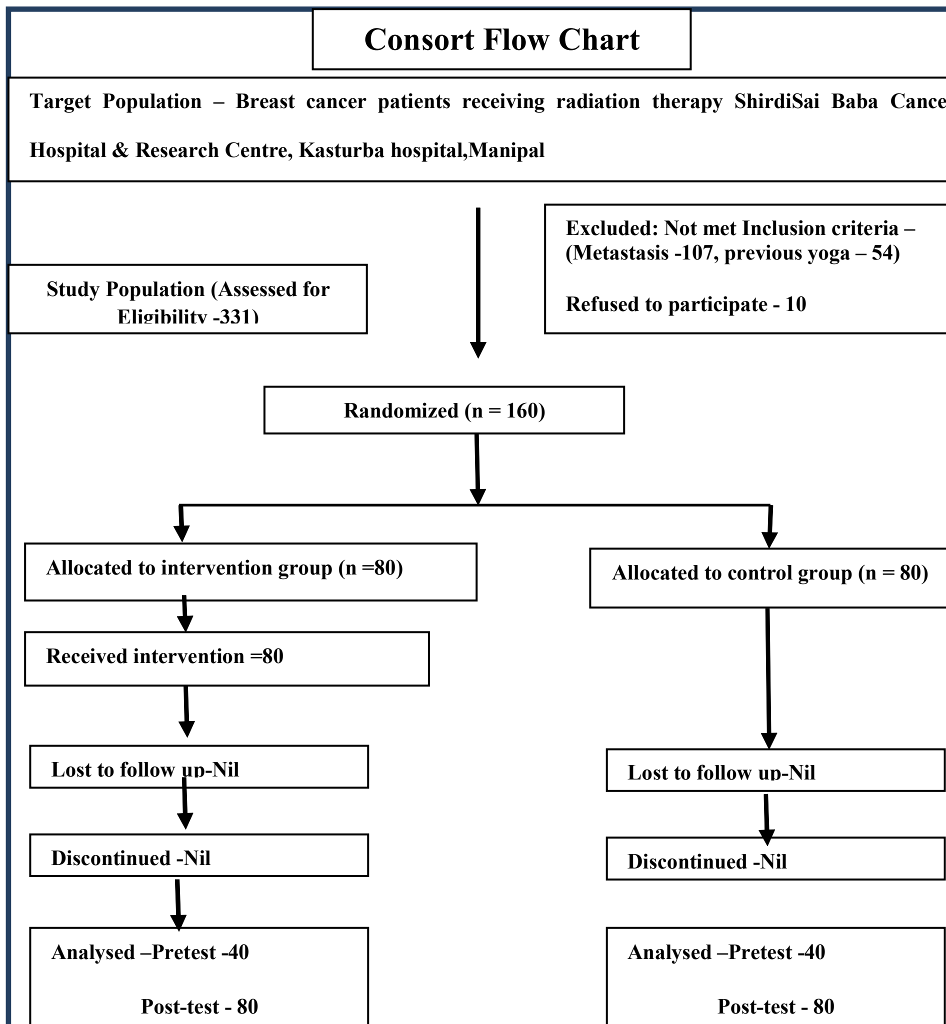
Preparation of packed cell

Blood samples were collected in EDTA bottle from patients. Blood was centrifuged at 3000 rpm for 10 minutes in refrigerator centrifuge without delay. Plasma was separated; buffy coat was carefully removed and separated, erythrocytes were washed thrice with cold normal saline. The haemoglobin content of the erythrocytes was determined by cyanmethemoglobin method. Erythrocytes enzymes were estimated in appropriately diluted haemolysates prepared by addition of distilled water.

Procedure for glutathione estimation

Glutathione, a tripeptide, γ-glutamylcysteinyl glycine, is the most abundant non-protein thiol in mammalian cells. Glutathione exists in two forms; the thiol-reduced (GSH) and disulphide oxidized (GSSG). Glutathione plays an important role in the detoxification of xenobiotic compounds and in the anti-oxidation of reactive oxygen species and free radicals (10).

1.810 ml of distilled water is added to 0.2 ml of the haemolysate. 0.01 ml of lysate is added to 3 ml of ferricyanide-cyanide



reagent (Drabkin's reagent). Reading is taken within 3 minutes at 540nm for hemoglobin estimation. Three ml of precipitating solution is added to the remaining haemolysate. After standing for 10 minutes, the mixture is filtered through a medium or coarse grade of filter paper. One milliliter of filtrate and 0.5 ml of DTNB is added to 4 ml of 0.3 M Na_2 HPO_4 solutions. It is read at 412 nm against a blank prepared by adding one ml of distilled water and 0.5 ml of DTNB to 4 ml of 0.3 M Na_2 HPO_4. (11).

Statistical analysis

Data were analyzed using SPSS package (version 16). Pretest and post test levels of serum protein thiols are expressed as mean and standard deviation. Since data were following normality, for comparison of serum protein thiol values between the experimental group and control group, an independent 't' test was used. The data for glutathione was compared using Mann Whitney U test as it was not following

normality. Statistical significance was fixed at p = 0.05.

RESULTS

Sample characteristics

The characteristics of the participants have been summarized in Table I. Data in the table show that majority of the women (140 out of 160) had locally advanced breast cancer and undergone modified radical mastectomy (123 out of 160) as the surgical treatment.

TABLE I: Sample Characteristics.

Sl. No.	*Experimental (n=80)*	*Control (n=80)*
Age in years		
≤ 45	46	35
> 45	34	45
Stages of breast cancer		
Stage 1	12	8
Stage 2	38	40
Stage3	30	32
Haemoglobin levels		
≤ 12	50	58
> 12	30	22
Surgery		
Modified radical mastectomy	63	60
Breast conservation	17	20

Comparison of serum protein thiols between the control group and the experimental group

There was no significant difference in the level of protein thiols between the experimental group and the control group at the beginning of radiation therapy. The serum concentration of protein thiols in the experimental group was 235.83±74.60 µmol/lit and in the control group was 213.20±88.03 µmol/lit with a p value of 0.206. Since the data were following normality, an independent 't' test was used to compare the mean difference of protein thiols between the control group and the experimental group. During the post test, i.e., at the end of radiation therapy, the serum concentration of protein thiols was significantly higher in the group who performed Pranayama (271.20 ±91.28 ìmol/lit) than in the control group (216.13±62.86 µmol/lit) (p 0.001). The patients who performed Pranayama along with radiation therapy had higher levels of serum protein thiols at the end of radiation therapy. As mentioned earlier, protein thiols get oxidised mainly by the formation of disulphide bonds in the plasma during oxidative stress. The elevated levels of protein thiols in the experimental group indicate that Pranayama influences the

TABLE II: Comparison of the mean values of serum protein thiols (µmol/lit) between experimental group and control group at the beginning of radiation therapy.

Groups (80)	*Mean±SD*	*'t' value*	*95% Confidence interval of the difference*	*P-value*
Experimental (40)	235.83±74.60	1.274	–12.70 to 57.95	0.206
Control (40)	213.20±88.03			

µmol/lit–micromoles per litre.

TABLE III: Comparison of the mean values of serum protein thiols (µmol/lit) between experimental group and control group at the end of radiation therapy.

Groups (160)	*Mean±SD*	*'t' value*	*95% Confidence interval of the difference*	*P-value*
Experimental (80)	271.20±91.28	4.43	30.53 to 79.60	0.001
Control (80)	216.13±62.86			

µmol/lit–micromoles per litre.

formation of serum protein thiols and reduces the toxicities to normal tissues related to radiation therapy.

Comparison of glutathione between the control group and the experimental group

With regard to glutathione, there was no statistically significant difference in the level of glutathione between the experimental group (26.61±15.99 mg/g of Hb) and the control group (27.03±11.63 mg/g of Hb) before starting the radiation therapy (p 0.857). Since the data were not following normality, a Mann Whitney U test was done to assess the difference between the two groups with regard to the level of glutathione. At the end of radiation therapy, the mean value of glutathione for the experimental group was (26.14±10.46 mg/g of Hb) which is higher than that of the control group (21.06±6.06 mg/g of Hb) (p 0.002). This also may probably indicate a less oxidative stress in breast cancer patients performing Pranayama when undergoing radiation therapy.

TABLE IV: Comparison of the median values of glutathione (mg/gHb) between experimental group and control group at the beginning of radiation therapy - Mann Whitney U test.

Groups (80)	*Inter quartile range*	*Median*	*Z value*	*p-value*
Experimental (40)	18.24 to 30.2	24.4	–0.18	0.857
Control (40)	19.89 to 33.97	22.38		

TABLE V: Comparison of the median values of glutathione (mg/gHb) between experimental group and control group at the end of radiation therapy - Mann Whitney U test.

Groups (160)	*Inter quartile range*	*Median*	*Z value*	*p-value*
Experimental (80)	18.31 to 30.55	24.21	–3.07	0.002
Control (80)	18.02 to 24.6	19.1		

DISCUSSION

Radiation therapy acts by two different methods to kill the cancer cells. Radiation causes DNA strand breakage either directly or indirectly by generating free radicals. One of the important radiation induced free radical species is the hydroxyl radical which results in the generation of other species of free radicals causing oxidative stress (12). Protein thiols are targets of oxidative stress. During oxidative stress, the protein thiols which are plasma sulfhydryl groups associated with protein get converted to disulphides resulting in a fall in their levels and antioxidant activity (8).

In the present study, experimental group of patients performed Pranayama, morning and evening for 5 days a week for 6 weeks (from the day of starting radiotherapy till the last day of radiotherapy). Patients performed Nadisodhana for approximately 5 minutes (21–25 cycles), Sheethali for approximately 5 minutes (50–60 cycles) and Brahmari for approximately 8 minutes (10 cycles). The levels of glutathione (GSH) and protein thiols two important antioxidants were significantly higher in patients with breast cancer undergoing radiation therapy and practising Pranayama, when compared to controls (breast cancer patients undergoing chemotherapy but not practising pranayama). The higher GSH and protein thiols may be protective for these patients in reducing the toxicities caused by radiation therapy.

Published studies to compare the antioxidant effects of Pranayama on breast cancer patients were not available. However, Sinha S et. al reported similar findings in a study conducted among the healthy male volunteers of Indian Navy. There were 30

volunteers in the experimental group who practised yoga, pranayama and meditation for one hour in the morning, five days a week for six months. There was a significant increase in the total antioxidant status and glutathione in healthy male volunteers from Navy who practised yoga and pranayama at the end of six months (13). Further Yadav et al. in their study concluded that yoga–based life style modification program was effective in reducing oxidative stress among a group of healthy volunteers and chronic patients (14). The present study confirms the results of these available studies.

As mentioned earlier, radiation therapy is known to induce free radicals in an effort to kill cancer cells. The exact physiological mechanism of Pranayama on antioxidants is not clear. It is encouraging to note that the level of protein thiols and glutathione, two important antioxidants have increased among this population in spite of the free radical injury. The elevated levels of protein thiols and glutathione may help in relieving the toxicities associated with radiation treatment among breast cancer patients. To understand the exact physiological mechanism of Pranayama on antioxidants, genetic studies on larger samples may be required. With the findings of the present study, it may be concluded that Pranayama may be employed as a supportive treatment to breast cancer patients undergoing radiation therapy.

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A STUDY OF PUPIL CYCLE TIME (PCT) IN ALTERED STATE OF NUTRITION IN INDIAN MALE

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Abstract : Pupil cycle time (PCT) is a simple way to measure the parasympathetic activity in the eye. This is a sensitive and specific test for the study of parasympathetic function. The cardiac parasympathetic tone is a good indicator to predict morbidity and mortality in an individual. The PCT is correlated with cardiac parasympathetic tone. In the present study we have aimed to look at the PCT in different nutritional status in human subjects. Sixty-three healthy adult male volunteers in the age group of 18–50 yrs were studied. They were divided in three groups based on their BMIs as undernourished, normal and preobese/obese. The mid-arm circumference (MAC) and waist- hip ratio (W:H) are incorporated as adjuvant to strengthen that they are divided into three different nutritional groups. The PCTs of undernourished (818±145 ms), normal control (904±63 ms) and overweight (991±106 ms) is expressed as mean and SD ($P<0.001$). A linear regression analysis was performed taking BMI, MAC, W:H and PCT as parameters, which shows a positive linear correlation. The results showed that the PNS activity in the eye is greatly modulated in different states of nutrition. Pupil cycle time is a simple, noninvasive investigative tool to assess and differentiate the PNS function in obese and chronic undernourished subjects.

Key words : autonomic function, pupil cycle time, body mass index, nutrition, mid arm circumference, waist hip ratio

INTRODUCTION

Nutritional health is maintained by a state of equilibrium when there is a balance between energy intake and expenditure. Loss of this equilibrium can lead to undernutrition i.e. negative energy balance (1, 2) and can/ or result in obesity i.e. positive energy balance (3, 4, 5, 6). This alteration in the energy balance which leads to malnutrition can cause changes in sympathetic (7) as well as parasympathetic activity in animals and human beings (8). Changes in the parasympathetic nervous system are evidenced by a decrease in Acetylcholinesterase in tissues (9) and cardiovascular parasympathetic

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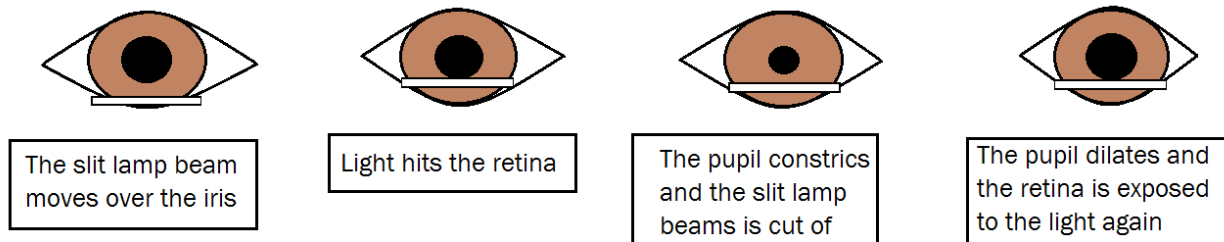

tone is increased in energy deficient subjects (10). Alteration of activity of the parasympathetic system has also been reported in obese humans (11).

The most commonly used tests for autonomic function are tests of cardiovascular system, which are well established (12). Another organ system that is tested for autonomic dysfunction is the eye. In the eye, pupil cycle time (PCT) is a sensitive measure of dysfunction of the parasympathetic efferent limb of the papillary light reflex arc (13) e.g. leprosy, diabetes etc. PCT is measured using slit-lamp that induces persistent oscillations of the pupil of the eye.

Autonomic dysfunction may be present in the eye without any clinical sign as has been shown in diseases affecting the eye e.g. Hansen disease and diabetes (14, 15). In 1990, Lanting et al showed that pupillary parasympathetic dysfunction occurred earlier than cardiovascular system when he considered darkness pupil diameter and pupil latency as ophthalmologic tests and response to standing and response to deep breathing as parasympathetic cardiovascular tests (13, 16). Martyn et al correlated well with the cardiac autonomic function test and opined that compared to CVS tests PCT is 59% sensitive and 70% specific (17).

It is well known that the nutritional status is a determinant of autonomic function may be an adaptation to the nutritional status of a subject and sympathetic tone is affected by the nutritional status (7, 17). Though pupillary oscillation is studied before using oscillometer and various drugs affecting ANS in the eye to establish parasympathetic dysfunction in obesity but PCT was not used before to investigate the effects of nutritional status, especially in Indian male subjects.

MATERIALS AND METHODS

Sixty-three males between the ages of 18 and 50 years were studied. They were recruited from general population and male staffs and students of St. Johns' Medical College. A brief history, including a system review and clinical examination was performed. The subjects who had history of diabetes, hypertension, hypotension, asthma, other cardiac and ophthalmologic diseases like cataract, optic neuritis, on chronic medication were excluded from the study. All of them were non-smoker, non-alcoholic and weight stable: those with a noticeable weight gain or loss over the preceding six months were also excluded (10). This was judged by taking detailed past history. They were instructed to have their last meal at 8 pm, the night before and avoid caffeinated

beverages for 12 hours prior to the study. Detail of the experimental procedure was outlined to the participants and an informed written consent was obtained. The ethics committee of St. Johns' Medical College approved the study.

Anthropometric measurements

Body mass index (BMI): Body weight of the subjects was measured to the nearest 0.001 kg using a sensitive electronic scale (Fitness, Edryl, India) and height was measured using a stadiometer (Nivostise Brivete Depose). Body mass index (BMI) was calculated using Quetlet's index (weight in kilogram/height in meter squared).

Now, Sixty – three healthy male subjects were divided in three groups according to their BMI as undernourished (N=22, BMI<18.5 kg/m^2), normal (N=21, BMI = 18.5 kg/m^2–24.9 kg/m^2) and preobese/obese (N=20, BMI > 24.9 kg/m^2).

Mid arm Circumference (MAC)

Mid arm circumference (MAC) was measured in the right arm. Firstly measurement was taken from tip of the coracoid process to the tip of the olecrenon process as the anatomical landmark and the midpoint was decided on the lateral side. Mid arm circumference was measured on the midpoint around the arm by a simple measuring tape (18).

Waist Hip ratio (W:H)

The midpoint was selected between lowest point of the costal cartilage and the highest point of the iliac crest on the midaxillary line in standing position in the right side (18). The circumference was measured around the point by a simple measuring tape. Hip was considered as the maximum bulge over the gluteus maximus muscle at the levels of the trochanters. The ratio between these two is calculated as W:H.

Mid arm circumference and W:H was measured to differentiate the nutritional status more accurately to avoid the bias of regional or uneven distribution of body fat as they are considered as adjuvant to assess malnutrition.

Slit-lamp

A Haggstreit-type of slit-lamp was used to measure the PCT, in the Department of Ophthalmology at St. Johns' Medical College Hospital. The same lamp was used for all the subjects studied. The subjects placed their chin on the chin rest. The height of the chin-rest from the base of the table is adjusted in order to view the eye adequately for all the subjects with different heights. The slit beam was horizontal axis and a thickness of 1mm as depicted in the picture below. The white diffuse horizontal light was used.

Measurement of pupil cycle time

The subject was made to sit in a dimly lit room and stopwatch with a least count of 1/1000. Pupil cycle time was measured in a dimly lit room to get maximum dilatation of pupil before starting of the study. The subjects are asked to gaze at a fixed point to

get accurate measurement. A horizontal beam of light of width of 1 mm was projected on the edge of the pupil. As soon as the light falls, the pupil constricts and again dilates to original position; when it dilates the beam of light again hit the pupil and pupil constricted. The time the pupil takes to constrict and dilate once is measured using a 'timed counter' with accuracy of 0.001 secs. The time taken for 90 cycles in 3 sittings with a gap of 15 minutes between them and PCT is calculated.

The stopwatch has an oscillator of 1 kHz, which gives an accuracy of 1 millisecond. The counter started the stopwatch at the beginning and at the end of the preselected number of cycles; which reduced human error to a minimum. The PCT is measured in both the eyes and the longer cycle is considered (measurements is obtained in both the eyes). The PCT which was of longer duration was in the two eyes were taken into consideration for analysis (20). Data is rejected if there was blinking more than 2 times in one sitting or too much of watering in the eye due to intensity of light.

Statistical analysis

Data were expressed as mean±SD (Table I) only. Fig. 1 depicts the true value of BMI and PCT in various data points and $P<0.05$ as criteria for statistical analysis with 95% confidence interval (Fig. 2, 3, 4). Statistical analysis is performed by linear regression analysis.

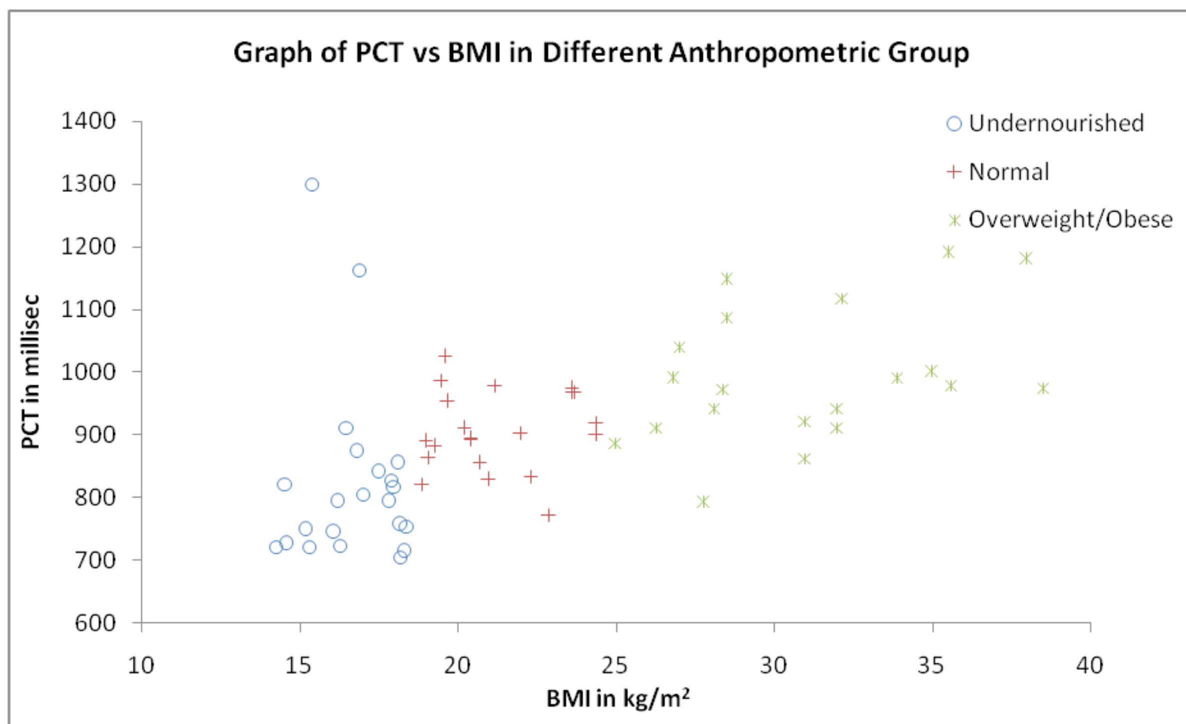

Fig. 1 : Graph showing the true values of PCTs and BMIs.

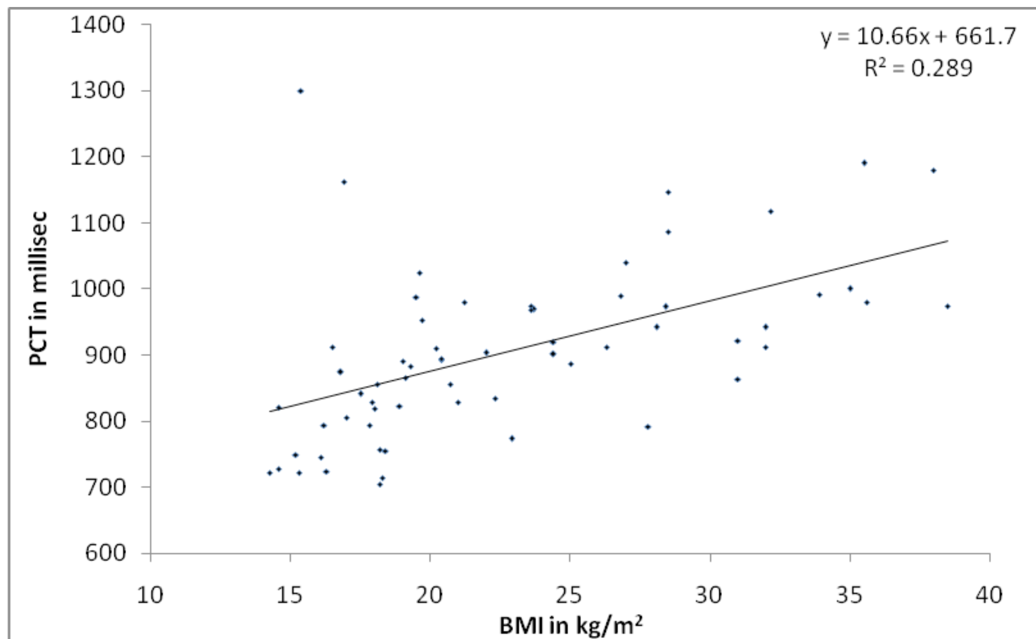

Fig 2 : A graph of statistical fitment is carried out through linear regression analysis to establish existence of linear relation between BMI and PCT in all three groups namely undernourished (BMI <18.5 kg/m²), normal (18.5-24.9 kg/m²) overweight/obese (24.9 kg/m²).

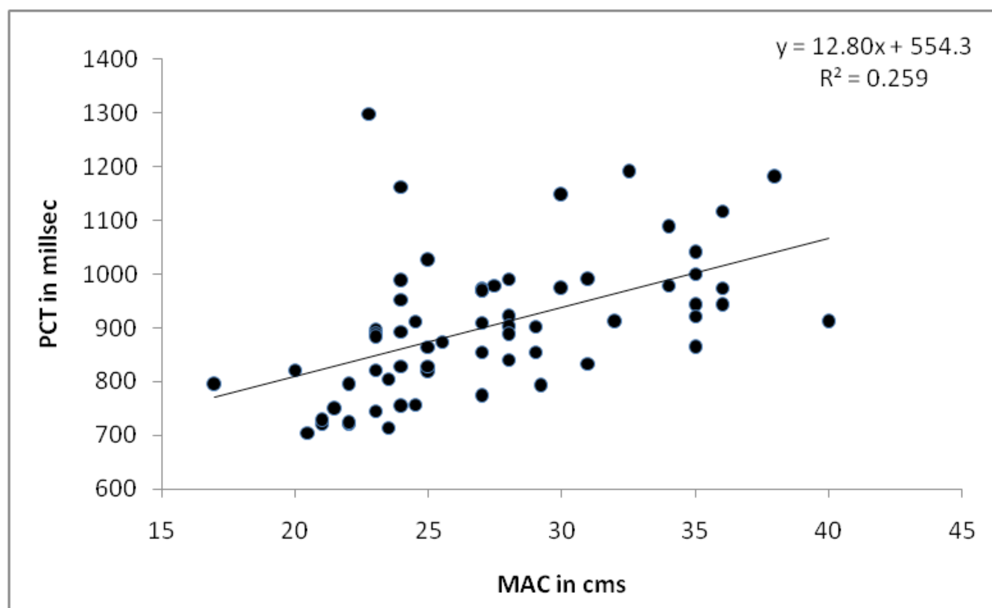

Fig 3 : A graph of statistical fitment is carried out through linear regression analysis to establish existence of linear relation between MAC and PCT in all three groups namely undernourished (23±2.61 cm), normal (25.8±2.20 cm) overweight/obese (33.4±3.39 cm).

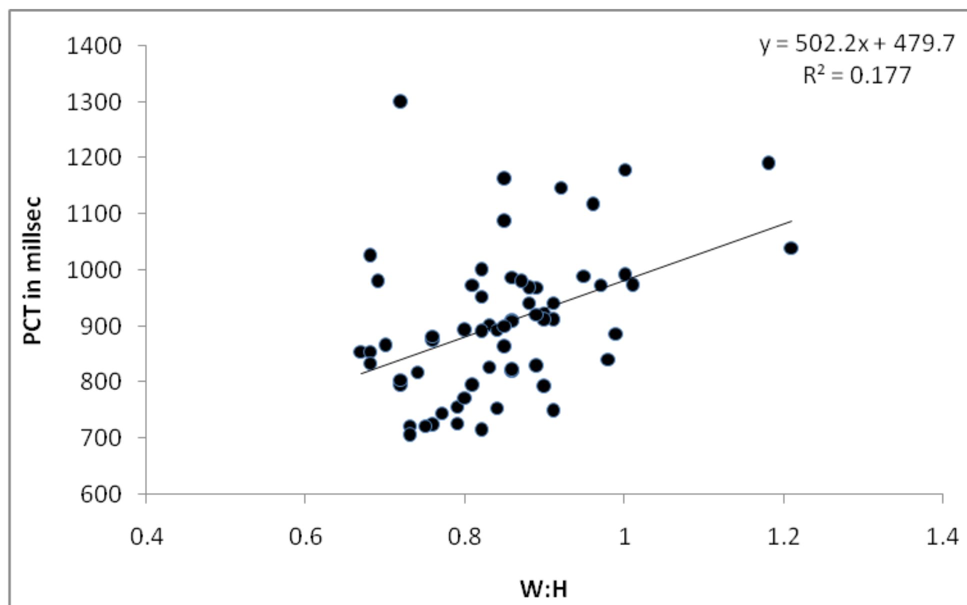

Fig 4 : A graph of statistical fitment is carried out through linear regression analysis to establish existence of linear relation between W:H and PCT in all three groups namely undernourished (o.79±0.07), normal (0.80±0.07) overweight/obese (0.94±0.1).

RESULTS

TABLE I : Subject characteristic of the three study groups with pct (Data is expressed as mean±SD).

Subject characteristic	*Under-nourished (n=22)*	*Well-nourished (normal, n=21)*	*Overweight/ Obese (n=20)*
Age (yr)	26±7.60	33±9.30	27±8.70
Weight (kg)	44.9±4.87	59.63±7.46	88.7±12.10
Height (M)	1.63±0.05	1.67±0.06	1.68±0.47
BMI (kg/M²)	16.7±0.02	21.2±1.83	31±4.00
MAC (cm)	23.1±2.61	25.8±2.20	33.4±3.39
Waist : Hip	0.79±0.07	0.80±0.07	0.94±0.10
PCT (milli sec)	818±145	904±63	991±101

DISCUSSION

In this cross sectional study we found that the PCT is directly and positively correlated with BMI; that means there is an increase in the duration of PCT with increase of BMI (16). In 1986, functional correlates of PCT were studied by Martyn and Ewing (19). Miller and Thompson were among the first to investigate the method of PCT in 1978 (20). They incorporated a method that we have followed closely in our study. We have used more accurate timer which can measure the duration with 0.001-second accuracy against 0.01-second used by them. Our study required 2 minutes as against 5 minutes taken by the earlier studies. Thus the ambient light is maintained in the same subject and we have conducted the study in same time (24). In 1993, again interest in PCT has also been raised by the exploration of the hypothesis that open angle glaucoma (OAG) has an autonomic neuropathy component which also investigated the cardiovascular autonomic function and found that there was presence of prolonged PCT in patients with OAG compared to normal

people without OAG and that is comparable with the cardiovascular autonomic function test (22). Using this approach, PCT measurements have since been made in wide variety of optic neuropathies (optic neuritis, compressive optic neuropathy, glaucoma, atrophic papilloedema, traumatic optic neuropathy, and ischaemic optic neuropathy) (24). So, we have taken care to choose the subject devoid of any of these diseases.

It is well known that quantifying the autonomic function is very difficult though lots of autonomic function tests are available. In contrast to that, PCT is a quantifiable test and can be conducted easily if some precautions can be taken as other CVS tests to investigate undernutrition (9). In the present study, we have taken measures, as far as possible, that minimal confounding factor would not interfere with the result. The physiological factor such as physical activity (ongoing study), gender (21) age (23) and pathological, such as diseases affecting autonomic nervous system, can affect PCT also. The PCT represent the only parasympathetic component of the autonomic nervous system in the eye. It was evidenced by blocked study or the sympathetic stimulation does not affect the PCT by Martyn et al (18). When the PCT increases the parasympathetic tone in the eye decreases and opposite happens when PCT decreases. As PCT is well correlated with the other autonomic function tests and we concluded that the ocular parasympathetic tone is increased in undernourished state and decreased in obesity.

As PCT is a quantitative index it would definitely assess the parasympathetic change and its extent in altered state of nutrition as other studies suggested (10). We have observed though two of the subjects were undernourished but they have very long PCT of 1162 millisecs and 1299 millisecs. This is considered long in terms of normal PCT which ranges from 800 millisecs – 900 millisecs (21). Though they are outliers we have included those data in the study. Except the fact that they were over 40 years of age they are fit to be included otherwise. The PCT becomes longer after 50 years of age (23) and age range of our study is 18-50 years. As Bremmer FD suggests that the measurement of pupil cycle time is the only clinical test that does not rely on comparison with the fellow eye, but it can only be measured in mild to moderate optic nerve dysfunction (24). But then also optic neuropathy can occur in chronic undernutrition. So, we have chosen carefully the subjects those who do not have the mildest form of optic neuritis. The caveat of the study may be, we did not consider the pupil size to measure the 'edge-light pupil cycle time' as described by Howarth et al (25). But, they have recruited only 22 subjects (10 male, 12 female) and intra-individual variation can occur especially in females. In female, there is a difference in PCT values in different phases of menstrual cycle as studied by Moodithaya et al. That is another reason that we have chosen the male subjects only to investigate the effect of nutrition on pupil cycle time (21). Our data was collected from the same setting and laboratory and same technique is quoted (25).

We could not depend on any available data, which had investigated directly the nutritional status by this simple but specific test. We have observed in our study, PCT is

reduced in undernourished subjects (818±145 ms) whose BMI ranged from 14.26 kg/m^2 to 17.5 kg/m^2 (average 16.7 kg/m^2) compared to normal with BMI range of 18.5 kg/m^2 to 24.9 kg/m^2 (average 21.2 kg/m^2). On contrary to that fact, PCT of the preobese/obese subject has increased with a BMI range of 25 kg/m^2 to 38 kg/m^2 (average being 31 kg/m^2)(Fig. 1). Through linear regression analysis we got that a fitment line can be drawn easily with BMI and PCT as parameter and they are positively correlated with $R^2 = 0.29$ (Fig. 2). In order to reduce the error of misclassifying individuals as undernourished or overweight/ obese the assessment of mid-arm circumference and waist-hip ratio was carried out. The cut-offs for MAC in men is 24 cm which is considered normal (3). In our cross sectional study we have the MAC value averaging 23.1±2.61 cm for the undernourished, 25.8±2.20 cm for the normal, and 33.4±3.39 cm for the overweight/obese subjects. The MAC value can change dramatically also within small range of change of BMI (5). A regression analysis is run with MAC and PCT as parameter and a linear positive correlation is obtained with $R^2 = 0.26$ and a fitment is drawn easily (Fig. 3).

Abdominal fat mass can vary dramatically within narrow range of body fat or BMI. Our data suggest that there is an increase of W:H with increase of BMI with a result of undernourished 0.79±0.07, normal0.80±0.07 and preobese/obese 0.94±0.1 which is directly correlated with cardiovascular risk factor where the cardiac parasympathetic activity is altered (5, 6). A linear fitment can be drawn easily with the W:H and PCT as parameter with $R^2 = 0.18$ which proves the linear positive correlation between the two (Fig. 4).

Summary and conclusion

In conclusion, the present finding demonstrate that in obesity, the ocular parasympathetic tone is decreased and there is an enhanced tone in undernourished people and this increase follows a steady rate supported by data for the first time in Indian males. As this result is corroborating the finding of cardiac parasympathetic tone, measurement of pupil cycle time can be tested as parasympathetic alteration in the eye in altered state of nutrition although further study is required to compare both of them in these particular conditions.

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The authors would like to acknowledge staffs of the Department of Ophthalmology of St. Johns' National Academy of Health Sciences for providing us slit-lamp, primarily used for the study. We also like to thank Dr. K.N. Maruthy of the Department of Physiology to develop cost-effective accurate timed counter with stopwatch for the present study and Biostatistician Dr. Rashi Gupta of ICGEB for statistical feedback.

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DIURNAL VARIATION IN PERFORMANCE BY ORCHESTRAL VIOLINISTS – PILOT STUDY

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Abstract : Human physiological parameters and performance depend on circadian rhythm. However, no information exists about diurnal variation of musicians' performance characteristics. In a pilot study 6 professional violinists (aged 38–57 years) presented a standard piece of music (Johann Sebastian Bach, Partita Nr. 2 a-moll, BWV 1004, 4. Satz „Gigure") and were assessed for body temperature, vital signs and musical performance criteria at 8.00 H, 12.00 H, 16.00 H and 20.00 H. There was no uniform variation but artistic presentation appear to have an optimum between 12.00 and 16.00 H, sound instability being most pronounced in the morning hours.

Key words : diurnal variation performance characteristics musician violinist

INTRODUCTION

Circadian rhythm influences physiological functions and performance of humans substantially 1, 2, 3. There is no unequivocal peak of physical performance, but coordination, reaction time or vigilance appear to have acrophases around 16.00 H. Heretofore, diurnal variation of musicians' performance has not been subject of research and almost no specific data of controlled studies are available with regard to musicians´s performance and presentation. Mulcahy and colleagues (4) monitored circadian heart rate in members of a symphony orchestra and found a substantial influence of environmental factors such as live presentations on the rhythm. In analogy, Monk et al. (5) studied diurnal variation of human performance and found maxima of dexterity during evening hours between 17.00 H and 21 H. Cognition (reasoning speed) revealed a maximum in the late afternoon hours. In general, performance characteristics were closely related to body temperature showing minima at low body temperatures (6). In order to assess circadian rhythms of musicians, the objective of this investigation was to first get data on diurnal variation of performance characteristics in professional violinists and stimulate further research in the field.

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METHOD

Six orchestral violinists (3 male and 3 female, aged 38 to 57 years, median 50; median BW 50 kg, range 19; median height 165.5 cm, range 17) arrived at the study centre at 07.30 H and gave their written informed consent. The study was approved by the ethics committee of the University of Dresden. Standard meals (breakfast, lunch, dinner) were served. At 08.00 H, 12.00 H, 16.00 and 20.00 H temperature (BT), vital signs (BP, HR) were controlled as marker rhythms (BT: Braun Thermoscan ear thermometer; BP/HR: Boso Medicus Family sphygmomanometer). Each volunteer presented the same standard piece of music (Johann Sebastian Bach, Partita Nr. 2 a-moll, BWV 1004, 4. Satz „Gigure") following recording of vital signs. In order to minimize learn effects all participants started to train the presentation 4 weeks before the study.

The tempo (bpm) of the presentation was objectively measured using a metronome (Cubase Studio 4). Agogic, tempo fluctuations, clarity of sounds, dynamics, articulation, expression, technical problems (score: 10-no of problems) and overall artistic presentation were scored by a blinded rater (between 9.00 and 11.00 a. m.) using a visual analogue scale (0 = bad to 10 = excellent) (7). All data were analysed descriptively (mean, SEM) and explorative Friedman ANOVA (Friedman test FT) was calculated (8). In order to estimate relation between body temperature and music characteristics Pearson correlation and cross-correlation techniques were applied (9). All calculations were done using commercially available software (NCSS 2000, NCSS, Kaysville, Utah, USA).

RESULTS

Vital signs (blood pressure, heart rate) showed a marked individual variability with mean maximum values at 08.00 H and 16.00 H. An expected acrophase of body temperature (BT) in the evening hours (16.00 H and 20.00 H) could be ascertained ($P<0.05$). The circadian characteristics of music presentation characteristics and BT are summarized descriptively in Table I. The maximum values of musical performance with regard to artistic presentation (tempo, articulation, dynamics, expression and overall performance)

TABLE I: Diurnal variation of mean values (±SEM) of musical characteristics and Body Temperature (*) p<0.05).

Characteristic	*08.00 H*	*12.00 H*	*16.00 H*	*20.00 H*
Tempo [bpm]	111.3 (2.11)	115.8 (2.11)	110.3 (2.11)	114.3 (2.11)
Fluctuation of tempo [sc]	6.3 (0.74)	5.2 (0.74)	5.4 (0.74)	5.3 (0.74)
Clarity of sounds [sc]	5.3 (0.73)	5.1 (0.73)	4.9 (0.73)	4.5 (0.73)
Articulation [sc]	3.3 (0.34)	3.4 (0.34)	3.8 (0.34)	3.1 (0.34)
Dynamics [sc]	4.6 (0.57)	4.8 (0.57)	4.8 (0.57)	3.7 (0.57)
Expression [sc]	4,2 (0.72)	4.5 (0.72)	4.8 (0.72)	3.8 (0.72)
Agogic [sc]	4.2 (0.66)	4.1 (0.66)	4.1 (0.66)	3.1 (0.66)
Technical problems [sc]	7.3 (0.65)	7.5 (0.65)	7.8 (0.65)	7.7 (0.65)
Overall Presentation [sc]	4.3 (0.66)	4.4 (0.66)	4.7 (0.66)	3.8 (0.66)
Body temperature (°C)*	36.5 (0.12)	36.3 (0.12)	36.7 (0.12)	36.7 (0.12)

showed a maximum at 12.00 H or 16.00 H. However, criteria referring to the stability of the sounds (clarity of sound or fluctuation of tempo [p=0.07, FT] and agogic) were slightly pronounced in the morning at 08.00 H. The error bar plot of diurnal variation of the variable "fluctuation of tempo" with a maxium in the morning is depicted in Figure 1. Technical problems were more frequently observed in the morning.

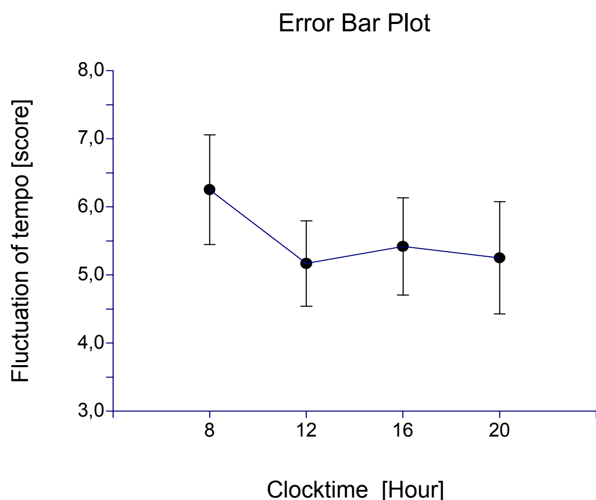

Fig. 1 : Diurnal variation of the parameter "Fluctuation of the tempo" [mean, SEM] which tends to result in a maximum in the morning hours (p=0.07).

Correlation analysis showed associations of presentation characteristics with body temperature, Pearson correlation coefficient – corresponding to a time lag of zero - being most marked for the variable sound clarity (r=0.36, p=0.08), which is given in Figure 2. Agogic, expression and overall presentation were correlated by trend with body temperature between 0.2 and 0.3. Cross-correlation technique revealed the highest correlation coefficient for the parameter expression at a time lag of –1 with 0.49 (P<0.05) with regard to body temperature

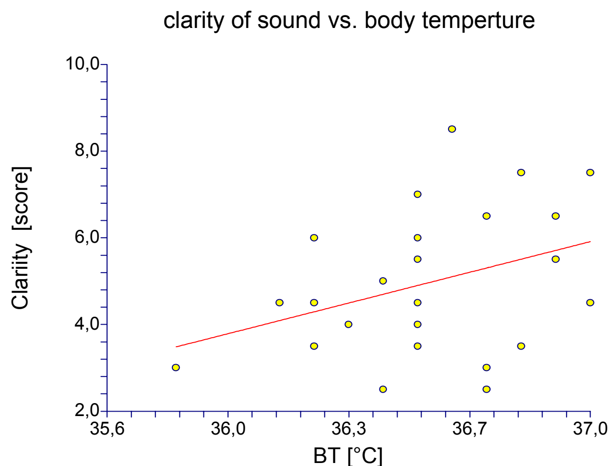

Fig. 2 : Scattergram of "Clarity of sound" versus BT with a positive trend (p=0.08, r=0.36).

(P<0.05) with the corresponding correlogram in Figure 3. All music presentation characteristics apart from technical problems and fluctuation of tempo – no clear cross-correlation - were positively correlated (between 0.3 and 0.45) at time lags between –1 and +1, i. e. with a slight temporal shift.

Conclusion

These preliminary results speak in favour of a differentiated approach of musician's presentation with less stability of sounds in the morning and overall trend to better artistic presentation in the afternoon. Previous studies postulated a correlation between body temperature on the one hand and alertness or performance on the other hand (10). As a result of this pilot study body temperature is also associated with music presentation, i. e. these characteristics appear to be coupled with body temperature. These preliminary results cannot be generalized but are in keeping with the

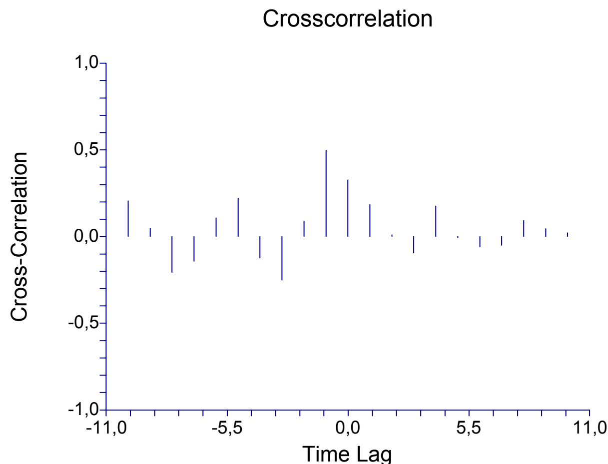

Fig. 3 : Cross-correlation diagram of the parameter "expression" versus BT. Maximum value is found at a time lag of –1 with 0.49.

physical exercise study of Monk et al. (5) that afternoon may be a favourable time span for musical presentation. Due to the pilot conditions of the investigation only a low number of musicians and co-workers were available for the present project. Therefore, further studies with orchestral musicians are necessary to substantiate the results and conclusions.

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CARDIAC AUTONOMIC ACTIVITY IN YOUNG FEMALES WITH PRIMARY DYSMENORRHEA

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Abstract : Various studies have reported the effect of different phases of normal menstrual cycle on the autonomic control over the cardiac activity using heart rate variability (HRV) analysis. However, information on cardiac autonomic activity in young females suffering from primary dysmenorrhea (PD) is scant. Hence, the aim of the present study was to assess the HRV and blood pressure (BP) in different phases of menstrual cycle in women with PD and to compare these findings with eumenorrheic females. Sixty healthy unmarried female subjects (30 females with PD and 30 control subjects), in the age group of 18-25 years, having regular 28-32 days menstrual cycle were recruited for the study. Anthropometric measurements such as height, weight, BMI and WHR were measured. Blood pressure was measured with automatic blood pressure monitor and lead II ECG recordings were done to obtain HRV, during menstrual, follicular and luteal phases of menstrual cycle in both groups. The data recorded were subjected to time and frequency domain analysis. The results showed that dysmenorrheic women had significantly high BMI ($P<0.05$). Analysis of HRV during the different phases of the menstrual cycle between two groups revealed significantly increased ($P<0.01$) mean HR and significantly reduced ($P<0.01$) mean RR and RMSSD in all the 3 examined phases, SDNN significantly less ($P<0.05$) and systolic BP significantly higher ($P<0.001$) during the luteal phase in study group compared to control. Also, we observed a statistically significant decrease in TP and LF ms^2 ($P<0.05$), HF ms^2 & nu ($P<0.01$) in menstrual phase, HF ms^2 ($P<0.05$) and HF nu ($P<0.01$) in luteal phase, while LF nu and LF-HF ratio were found to be significantly higher ($P<0.01$) in menstrual and luteal phases in women with PD compared to control group. This study has shown decreased HRV in the form of increased sympathetic tone and decreased parasympathetic activity in dysmenorrheic women which may be considered an important cardiovascular risk factor.

Key words : heart rate variability; primary dysmenorrhea; parasympathetic activity; sympathetic activity; young females

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INTRODUCTION

Primary dysmenorrhea (PD) is a common gynaecological problem, affecting more than 70% of young women and refers to painful menstrual periods in the absence of any underlying pathology (1). It is characterized by lower abdominal colicky pain which starts with the onset of menstrual flow or a few hours following onset and may last for a few hours up to 2 days. The risk factors for PD are early menarche, long and heavy menstrual flow (2) as well as positive family history (3). Primary dysmenorrhea is seen only in ovulatory cycles usually developing within 6 to 12 months of menarche (4) and peaks in late adolescence and the early 20s (5). The incidence falls with increasing age and with increasing parity (6). Studies suggest that severe menstrual pain is associated with absence from school or work and restricts other activities of daily life (7, 8).

However, the underlying pathophysiological mechanisms of PD remain unknown. Few studies have reported that it may be associated with some degree of autonomic imbalance (9). The sympathetic and parasympathetic branches of the autonomic nervous system (ANS) regulate the activity of the sinoatrial node, the cardiac pacemaker. The beat-to-beat variation in heart rate therefore reflects the time varying influence of the ANS and its components, on cardiac function. Heart rate variability (HRV), a non invasive tool, can assess the balance between sympathetic and parasympathetic regulation on cardiac activity (10). Increased regularity of heart beat activity corresponds to decreased HRV and vice- versa (11). Decreased HRV reflects the increased sympathetic tone or decreased parasympathetic activity and is considered an important cardiovascular risk factor (12).

Various studies have reported greater sympathetic activity in the luteal phase whereas dominant parasympathetic activity in the follicular phase of the menstrual cycle (13, 14, 15). Gonadotropic and ovarian hormones have been known to affect this balance in women (16).

Recent study suggests that primary dysmenorrhea is a significant health problem and may be associated with cardiac arrhythmia especially atrial fibrillation and ventricular arrhythmia (17). Although, many studies have assessed the effect of different phases of menstrual cycle on HRV in eumenorrheic women, there have been very few reports on HRV in dysmenorrheic women. Therefore, the objective of the present study was to assess the HRV in young women suffering from PD and to compare these findings with eumenorrheic women.

MATERIALS AND METHODS

The present study was conducted in the Department of Physiology, Subharti Medical college and associated Chattrapati Shivaji Subharti Hospital, Meerut. The study was approved by the research and ethical committees of the institute. Sixty healthy unmarried female volunteers between the age group of 18-25 years, having regular 28-32 days menstrual cycle for at least last 6 months prior to the study, were recruited from the Subharti University campus and OPD of obstetrics and gynaecology

department. Written informed consent was taken from all the participants prior to the beginning of the study.

The menstrual distress questionnaire (MDQ) was used to assess the physical symptoms associated with the menstrual cycle. The MDQ consisted of 47 items providing eight subcategories of symptoms: pain, concentration, water retention, behavioural changes, autonomic reactions, negative effect, arousal and control (18). All the examined subjects completed the MDQ for the menstrual, follicular and luteal phases of her most recent menstrual cycle. Intensity of pain was assessed by visual analogue scale (VAS). It consists of a horizontal line, 10 cm in length, with the two end points labelled "no pain" and "worst pain". The subject marks on the line at a point that they feel represents their perception of pain. The VAS score in numerical index is determined by measuring the distance in cm from the low end of the line to the point that the subject marks.

The subjects were divided in two groups:

Group I (study) consisting of 30 females suffering from primary dysmenorrhea having VAS score ≥ 4.

Group II consisting of 30 females with few premenstrual symptoms and VAS score ≤ 3 were taken as control.

In the dysmenorrheic group, family history of PD, effect of dysmenorrhoea on daily activity and analgesic requirement was also noted.

Exclusion criteria

After a detailed medical history and thorough clinical examination of the subjects, those with history of secondary dysmenorrhea, premenstrual syndrome, smoking, alcoholism, diabetes, hypertension, thyroid disorders, cardiovascular diseases, taking oral contraceptive pills were excluded from the study.

Experimental protocol

The subjects were asked to report to the research laboratory of the department in the morning between 9-11 AM with following instructions :

i. To avoid food, coffee, tea and strenuous physical activity 2 hours prior to testing

ii. To avoid taking any anti-cholinergic drugs 2 days prior to testing.

Anthropometric measurements

Height was measured by stadiometer to nearest 1 cm and weight by weighing machine (Krups) to the nearest 1 kg with subjects standing without shoes and wearing light clothes. Body mass index (BMI) in kg/m^2 was calculated by Quetelet's index. Circumferences at waist (at the level of umbilicus) and hip (at the level of maximum extension of hips) were measured with a tape measure nearest to 0.1 cm. Waist-Hip ratio (WHR) was calculated.

Recording of blood pressure and HRV

Experiments were done in a quiet room

during which subjects lay supine, awake and breathing normally. Blood pressure and ECG were recorded during the following 3 phases - menstrual phase (M) - 1st to 5th day of bleeding, follicular phase (F) - 6th to 14th day and luteal phase (L) - 15th to 28th day of menstrual cycle. Systolic (SBP) and diastolic blood pressure (DBP) were recorded in the left arm after 10 minute of rest using automatic blood pressure monitor (OMRON HEM -712C, Omron Healthcare, Inc., Illinois, US).

For recording of short term HRV, lead II ECG recordings were done at (25 mm/s & voltage at 10 mm/mV) for 330 seconds to obtain HRV, using data acquisition system, RMS Polyrite D (Chandigarh, India). Recommendation of Task Force on HRV was followed (10).

The ECG signals were converted through a 14-bit A/D converter at a sampling frequency of 256 Hz to PC and were analyzed offline after visual checking of abnormal ECG. High and low filters were set at 99 and 0.1 Hz respectively. The screen sweep speed was kept at 30 mm/sec. HRV software detects the 'R' wave by using tall peak detection algorithm and computes R-R interval. The data recorded was subjected to time domain and frequency domain analysis using the HRV analysis software RMS Polyrite D version 3.0.7 (Chandigarh, India). Frequency domain analysis was performed using non-parametric method of Fast Fourier Transformation.

Time domain indices such as mean RR was measured in seconds, mean heart rate (HR in beats per minute), SDNN (standard deviations of the averages of normal to normal (N-N) intervals), RMSSD (root mean square of differences of successive N-N intervals) and different frequency domain indices such as total power (TP) in absolute values (ms^2), low frequency (LF) component (0.04–0.15 Hz), high frequency (HF) component (0.15–0.4 Hz) in absolute (ms^2) and normalized units (nu) and LF-HF ratio were recorded.

Statistical analysis

SPSS version 11.5 software for windows and Microsoft excel were used for statistical analysis. All values were expressed as mean±SD. One-way analysis of variance (ANOVA) followed by post-hoc Tukey test was used in analysing the data among the phases of menstrual cycle in both groups. Unpaired Student's t-test was used to find out the level of significance between the two groups. $P<0.05$ was considered statistically significant.

RESULTS

General parameters

In the present study, there was no significant difference ($P>0.05$) with respect to age and WHR between the two groups. Although, the mean BMI was found to be within the normal range in both the groups but on statistical analysis it was significantly higher ($P<0.05$) in dysmenorrheic women compared to control group (Table I). In study group, SBP was significantly higher ($P<0.001$) during luteal phase when compared with menstrual and follicular phases (Table II) and also it was higher ($P<0.001$) in comparison with luteal phase of control subjects (Table IV). However, changes in DBP were not

TABLE I : Age and anthropometric parameters of the subjects of dysmeorrheic (Group I) and eumenorrheic (Group II).

Parameters	*Group I (n=30)*	*Group II (n=30)*	*P value*
Age (years)	19.13±1.27	18.80±0.99	0.265
BMI (kg/m^2)	23.90±4.10	22.08±2.73	0.046*
WHR	0.84±0.05	0.82±0.06	0.236

Values are expressed as Mean±SD; Data was analysed using Student' s unpaired t test.
*(P<0.05) statistically significant. BMI: Body Mass Index; WHR: Waist-Hip Ratio.

significant (P>0.05) amongst all the phases in both groups (Table II and III).

HRV Parameters

Time domain indices

Table II shows comparison of HRV indices among the phases of menstrual cycle in group I. By applying ANOVA, we found no statistically significant differences in mean R-R interval, mean HR, SDNN and RMSSD among the phases of menstrual cycle.

Table III shows HRV indices in different phases of menstrual cycle in Group II. Results of ANOVA showed that mean RR, mean HR and RMSSD differ significantly amongst all 3 phases. When post- hoc test was applied, mean RR and RMSSD were found to be significantly reduced (P<0.001 and P<0.05 respectively) and mean HR was found to be significantly increased (P<0.01) in the luteal phase as compared to the menstrual phase, while SDNN was not significant among all the examined phases.

Analysis of HRV during the different phases of the menstrual cycle between group I and group II (Table IV) showed that mean

TABLE II : Comparison of HRV indices and basal cardiovascular parameters in different phases of menstrual cycle of the subjects of Group I (dysmenorrheic n=30).

Parameters	*Phases of menstrual cycle*			
	M	*F*	*L*	*P value*
Mean RR (s)	0.751±0.08	0.759±0.09	0.716±0.08	0.166
Mean HR (bpm)	81.0±9.29	79.53±8.9	85.40±10.70	0.055
SDNN (ms)	49.15±21.06	47.40±18.63	41.14±16.12	0.148
RMSSD (ms)	35.69±15.95	37.50±17.18	30.33±13.01	0.182
TP (ms^2)	402.15±242.33	470.61±213.95	451.32±304.57	0.568
LF (ms^2)	100.66±77.97	146.72±76.63	124.28±96.15	0.111
HF (ms^2)	87.83±81.56	148.77±127.31	72.53±47.1$^{\#\#}$	0.004
LFnu	57.49±9.88	51.35±12.40	63.20±12.65$^{\#\#}$	0.001
HFnu	42.59±9.74	48.87±12.42	36.79±12.98$^{\#\#\#}$	0.001
LF-HF ratio	1.49±0.70	1.20±0.58	2.11±1.04$^{\pounds\ \#\#\#}$	0.000
SBP (mmHg)	107.50±6.44	107.66±8.27	116.86±8.36$^{\pounds\pounds\pounds\#\#\#}$	0.000
DBP (mmHg)	74.20±5.85	73.30±6.63	74.93±4.67	0.550

Values are expressed as Mean±SD; statistical analysis was done by one-way ANOVA test followed by post-hoc Tukey test among 3 phases. The (£) depicts comparison between M & L phases; $^{\pounds}$(P<0.05), $^{\pounds\pounds\pounds}$(P<0.001). The (#) depicts comparison between F & L phases; $^{\#\#}$(P<0.01), $^{\#\#\#}$(P<0.001). M-menstrual phase, F-follicular phase, L-luteal phase, Mean RR: mean RR interval, Mean HR (bpm) : mean heart rate in beats per minute, SDNN: standard deviations of averages of normal to normal (N-N) intervals, RMSSD: root mean square of differences of successive N-N intervals, TP: total power in absolute values (ms^2), LF: low frequency and HF: high frequency in absolute values (ms^2) and normalized units (nu), SBP: systolic blood pressure, DBP: diastolic blood pressure.

TABLE III : Comparison of HRV indices and basal cardiovascular parameters in different phases of menstrual cycle of the subjects of Group II (control n=30).

Parameters	*Phases of menstrual cycle*			
	M	*F*	*L*	*P value*
Mean RR (s)	0.866±0.10	0.818±0.06	0.779±0.07£££	0.000
Mean HR (bpm)	70.13±8.19	73.66±5.67	77.16±7.35££	0.001
SDNN (ms)	55.74±19.25	55.18±17.69	54.51±23.14	0.966
RMSSD (ms)	56.40±23.58	51.07±15.52	43.86±10.16£	0.039
TP (ms^2)	562.92±255.10	581.22±279.14	523.66±238.68	0.677
LF (ms^2)	145.21±75.64	161.20±107.87	145.13±117.06	0.780
HF (ms^2)	205.42±102.47	170.97±119.32	120.89±92.95££	0.009
LF nu	42.48±11.08	47.91±18.34	54.10±11.11££	0.007
HF nu	57.40±10.92	52.02±18.27	45.88±11.10££	0.007
LF-HF ratio	0.81±0.36	1.21±0.93	1.30±0.56£	0.012
SBP (mmHg)	107.33±6.56	108.93±8.25	109.66±7.14	0.456
DBP (mmHg)	71.06±6.61	71.73±7.91	74.03±7.04	0.252

Values are expressed as Mean±SD; statistical analysis was done by one-way ANOVA test followed by post-hoc Tukey test among 3 phases. The (£) depicts comparison between M & L phases; £(P<0.05), ££(P<0.01), £££(P<0.001). M-menstrual phase, F-follicular phase, L-luteal phase, Mean RR: mean RR interval, Mean HR (bpm) : mean heart rate in beats per minute, SDNN: standard deviations of averages of normal to normal (N-N) intervals, RMSSD : root mean square of differences of successive N-N intervals, TP: total power in absolute values (ms^2), LF: low frequency and HF: high frequency in absolute values (ms^2) and normalized units (nu), SBP: systolic blood pressure, DBP: diastolic blood pressure.

TABLE IV : Comparison of HRV indices and basal cardiovascular parameters in different phases of menstrual cycle between two groups (n=30 in each group).

Parameters	*Menstrual Phase*		*Follicular Phase*		*Luteal Phase*	
	Group I	*Group II*	*Group I*	*Group II*	*Group I*	*Group II*
Mean RR (s)	0.751±0.08**	0.866±0.10	0.759±0.09**	0.818±0.06	0.716±0.08**	0.779±0.07
Mean HR (bpm)	81.0±9.29**	70.13±8.19	79.53±8.9**	73.66±5.67	85.40±10.70**	77.16±7.35
SDNN (ms)	49.15±21.06	55.74±19.25	47.40±18.63	55.18±17.69	41.14±16.12*	54.51±23.14
RMSSD (ms)	35.69±15.95**	56.40±23.58	37.50±17.18**	51.07±15.52	30.33±13.01**	43.86±10.16
TP (ms^2)	402.15±242.33*	562.92±255.10	470.61±213.95	581.22±279.14	451.32±304.57	523.66±238.68
LF (ms^2)	100.66±77.97*	145.21±75.64	146.72±76.63	161.20±107.87	124.28±96.15	145.13±117.06
HF (ms^2)	87.83±81.56**	205.42±102.47	148.77±127.31	170.97±119.32	72.53±47.1*	120.89±92.95
LFnu	57.49±9.88**	42.48±11.08	51.35±12.40	47.91±18.34	63.20±12.65**	54.10±11.11
HFnu	42.59±9.74**	57.40±10.92	48.87±12.42	52.02±18.27	36.79±12.98**	45.88±11.10
LF-HF ratio	1.49±0.70**	0.81±0.36	1.20±0.58	1.21±0.93	2.11±1.04**	1.30±0.56
SBP (mmHg)	107.50±6.44	107.33±6.56	107.66±8.27	108.93±8.25	116.86±8.36***	109.66±7.14
DBP (mmHg)	74.20±5.85	71.06±6.61	73.30±6.63	71.73±7.91	74.93±4.67	74.03±7.04

Values are significant; *(P<0.05), **(P<0.01), ***(P<0.001). Mean RR: mean RR interval, mean HR (bpm): mean heart rate in beats per minute, SDNN: standard deviations of averages of normal to normal (N-N) intervals, RMSSD: root mean square of differences of successive N-N intervals, TP: total power in absolute values (ms^2), LF: low frequency and HF: high frequency in absolute values (ms^2) and normalized units (nu), SBP: systolic blood pressure, DBP: diastolic blood pressure.

RR and RMSSD were significantly reduced (P<0.01) and mean HR was significantly higher (P<0.01) in all 3 phases while SDNN was significantly lower (P<0.05) in luteal phase in dysmenorrheic subjects.

Frequency domain indices

In the present study, TP and LF ms^2 showed no statistically significant difference among the three phases of menstrual cycle

in group I (Table II) and group II (Table III). However, significant differences were observed in LF nu, HF (ms^2 and nu) and LF-HF ratio. In dysmenorrheic women, post-hoc analysis revealed significant reduction in HF ms^2 ($P<0.01$) and HF nu ($P<0.001$) and significant increase in LF nu ($P<0.01$) in luteal phase as compared with follicular phase. LF-HF ratio was significantly increased in luteal phase in comparison with menstrual ($P<0.05$) and follicular ($P<0.001$) phases (Table II).

In group II (control), Post-hoc analysis revealed significant reduction ($P<0.01$) in HF (ms^2 and nu) and significant increase in LFnu ($P<0.01$) and LF-HF ratio ($P<0.05$) in luteal phase when compared with menstrual phase (Table III).

Analysis of HRV during the different phases of the menstrual cycle between group I and group II (Table IV), revealed a statistically significant decrease in TP and LF ms^2 ($P<0.05$), HF ms^2 & nu ($P<0.01$) in menstrual phase, HF ms^2 ($P<0.05$) and HF nu ($P<0.01$) in luteal phase, while LFnu and LF-HF ratio were found to be significantly higher ($P<0.01$) in menstrual and luteal phases in women with PD compared to control group.

DISCUSSION

In the present study, we found increased sympathetic activity reflected by decreased total power, increased LFnu and reduced parasympathetic (vagal) tone in the form of decreased SDNN, RMSSD and HF (ms^2 and nu) in the luteal phase compared to follicular phase of the menstrual cycle in both groups. Similar findings have been reported by other authors in normal menstrual cycle (13, 19). Also, significantly higher systolic blood pressure was found in the luteal phase in group I indicating sympathetic dominance.

Earlier studies have shown that there appears to be a correlation between the hormonal levels in female hypothalamo-pituitary-gonadal axis and the ANS control of their cardiac activity (16, 20). Estrogen has a role in increasing vagal and reducing sympathetic activity by enhancing the cholinergic muscarinic activity at central and peripheral levels (21). Elevated progesterone may be responsible for the increase in systolic blood pressure in luteal phase of the menstrual cycle by increasing the fluid and salt retention. Physiological and psychological stress contributes to the blood pressure rise during luteal and menstrual phases. Mehta et al have studied the autonomic functions in the different phases of menstrual cycle and reported significantly higher SBP and increased sympathetic activity in luteal phase compared to menstrual and follicular phases without significant differences in parasympathetic activity (22). One study has reported that, in the menstrual phase, the pain ratings, SBP and DBP were significantly higher in dysmenorrheic group than non dysmenorrheic group and strongly suggests that there is activation of the sympathetic-adrenal-medullary axis by painful stress (23). Little et al also showed higher heart and respiration rates during the luteal phase compared with other phases (24).

In this study, a significant difference was observed between the two groups with respect to their overall HRV status. It was found that the young women with primary

dysmenorrhea had a significantly reduced heart rate variability throughout the menstrual cycle in the form of decreased vagal and increased sympathetic activity reflected by lower total power, HF (ms^2 and nu), SDNN, RMSSD and increased mean heart rate. This is in accordance with the previous study by Hegazi et al (25). In contrast, Matsumoto et al found no differences in HRV between the phases of the menstrual cycle (26).

Further LFnu, which reflects the fluctuation in sympathetic tone and LF-HF ratio, a marker of sympathovagal balance, was found to be increased during menstrual and luteal phases in group I. This suggests that primary dysmenorrhea is associated with shifting of cardiac autonomic activity towards sympathetic dominance along the whole cycle as compared to women with eumennorheic cycle.

The present study showed significantly high ($P<0.05$) BMI in group I compared to the control group, although the mean BMI of the subjects of both the groups was within the normal range. Anthropometric measurements BMI and WHR were used to assess central obesity. Previous studies have reported that central obesity is associated with menstrual disorders (27) and cardiovascular diseases (28).

Our study has limitations. Sample size was small in both groups. HRV was recorded in different phases for one menstrual cycle only. Follow up should be at least for 3 consecutive months. Also, we have not recorded HRV with 24 hours holter monitoring which is a better choice to measure the autonomic activity. Further, the study can be expanded by correlating cardiac autonomic activity with body composition, plasma levels of cortisol, estrogen, progesterone hormones and serum lipid profile and may be extended in obese subjects.

We concluded that women with primary dysmenorrhea have more sensitive responses to the sympathetic-adrenal-medullary axis system than eumennorheic women throughout the whole menstrual cycle. Prospective study is needed to explore the effect of non pharmacological means like slow breathing, as the reports indicate that it decreases the sympathetic nervous system activity (29) and increases the baroreflex sensitivity in normal subjects and patients with heart failure (30).

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EFFECT OF FORMALIN VAPOURS ON PULMONARY FUNCTIONS OF MEDICAL STUDENTS IN ANATOMY DISSECTION HALL OVER A PERIOD OF ONE YEAR

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Abstract : Formalin is extensively used for preservation of cadavers in department of Anatomy. However it is a noxious chemical which vaporises at normal room temperature in the air and may cause respiratory health problems among first year medical students. The study was planned to observe the effect of formalin vapours on lung function tests of first year medical students who are exposed routinely for 2 hrs every day for 6 days per week throughout the year. Following written informed consent clinically healthy 100 medical students between age group 18-23 yrs were subjected to pulmonary function testing by computerised spirometery. The dynamic lung function tests (FVC, FEV1, FEV1%, FEF_{25-75}, PEFR) were measured on four occasions-basal (before exposure), 1 month, 6 months, 11 months of exposure to formalin vapours in anatomy dissection hall. The study revealed statistically significant ($P<0.0001$) decreases in FVC, FEV1%, FEF_{25-75}, PEFR except FEV1 after 1 month of exposure to anatomy dissection hall. The decrease in all the parameters slowly reverted back towards normal basal values across 6 and 11 months and was statistically significant in all ($P<0.0001$) except FVC. Acute exposure to formalin vapours at anatomy dissection hall decreases the respiratory functions, however on long term exposure the body corrects the damage. Further studies are required to see the changes at cellular levels and the extent of damage to respiratory system.

Key words : formalin, pulmonary functions, anatomy, dissection hall

INTRODUCTION

Formalin is a colorless and irritative fluid that contains 37% of formaldehyde and is widely used as a preserving agent of biological specimen. At the medical and dental colleges formalin has been used for years to preserve cadavers. Recently, however formaldehyde has attracted much attention due to its health hazards. The

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primary route of exposure to formaldehyde is by inhalation, where it is absorbed by the lungs and also through gastrointestinal tract and to much lesser extent through the skin (1). Several published reports, research papers and industrial experience suggest that exposure to formaldehyde is associated with adverse effects on respiratory health (2). Recent review of the studies have indicated that upper respiratory tract is the critical target of the toxicity of air borne formaldehyde (3-6). Symptoms of upper airway irritation, including dry and sore throat, itching, burning sensation of the nose and nasal congestions have been reported by the workers (7). Studies in rats and mice using high concentration of formaldehyde over an extremely long period have reported to result in squamous carcinoma of nose (8). Occupational data suggests that small but significant changes may occur in lungs following prolonged exposure in the work place (7). It is also utilised by manufacturer of resins, plywood, leather good but exposure to formaldehyde in department of anatomy is continuous and higher than its use in other areas. The evaporation of formaldehyde from cadavers in anatomy dissection hall can produce high exposure among medical students and instructors (9). There has been an increasing number of reports that students suffer from various physical symptoms including burning eyes, lacrimation, irritation of airways, dermatitis with a high prevalence during gross anatomy dissection (3). In view of its widespread use, toxicity, and volatility, exposure to formaldehyde is a significant factor affecting human health (10). However only few studies have mentioned the effect of formalin vapours in anatomy dissection hall on pulmonary function tests of medical students. Therefore the study was planned with the aim to see effects of formalin on pulmonary functions in medical students who are exposed to formalin vapours for two hours every day for 6 days a week in anatomy dissection hall.

MATERIALS AND METHODS

The present study was carried out on 100 healthy medical students between age group 18-23 years. Prior approval from Research Committee and Institutional ethical committee was obtained. Subjects were recruited based on the subject proforma. Inclusion criteria being clinically healthy, non-smokers, without any chronic respiratory disease, systemic illness like diabetes, hypertension The pulmonary function tests were carried out using computerised spirometer (Spiro Lab-II). The flow, volume/ time graphs were taken out in accordance to the criteria based on American Thoracic Society (11). Following informed and written consent, the anthropometric measurement like height, weight were recorded. The pulmonary function were tested on four occasions –

1) Basal (before exposure to formalin)
2) 1month of exposure to formalin vapours
3) 6 months of exposure to formalin vapours
4) 11 months of exposure to formalin vapours.

The dynamic function tests-FVC, FEV1, FEV1%, FEF_{25-75}, PEFR were recorded. The lung function tests were repeated thrice on each occasion for each subject and the maximum reading was selected for analysis.

Statistical analysis

The data was collected and subjected to statistical analysis using SPSS ver19. After the anthropometric profile, the basal values of the dynamic lung function tests were compared with 1 month exposure to anatomy dissection hall by Paired Student's t test to look for acute changes. Variability in all dynamic lung function test within the subjects and across the time were analysed by repeated measures ANOVA and Post Hoc Bonferroni test. The level of significance was set at $P<0.05$.

RESULTS

The mean age (years) of the subjects in the present study was 18.32±1.1 (mean±SD). The mean height of these subjects was 166.8±9.1 cm and mean weight was 65.63±16.3 kg. The mean BMI of the subject was 23.45±5 (Table-I).

TABLE I : Anthropometric profile and basal parameters of dynamic lung function tests (n=100).

Baseline parameters	*Values*
Age (years)	18.32±1.1
Height (cms)	166.8±9.1
Weight (kg)	65.63±16.3
BMI (kg/m^2)	23.45±5
Basal FVC (L)	3.41±0.87
Basal FEV1 (L)	4.60±11.8
Basal FEV1%	89.91±13.6
Basal FEF_{25-75} (L/S)	4.29±1.4
Basal PEFR (L/S)	5.73±2.14

Values in mean±SD.

The dynamic lung function tests of subjects 1month after exposure to anatomy dissection hall for 2 hours/day 6 days a week showed statistically significant decrease in mean values of all the dynamic lung function tests ($P<0.0001$) except for FEV1. There were subjective complaints of mild irritation in the nose, lacrimation, redness of the eyes, dry throat, headache and itching in the skin (Table-II).

Table II : Comparsion of lung functions in students before and after exposure for 1 month to formalin in anatomy dissection hall (n=100).

Parameters	*Before exposure*	*After exposure (1 month)*
FVC (L)	3.41±0.87	2.94±0.8***
FEV1 (L)	4.60±11.8	2.41±0.8^{ns}
FEV1%	89.91±13.6	78.82±14.9***
FEF_{25-75} (L/S)	4.29±1.4	2.78±1.38***
PEFR (L/S)	5.73±2.14	3.61±1.95***

Values in mean±SD. *P value<0.05, **P<0.01,. ***P<0.001, ns-not significant.

Duration (in months) of stay in formalin vapours of anatomy dissection hall had statistically significant effect on the dynamic lung function tests (Table-III).

The mean value of FVC for the subjects at time of entry into the study was 3.41±0.87 L as compared to FVC at 1 month (2.94±0.8 L) 6 month (3.19±0.71L) and at 11 month (3.22±0.76 L). Comparsion of mean values of FVC reported statistically significant difference between basal and 1 month ($P<0.0001$), however rest all the values were statistically insignificant. The mean value of FEV1 for the subjects at time of entry into the study was 4.60±11.8 L which decreased drastically at 1 month and remained so by 11 month, and this decline was statistically significantly lower ($P<0.0001$). The mean of FEV1% of the subjects at time of entry into the study was 89.91±13.6 which decreased at 1 month (78.82±14.9), but was later

TABLE III : Comparison of dynamic function tests in medical students before exposure and after exposure to formalin in anatomy dissection hall (n=100).

Parameters	*Before exposure*	*After exposure (1 month)*	*After exposure (6 month)*	*After exposure (11 month)*
FVC (L)	3.41±0.87	2.94±0.8$^{###}$	3.19±0.71	3.22±0.76
FEV1 (L)	4.60±11.8	2.41±0.8	2.72±0.73	2.94±0.62$^{\$\$\$}$
FEV1%	89.91±13.6	78.82±14.9$^{###}$	83.60±14.35	89.72±6.81$^{\$\$\$,\wedge\wedge\wedge}$
FEF_{25-75} (L/S)	4.29±1.4	2.78±1.38$^{###}$	3.33±1.24$^{###}$	3.76±1.24$^{\$\$\$}$
PEFR (L/S)	5.73±2.14	3.61±1.95$^{###}$	5.15±2.14$^{\$\$\$}$	5.16±1.97$^{\$\$\$}$

The values are mean±SD Statistical analysis done by Repeated Measures Anova and Post Hoc Bonferroni comparsion test for the variability in dynamic lung function tests within the subjects and across the time. The mark # indicate comparsion of values from basal reading; $ for comparsion with 1 month; ^ for comarsion with 6 month. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.0001$, $^{\$}P<0.05$, $^{\$\$}P<0.01$; $^{\wedge}P<0.05$; $^{\wedge\wedge}P<0.01$, $^{\wedge\wedge}P<0.001$.

restored back to normal by 11 months (89.72±6.81).

The mean of FEF_{25-75} of students at time of entry into study was higher (4.29±1.4 L/S) as compared to 1 month (2.78±1.38 L/S), 6 month (3.33±1.24 L/S) and 11 months (3.76±1.24 L/S). The values of FEF_{25-75} also became back towards normal by 11 months. The mean value of PEFR of the students at the time of entry into study was higher (5.73±2.14 L/S) which decreased at 1 month (3.61±1.95 L/S), but was later found to be increased by 11 months (5.16±1.97 L/S).

DISCUSSION

First year Medical students during their anatomy dissection classes are exposed to formaldehyde vapours, whose exposure is considered to be one of the cause of multiple chemical sensitivity. The binding of formaldehyde to endogenous proteins creates haptens that can elicit an immune response. Chronic exposure to formaldehyde has been associated with immunological hypersensitivity as measured by elevated circulating IgE and IgG autoantibodies to human sernum albumin. In addition, a decrease in the proportion of T cells was observed indicating altered immunity (12). Most of the students in our study complained of irritation in eyes, nose lacrimation, itching in skin.

In the present study acute exposure to formalin for 2 hrs/day for 6 days/week resulted in decrease in FVC, FEV1%, FEF_{25-75}, PEFR except FEV1 indicating mild bronchoconstriction. The study done earlier revealed that FVC decreased in subjects immediately after their first exposure, while all other lung function parameters remained unchanged indicating bronchoconstriction on acute exposure to formalin(7). Akbar-Khanzadeh et al evaluated acute pulmonary response in group of 34 workers exposed to formalin in gross anatomy dissection hall, also reported decrease in FVC but FEV1/FVC ratio increased during exposure (13). Farah Khaliq et al reported decrease in FVC immediately after 2 hours exposure to formalin. A trend towards decrease in values of FEV1 immediately after exposure was observed but it was not statistically significant (7). In the present study we also observed decease in FEV1 on acute exposure to formalin but it was not statistically significant (Table-II).

On the contrary, Chia et al reported no significant difference in the pre and post exposure in mean values of FVC and FEV1 (14). Alexanderson and Hedenstierna, evaluated lung function tests and immunoglogulin levels in 34 wood workers who were exposed to formaldehyde. A significant decrease in FVC, FEV1, FEF_{25-75} was reported (15). Mean while, the effect of formaldehyde exposure in plywood workers resulted in significantly reduced FEV1, FEV1/FVC ratio, FEF_{25-75} but not FVC (16).

In the present study there was a sharp decrease in dynamic lung function tests following 1 month of exposure, however the basal values were restored after 11 months of exposure to formalin vapours. A prospective study by Alexanderson R et al have reported significant decrease in FVC, FEF_{25-75}, FEV1% in wood worker who were exposed to formaldehyde since 4 years, however these decrements returned to normal after 4 weeks of nonexposure showing reversibility of pulmonary function tests following cessation of exposure (17). The exact concentration of formaldehyde to which our subjects were exposed in dissection hall could not be determined, which is the limitation of the study, but it is definitely at a concentration (2-3 ppm) causing severe eyes and nose irritation which was reported by the students following acute exposure (14).

For the numerous health challanges that formaldehyde causes on the students in anatomy dissection hall, it cannot be considered as an ideal chemical for embalming of cadaver. The laboratory attendents working in anatomy dissection hall for years are being continuously exposed to formalin vapours. They should be informed of potential health hazards of formalin and attempts should be done to reduce the concentration of formaldehyde by using other chemicals like glutaraldehyde, which can serve a good substitute for formaldehyde (18). As quoted by BS Mitchell "Reduction in formaldehyde concentration is not deleterious to specimen preservation, but leads to a safer working environment" (19).

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NO DIFFERENCE IN THE OMITTED STIMULUS REACTION TIME TASK DURING THE MENSTRUAL CYCLE : A MULTISENSORY STUDY

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Abstract : Pharmacological studies are currently performed in men and not in women due to the absence of methodological or conceptual information concerning the effects of the menstrual cycle. This is the first study in which three different sensory stimuli were applied to females in an omitted stimulus reaction time (OSRT) task during three phases of the menstrual cycle. Eleven college volunteers with regular menstrual cycles responded to lateralized trains of visual, auditory, and somatosensory stimuli. A participant's OSRT was recorded for each trial performed during the menstrual (day 2-3), follicular (day 7-8), and luteal (day 21-22) phases. The results showed that the ovarian cycle has no effects on the OSRT task in any of the sensory modalities, but the reaction to the missing auditory stimuli was found to be systematically faster than the reactions to the visual and somatosensory stimuli (P<0.002). Thus the OSRT is affected by sensory modality but not by the normal menstrual cycle.

Key words : menstrual; reaction time; omitted stimulus; multisensory

INTRODUCTION

An increased awareness of the inclusion of the female in drug abuse research has brought increased scientific interest in the potential influence of the menstrual cycle phase on the responses to neuroactive drugs (1, 2). Many studies have used reaction time (RT) tasks to test the effects of sex hormones or the menstrual cycle (3, 4). Some evidence supports the hypothesis that a simple auditory RT is influenced by the fluctuating levels of sexual hormones across the normal menstrual cycle, which is highest during the premenstrual phase and lowest during the mid-secretory phase (5). Nevertheless, many

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papers report no differences in performance between the different phases of the menstrual cycle (6, 7, 8, 9, 10, 11, 12).

An omitted stimulus task presents a recurrent stimulus that requires an immediate response to the omission of the stimulus. Unlike the reaction to the presentation of a stimulus, the reaction to an omitted stimulus is generated by an endogenous process and is considered to require additional cognitive processes (13, 14). To date, no studies have analyzed the effect of the menstrual cycle on an omitted stimulus reaction time (OSRT) task through the application of three sensory modalities to the same person (14). Multisensory experiments are important because these could provide valuable new information on the possible differences in the sensitivity to steroids that fluctuate during the menstrual cycle between different sensory modalities. Studies that utilize reaction time tasks have showed that auditory stimuli produce faster responses than visual or tactile stimuli (14, 15, 16, 17, 18, 19, 20).

Previous research in males indicates that moderately increasing blood alcohol concentrations can impair the visual and auditory OSRTs without affecting the somatosensory OSRT (18). These interesting results are missing in females because, in part, we do not know how the OSRT task could be influenced by the menstrual cycle. Although previous research has reported that hormonal fluctuations have important impacts on the functional cerebral asymmetries (21, 22, 23), other studies have not observed this laterality (24, 25). To date, no research study has applied lateralized sensory stimuli in the OSRT task.

The aim of the present research study was to determine whether there is any alteration of OSRT task across the normal menstrual cycle when trains of lateralized visual, auditory, and somatosensory stimuli are applied. According to previous research (11, 12, 14, 18, 19, 24, 25), we hypothesize that the auditory stimuli will produce a shorter OSRT than the visual and the somatosensory stimuli and that no changes or asymmetries will be observed between the phases of the menstrual cycle. This study will thus test the OSRT in response to the administration of acute doses of alcohol in women.

METHODS

Participants

Eleven healthy college students with regular menstrual cycles participated in the study (5). The right-handedness was assessed using the Shimizu and Endo (26) questionnaire. All of the participants were aged between 18 and 25 years (mean of 21.8±2), and none had any history of nervous system diseases or motor disability. Subjects with well-defined PMS, any gynecological problems, irregular cycles, or any drug consumption (e.g., hormonal treatment and psychotropic drugs) were excluded from the study. The subjects were instructed to abstain from the consumption of any stimulant drink or alcohol for at least 24 hours before their arrival at the lab and participation. All of the participants volunteered for the study and were informed of the procedures before completing an informed consent form and reporting their age and health history. The protocol was reviewed and approved by the Ethics

Committee of the University of Campeche.

Apparatus and materials

The OSRT task used in this study was similar to that used in prior research (14). In this task, trains of visual, auditory, and somatosensory stimuli were applied at 2-s intervals (0.5 Hz). Only few differences in the visual stimuli must be observed. Briefly, the visual stimuli consisted of light flashes (white light, 10 µs, 1.5×10^{-3} lumen/sec/cm^2) delivered by an optic fiber connected to a photic stimulator (GRASS PS33Plus). The center of the optic fiber tip (4 mm in diameter) was placed 30 cm in front and 30 cm lateral (left or right) from the participant's eyes to ensure the use of the peripheral visual field of each eye (as binocular vision). An electrical stimulator (GRASS S48) triggered the auditory stimuli, which were presented as 10-ms "clicks" to one of the ears through headphones. The auditory thresholds of each ear were determined and then set at 20 times the threshold to ensure that the clicks would be clearly heard. The somatosensory stimuli were administered by two disc electrodes (GRASS F-E5SH) that were placed on the medial finger of the left or the right hand and were connected to the electrical stimulator through a stimulus isolation unit (GRASS SIU5). The somatosensory thresholds were determined for each hand and then set at 1.2 times the threshold, which was well below the pain threshold.

The responses to the termination of a train of stimuli in each sensory modality were also measured. At the outset of a trial, a response key was depressed with the ipsilateral thumb to the side of the stimuli until the train of stimuli ceased, at which point the key was released. The response key was connected to an AC amplifier (GRASS P511). Each stimulus and the release of the response key generated clear changes in the voltage compared with the baseline, and these changes were collected online using a computer fitted with an analog-to digital converter and analyzed using the ACQKNOWLEDGE software (BIOPAC Inc.).

The OSRT was measured as the time between the occurrence of the first missing stimulus and the release of the key. A participant's OSRT was recorded during each visual, auditory, and somatosensory task that was performed during three phases of the menstrual cycle: the menstrual (day 2-3), follicular (day 7-8), and luteal (day 21-22) phases.

Procedure

The subjects who met the inclusion criteria were subjected to reaction time testing during three occasions within one month. The phase during which the first test occurred (menstrual, follicular, or luteal phase) was counterbalanced. After an individual was subjected to the first test, she was scheduled to return to the lab for the next test according to her cycle. The thresholds and intensities were then determined, and the participant was seated in front of a table, where the response key was within easy reach. The task instructions were identical for each sensory modality. The participants were told to hold down the key at the beginning of each trial until they realized that the train of stimuli had ceased, at which point they were to immediately release it.

The participants wore headphones during all of the tests and rested their heads on a chin support that fixed their sight on a blue spot, which was 6.5 cm in diameter, on the wall 79 cm in front of the head. The participants were instructed to maintain their gaze on the central fixation point at all times. Each trial was preceded by a verbal "ready" signal. The number of stimuli (visual, auditory, or somatosensory) in a train during a given trial varied between 5 and 20 in a predetermined pseudorandom fashion. A test of a sensory task included the administration of five trials on each side (left and right; for a total of 10), and the relationship between the side of the stimuli and the response hand was found to be always ipsilateral. The test was completed in approximately 10 minutes and was immediately followed by the next test, which presented a different sensory stimulus. Thus, the test of all three sensory tasks was completed in approximately 30 minutes. The administration of additional trials would extend the duration of a test and possibly introduce boredom and fatigue effects. The starting sensory modality and the side of the stimuli were counterbalanced across the entire sample.

Data analyses

The scores of any trial in which the response occurred before or coincided with the first missing stimulus in a train were discarded. In total, 2.6% of the trial scores were rejected. For each test of a sensory task, a participant's OSRT was averaged over the trials presented on each side. The SPSS software (SPSS, 2001) was used to analyze the OSRT measures using a 3 (cycle, i.e., menstrual, follicular, and luteal) × 3 (sensory, i.e., visual, auditory, and somatosensory) × 2 (sides, i.e., left and right) repeated measures analysis of variance (ANOVA). To correct for the chance occurrence of a result with $P<0.05$ when tests are repeated, a Bonferroni correction was used to adjust the alpha level.

RESULTS

All data were transformed to the log to meet assumptions of normality (Kolmogorov Smirnov test) and equal variance (Levene test). A 3 (cycle) × 3 (sensory) × 2 (side) ANOVA of the OSRT showed that the main effect was obtained through the sensory factors ($F_{2,20} = 18.81$, $P<0.0001$, $\eta^2 = 0.653$). No main effects were detected for cycle ($F_{2,20} = 3.44$, $P>0.052$, $\eta^2 = 0.256$) or the side of the presentation ($F_{1,10} = 0.151$, $P>0.706$, $\eta^2 = 0.015$), and no interactions were significant ($P>0.086$). The paired comparisons with the Bonferroni correction showed that the response to the auditory task was faster than those obtained with the visual task ($P<0.001$) and the somatosensory task ($P<0.002$). No difference was found between the visual and the somatosensory tasks ($P>0.551$). The non-significant cycle and side effects obtained through ANOVA showed that the mean (SD) OSRT to the tasks were 939.5 ms (278) during the menstrual phase, 959.1 ms (272.7) during the follicular phase, and 814.5 ms (218.8) during the luteal phase. The left and right side presentations were 903.1 ms (221.2) and 905.7 ms (238.5), respectively. Table I shows the mean (SD) of each of the measures.

DISCUSSION

The present research is an extension of

TABLE I: Mean (SD) measures in milliseconds of the omitted stimulus reaction time (OSRT) to different sensory stimuli in the three phases of the menstrual cycle and the two sides. N=11

Sensory Modality	*Side*	*Menstrual M (SD)*	*Follicular M (SD)*	*Luteal M (SD)*
Visual	Left	937.3 (270.0)	1,012.7 (307.6)	784.5 (237.3)
Visual	Right	991.8 (308.9)	980.9 (362.9)	879.1 (244.5)
Auditory	Left	851.8 (310.8)	910.0 (292.2)	714.5 (244.9)
Auditory	Right	819.1 (352.9)	833.6 (324.9)	743.6 (242.8)
Somatosensory	Left	1,034.5 (314.5)	1,030.9 (390.3)	851.8 (291.2)
Somatosensory	Right	1,002.7 (328.6)	986.4 (320.1)	913.6 (238.3)

M = mean; SD = standard deviation.

our previous studies on the OSRT task under drug-free and acute doses of alcohol in college volunteers using trains of sensory stimuli (14, 18, 19, 27, 28, 29). To understand the impact of alcohol or other drugs on the sensory motor system of the female, it is necessary to first determine whether the menstrual cycle is able to affect the performance of the subjects under drug-free conditions.

Although the OSRT paradigm is somewhat uncommon, it bears a resemblance to some real-life situations, such as those that require a reaction to the cessation of a flashing stoplight or to a missing beep on a heart monitor. The omitted stimulus task is considered to require additional cognitive functions, such as sustained attention and the discrimination of the cessation of a temporal stimulus sequence, that are not involved in simple or choice reaction time tasks (14). In this study, we tested whether the OSRT task is altered when trains of lateralized visual, auditory, or somatosensory stimuli are applied across the menstrual cycle. The results clearly showed that the ovarian cycle had no effects on the OSRT task in any of the three sensory systems used, but the sensory modality affected the OSRT. In support of the hypothesis, the auditory stimuli produced faster responses than the visual or the somatosensory stimuli with no hemispheric asymmetries (14, 18, 19, 20, 24).

An important challenge in neuroscience is the identification of how information from the external world, which is perceived through the different senses and processes in the sensory-specific cortical modules, is compared, integrated, and evaluated to obtain an accurate, meaningful, and coherent perception of the external events (17) and how this perception is disturbed by the presence of steroid hormones or drugs. To understand the exact role of the sensory processes on behavior, it is necessary to study the nervous system´s capacity to integrate information across the normal menstrual cycle. The brain integrates and converge signals of many sensory modalities at the brainstem and cortical sites, where individual neurons respond through specific patterns of activation that depend on the nature of the stimulus complex and the intrinsic properties of the neuron (30). The within-subject design in the OSRT paradigm with three sensory modalities during the menstrual cycle phases provided valuable information on the sensitivity of the sensory systems to female steroid fluctuations. This

information is important because the responses were evaluated under the same experimental conditions in each subject, which provided the opportunity to establish comparisons and/or hierarchies among the sensory modalities during the menstrual cycle phases. In addition, such data will be useful in future alcohol and neuroactive drug studies performed in females. The faster processing of auditory signals is in agreement with the results obtained by Rousseau and Rousseau (15), Naito et al. (16), Thesen et al. (17), and our previous work (14, 18, 19). It is hypothesized that this faster response time depends on differences in the timing operations of the sensory-specific modules that are required to reach a time criterion and ultimately trigger a behavioral response (15).

Nene and Pazare (5) found that the simple reaction time to auditory stimuli is highest during the pre-menstrual phase and lowest during the mild secretory phase. Other studies have also found faster RTs during the luteal phase of the cycle (9, 10). The present research does not support these findings but is in agreement with other studies that have shown that the menstrual cycle has no effects on behavior (6, 7, 8, 9, 10, 11, 12). The scientific reasons for this discrepancy in the effects of the menstrual cycle on performance or cognitive processing have not been fully established (31). As in the study conducted by Nene and Pazare (5), this research study did not measure the hormonal levels during the cycle, but, based on existing knowledge, it is possible that the OSRT task might be affected by the normal menstrual cycle. The absence of such effects supports the continuation of OSRT studies of alcohol and other drugs in female subjects.

It is known that ovarian hormones interact with neurotransmitters (dopamine and gamma-aminobutyric acid) to modulate the effects of ethanol (32), and previous studies have shown that the effects of acute alcohol in women are controversial and depend on the variable under study, e.g., Linnoila et al. (33) studied the psychomotor performance and reported that the dose-response relationships of alcohol differ between the different menstrual cycle phases. In contrast, no cyclic differences were observed in the pharmacokinetics (34), consumption (32), and absorption of alcohol (35), or in mood and behavior under intoxication (34, 35). At present, many toxicological research studies are performed only in males due to the absence of reports on the effects of the ovarian cycle on the biochemical, psychological, or physiological variables (36), although some authors argue that that female must not be excluded from studies on the effects of alcohol on behavior because certain behaviors affected by alcohol are not affected by the menstrual cycle phase (34). Studies on male subjects in our lab have shown that the impairment of the acute doses of alcohol on the OSRT is dependent on the sensory stimulus involved in the task (18).

The results obtained in the present research are in agreement with the findings reported by Brick et al. (34) and others (9, 10, 11, 12) because no menstrual effects were obtained in the omitted stimulus task performance. Thus, it will be interesting to perform alcohol experiments in female subjects through the application of multiple sensory stimuli in the OSRT task (36).

In this study, lateralized stimuli were applied because it is known that the

fluctuating levels of hormones during the menstrual cycle are able to affect the timing responses of the right and the left hemispheres (37, 38). It is widely accepted that the left hemisphere (right hand) exhibits greater expertise in both the production and perception of language and in motor control, whereas the right hemisphere (left hand) has a greater degree of engagement in non-verbal, spatial processing, attention, movement planning, and time estimation (39). In the present research study, no lateralized responses were observed, and this finding is in agreement with the results reported by Hausmann et al. (23). This finding is most likely because the greater motor speed performed by the right hand was balanced with the greater attention, time estimation, and movement planning from the right hemisphere to the left hand. However, the absence of asymmetry is not in agreement with the results obtained by Whitehead (40), who found faster responses to targets presented directly to the right hemisphere compared with targets presented to the left hemisphere when the subjects were required to maintain attention continuously for longer than 10 seconds, and the results obtained by Pardo et al. (41), who reported an increased cerebral blood flow in the right frontal lobe after the subjects maintained attention for long periods. Our OSRT task is highly demanding of all of these processes. Thus, additional experiments will be necessary to firmly establish the role of the asymmetric functions on the omitted stimulus tasks with different sensory stimuli in male and female subjects.

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REGION-WISE GRAY MATTER VOLUME ALTERATIONS IN BRAIN OF ADOLESCENTS WITH ATTENTION DEFICIT HYPERACTIVE DISORDER : A VOXEL BASED MORPHOMETRIC ANALYSIS

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Abstract : Attention Deficit Hyperactive Disorder (ADHD) is a behavioral neuropsychiatric disorder affecting an estimated 5% of school-age children worldwide with symptoms persisting into adulthood in 80% of cases. Presently clinical diagnosis and treatment of ADHD relies on behavioral disturbances than on detecting underlying defective brain regions. Therapeutic outcome in treatment of ADHD may be more positive if defective brain region in clinically diagnosed ADHD is detected by voxel based morphometric (VBM) analysis that measures voxel-wise global and regional focal volume differences in structural magnetic resonance images (sMRI) of brain. This study was designed to detect any region-specific gray matter (GM) volume defects in sMRI of ADHD adolescents by VBM analysis. Thirty sMRI datasets matched for sex, handedness of adolescents aged between 11.66 and 20.47 years (mean age 16.27±2.48 years) obtained from NeuroImage webpage, were selected (Control n=15; ADHD combined type n=15). These sMRI were analyzed by VBM technique and compared using statistical parametric mapping (SPM). Significant regional GM volume deficits ($P<0.05$) was specifically identified in left cuneus and middle occipital gyrus in ADHD, after voxel-wise false discovery rate correction over the whole brain compared to matched controls. Deficit of GM volume in occipital cortex detected by VBM analysis in ADHD children, suggests defects of visual processing affecting attention mechanisms.

Key words : ADHD occipital gyrus sMRI VBM analysis

INTRODUCTION

Attention Deficit Hyperactive Disorder (ADHD) is a behavioral neuropsychiatric disorder characterized by hyperactivity, inattention or both (combined type) (1) affecting up to 1 in 20 children in the USA (2). The estimated prevalence of ADHD in school-age children worldwide is approximately 5%. However, the prevalence

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of ADHD varies according to the diagnostic criteria used, geographical location and various other factors (3). The estimated prevalence of school-going children with ADHD in India is about 12.2%. A wide range of behavioral and cognitive impairment are found in ADHD individuals, which includes deficits in working memory, inhibitory control, altered motivation, motor restlessness and poor performance. ADHD is associated with significant comorbidity including anxiety, and bipolar disorders (4-6). In approximately 80% of ADHD children, symptoms starts in childhood and frequently persists into adolescence through adulthood (7).

Typically screening/assessment of ADHD involves more than one professional, since there is no single test to confirm ADHD. Screening tests include thorough medical and family history, general physical, neurological examination, comprehensive questionnaires/tools to parents, teachers and children to rule-out symptoms of anxiety, depression and or learning disabilities/other neurological problems, psychological tests to measure IQ, social and emotional behaviors. Subsequent to behavioral screening tests, various imaging tests like single photon emission computed tomography (SPECT) (8), functional magnetic resonance imaging (fMRI), structural magnetic resonance imaging (sMRI) (9, 10) and quantitative electroencephalography (qEEG) (11), in addition to genetic and genomic tests (12) may be used to detect any defect in the brain regions. Thus, a more accurate diagnosis of specific regional defects in gray matter (GM) of brain in ADHD and accuracy in treatment protocol requires state-of-the-art NeuroImaging techniques and analysis.

MRI analysis studies show that ADHD is associated with alterations in brain structure, particularly a decrease in total cerebral volume (13) and other studies on ADHD have also shown an association with anterior cingulate dysfunction which plays an important role in attention (14). Current neurobiological models of ADHD postulate fronto-striatal dysfunction as a key component of the disorder. Evidence from NeuroImaging studies shows the involvement of fronto-striatal circuit in ADHD (15). In a comparative study, the right caudate nucleus area in the ADHD group was found to be larger (16). However, studies comparing children with and without ADHD have found differences in the size and symmetry of the caudate nuclei (17).

The underlying specific regional defects in grey matter of brain in ADHD can be more accurately detected and gauged by using voxel based morphometric (VBM) analysis, an advanced NeurImaging analytical technique that measures voxel-wise changes of global and regional volume differences in sMRI, using statistical parametric mapping (SPM).

Thus, the objective of the present comparative study was to detect any region-specific GM volume alterations in brain sMRI from control adolescents matched for age, sex and handedness and those diagnosed with combined type of ADHD, by use of VBM analytical technique.

MATERIAL AND METHODS

sMRI datasets

Forty-eight, three dimensional T1

weighted sMRI datasets of adolescent children aged between 11.66 and 20.47 years (mean age 16.27±2.48 years) from the NeuroImage webpage (http://fcon_1000.projects.nitrc.org/indi/adhd200)- International NeuroImaging Data sharing Initiative (INDI), were obtained. INDI allows contributing or downloading MRI datasets, which are permitted to be used for publication under Creative Commons License (Attribution Non-Commercial license). Of forty-eight sMRI datasets of adolescent children, thirty datasets for age, sex and handedness were matched and selected (Table I) for comparative analysis of regional GM volume by VBM analytic technique. The selected data sets consist of 15 combined type ADHD and 15 typically developing children, designated as controls. The study was approved by our Institutional Research and Ethics Committee.

Diagnosis of ADHD

As given in the downloaded dataset, diagnosis of ADHD was based on parent and child responses to the Schedule of Affective Disorders and Schizophrenia for Children—Present and Lifetime Version (KSADS-PL) and the Conner's Parent Rating Scale-Revised, Long version (CPRS-LV). Psycho stimulant drugs were withheld at least 24 hours before scanning, and other psychotropic medications were withheld at least 3 days before scanning. Subjects with absence of any Axis-I psychiatric diagnoses per parent and child KSADS-PL interview, as well as T-scores below 60 for all the CPRS-R: LV ADHD summary scales were included as control. Estimates of full scale intelligent quotient (FSIQ) above 80, and absence of other chronic medical conditions were required for all children. Intelligence was evaluated with the Wechsler Abbreviated Scale of Intelligence (WASI). (http://fcon_1000.projects.nitrc.org/indi/adhd200)

TABLE I : Comparison of age, sex and handedness of the patients.

	Control n=15	*ADHD Patients n=15*	*P-value*
Age†	16.72±2.55	16.8±2.5	>0.05
Sex (Male : Female)‡	4:11	4:11	>0.05
Handedness (Right : Left)§	14:1	14:1	>0.05

†Comparison using independent sample t test, Values represented as Mean±SD; ‡,§Comparison using chi square test.

Image acquisition

According to the information obtained in the downloaded dataset, all thirty sMRI of each subject were taken using magnetic field intensity, with high resolution; Magnetom Symphony (Avanto syngo MR B17) scanner; sequence, T1 magnetization-prepared rapid acquisition of gradient echo; TR, 2730 ms; TE, 2.95 ms; TI, 1000 ms; voxel size, $1 \times 1 \times 1 mm^3$; flip angle, 7°; sagittal slices per slab, 176).

VBM analysis

In this study, by using these downloaded dataset, VBM analysis was performed. It is a method for investigating neuroanatomical alterations in an unbiased, objective way. VBM analysis involves measurement of voxels in the images, from which differences in volume of brain regions, especially GM can be obtained. Significant difference in the volume of brain regions is established by statistically comparing between two or more

regions of different experimental groups (18). VBM analysis was done by using VBM8 tool box software version 429 (cf. http://dbm.neuro.unijena.de/vbm8) and SPM8 software version 4667 (cf. ftp://ftp.fil.ion.ucl.ac.uk/spm) installed in MATLAB software version 7.12.0.635 (R2011a).

All the sMRI images used in this analysis were manually reoriented by setting anterior commissure as origin.

(a) Preprocessing

Since the present study was performed by using sMRI brain images from adolescents, Tissue Probability Map (TPM) was customized, appropriate for spatial normalization of sMRI data, by using the TOM8 toolbox version 9 (cf. https://irc.cchmc.org/software/tom/agreement.php). All other parameters remained default except TPM. Preprocessing of all reoriented T1 images resulted in modulated non-linear dartel warped segmented volumes of GM, WM and CSF and values for global volume of GM, WM and CSF for each image was automatically obtained.

(b) Quality check

By using VBM8 check quality option, all segmented T1 images were checked for image quality and presence of any artifact. All images selected were of good in quality and without artifacts.

(c) Smoothing

For VBM statistical analysis, Gaussian kernel of 8-mm full-width at half maximum (FWHM) was used to smooth the images.

(d) Statistical analysis

The final probability maps were obtained and automatically analyzed by voxel-wise testing using a two sample *t*-test with intra cranial volume as covariate in SPM8, to measure group differences in regional GM volumes. Effect of nuisance variables in selected sMRI used in this analysis were automatically removed by matching for age, sex and handedness of subjects. XJ View software version 8.4 (cf. http://www.alivelearn.net/xjview8/) was used for further analysis of results and all observed p-value distributions were corrected by applying voxel wise false discovery rate (voxel-FDR) ($P<0.05$). FDR is a new approach to multiple comparison problems and studies by Chumbley J et al (2010) has cited that voxel-FDR is more sensitive than extent-FDR, which in turn is more sensitive than peak-FDR and additionally conventional voxel-FDR discovers more local peaks than conventional FWE. Instead of controlling chance of any false positives (as in Bonferroni or random field methods), voxel-FDR controls the expected proportion of false positives among supra-threshold voxels. Values for GM, WM, and CSF volumes for each subject were automatically generated by this method and total brain volume/intracranial volume (TBV/ICV) for each subject was obtained by adding values of GM, WM and CSF volumes. SPSS software version 16 for paired *t* test to compare values of GM, WM, and CSF volume of control and ADHD patients was used in this study since all selected sMRI were matched for age, sex and handedness.

RESULTS

A mean difference in global GM, WM and

CSF volumes were found in sMRI brain images of ADHD patients when compared with the same in controls as shown in Fig. 1. Additionally, a significant decrease (P<0.05) after correction for voxel-FDR was found in region-specific GM volumes (Table II) of sMRI brain images from ADHD children as compared to the same in controls, particularly in regions like the occipital, cingulate, superior frontal cortices and other regions (Fig. 4), but also an increase in GM volume in the right thalamus (uncorrected P<0.001) (Fig. 3, Table III) which was not significant with voxel-wise FDR correction.

A significant deficit in the left cuneus and left middle occipital gyrus (Fig. 2) in addition to other regions detected in this

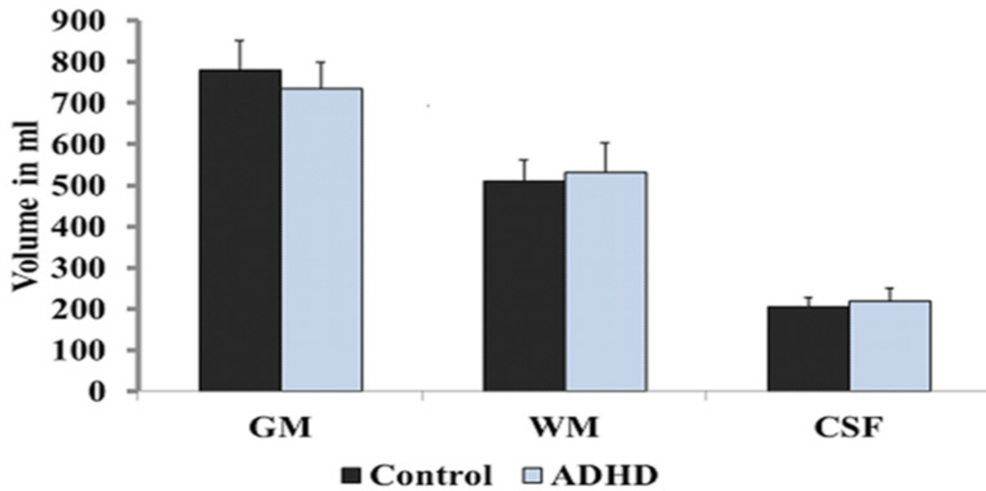

Fig. 1 : Comparison of global regional volume between ADHD patients (n=15) and controls (n=15). GM-gray matter; WM-white matter; CSF-cerebro spinal fluid.

TABLE II : Region specific GM volume deficits in ADHD compared to controls [n=15/group].

Brain regions	*BA*	*Coordinates (MNI)*[†]			*Number of voxels in cluster*	*t-score*[‡]
		X	*Y*	*Z*		
Left cingulate gyrus	–	–17	–40	36	2965	5.65*
Right middle cingulate gyrus	24	3	–13	39	2965	5.14*
Left superior frontal gyrus	–	–23	56	31	176	5.29*
Left medial superior frontal gyrus	9	0	54	5	212	5.10*
Right medial superior frontal gyrus	9	3	33	39	125	4.96*
Left temporal lobe - sub-gyrus	–	–38	–63	4	264	4.89*
Left middle occipital gyrus	–	–41	–75	4	264	4.76*
Left cuneus	18	–12	–88	12	125	4.66*
Left superior temporal gyrus	39	–45	–61	28	183	4.42*
Left temporal supra marginal gyrus	–	–50	–55	24	183	4.01*

[†]MNI-Montreal Neurological Institute coordinates of peak difference, [‡]t-score indicates significant deficits after false discovery rate correction over the whole brain. BA – Bradman area, *P<0.05.

TABLE III : Increase in GM volume of right thalamus in ADHD compared to controls (n=15/group).

Brain regions	*BA*	*Coordinates (MNI)*†			*Number of voxels in cluster*	*t-score*‡
		X	*Y*	*Z*		
Right Thalamus-Sub lobar region	–	23	–33	–2	36	3.98*

†MNI-Montreal Neurological Institute coordinates of peak difference. ‡t-score indicates significant deficits.BA – Bradman area, *P<0.001 (Uncorrected).

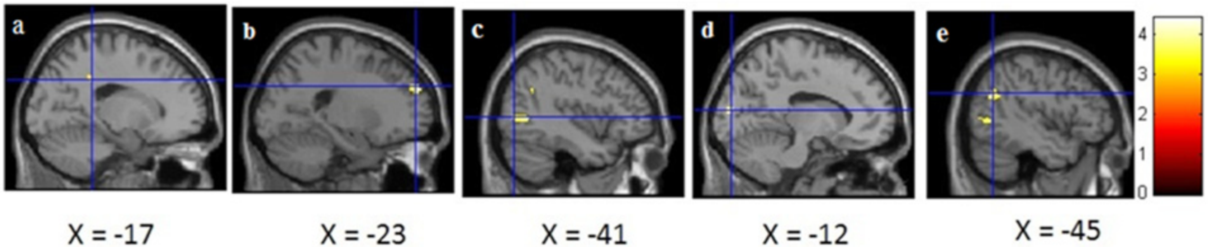

Fig. 2 : Representative sMRI sagittal views of ADHD patients showing regional GM volume deficits in the a. Left cingulate gyrus (X=–17), b. left superior frontal cortex (X=–23), c.Left middle occipital gyrus (X=–41), d. Left cuneus (X=–12), and e. Left superior temporal gyrus (X=–45). Color bar indicates t-score values; t-score values >0 indicates a deficit and <0 indicates an increase in GM volume.

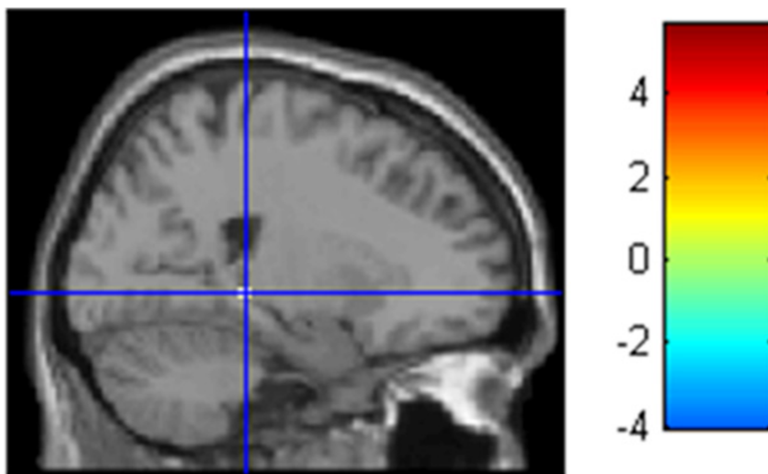

Fig. 3 : Representative sMR sagittal image of ADHD patient showing increase in GM volume at the right thalamus - sublobar region (X = 23). Color bar indicates t-score values; t-score values >0 indicates a deficit and <0 indicates an increase in GM volume.

study suggests involvement of occipital cortex also, in the development of behavioral signs and symptoms in ADHD children.

DISCUSSION

A deficit in the left middle occipital cortex

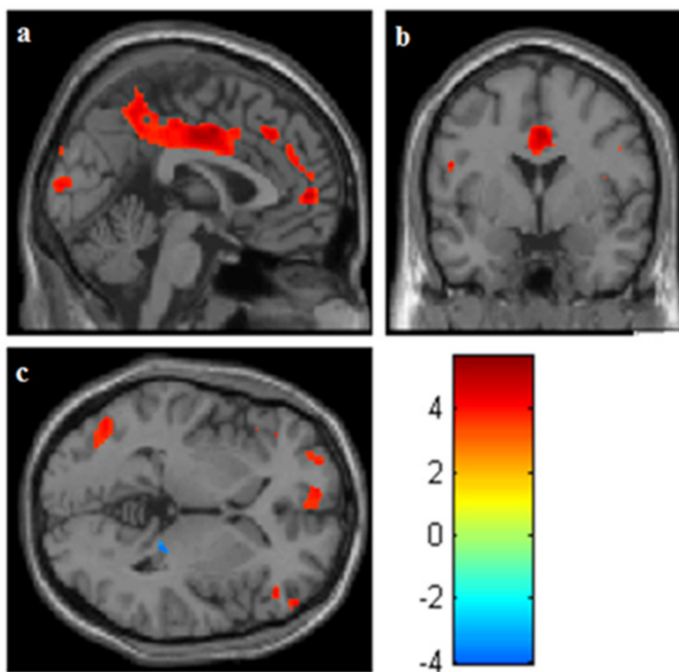

Fig. 4 : Representative sMRI a. sagittal view, b.coronal view, c. axial view overlapped with single subject MNI template showing overall regional GM volume deficits in ADHD patients compared to controls. Color bar indicates t-score values; t-score values >0 indicates a deficit and <0 indicates an increase in GM volume.

Region-wise gray matter volume alterations in brain of adolescents with attention deficit hyperactive disorder: A voxel based morphometric analysis.

and left cuneus region was detected by VBM technique in addition to other regions in this study, suggesting involvement of occipital region in development of signs and symptoms of attention deficit in combined type ADHD. Though behavioral data of the selected adolescent ADHD subjects in this study was not available from the downloaded dataset, the detected deficits in volumes of occipital and cuneus regions in these ADHD subjects suggests early signs of abnormalities in visual sensory processing, a possible cause for impairment in attention mechanism. Alternately, few NeuroImaging studies specifically indicate increase in occipital gray matter (GM) volume in ADHD (19, 20), though in the same studies regional volume deficits in other regions of brain was observed.

In support of our findings, Ahrendts et al (21) described visual cortex abnormalities in sMRI of adults and Durston S et al (22) observed left occipital GM and WM volume reduction in adolescents with ADHD and they suggest that these abnormalities may be related to pathophysiology of ADHD. Moreover, EEG studies on children with ADHD by Nazari et al (23) suggested early deficits in electrical activity and visual sensory integration in occipital cortex. Alternately, other studies on children with ADHD did not find EEG correlations for cued targets, suggesting a functional disconnection between frontal and occipital cortex in them (24).

In addition deficits in GM volume of bilateral cingulate gyrus and bilateral medial superior frontal regions in ADHD were also found in the present study. Many studies report that these regions are involved in alerting, executive attention functions and behavioral inhibition, which are necessary for working memory. These regions are also important in higher-level cognitive functions but basically deals with primary stimuli such as reward (25). A decrease in GM volume of left temporal region that is reported to be associated with verbal strategies was also observed in the present study similar to recent studies on ADHD children with history of low birth weight by Sasayama et al (26).

Alternately, an increase in GM volume of sub-lobar region of right thalamus was also found in ADHD subjects, consistent to other studies reporting regional increase in pulvinar volume following stimulant drug treatment (27). Studies by Cortese et al (28) suggest that low iron in the thalamus may contribute to ADHD pathophysiology.

Along with regional alterations, global GM volume deficits were also found in the present study. Individuals with ADHD tend to activate a more diffuse, wider system of brain region to perform a task (14, 29-31) whereas patterns of functional brain activity becomes less diffuse and more focal with maturation and is associated with development in children (20). Similar GM volume deficits have been reported in various other sMRI imaging studies (13, 30) on ADHD patients. The study by Castellanos et al. (32) on brain development in ADHD during childhood and adolescence report that developmental trajectories of all structures, except caudate showed smaller volumes suggesting that genetic and/or early environmental influences are fixed, non-progressive, and unrelated to stimulant treatment. Brain-imaging data in adults suggest that the pathophysiological principles of ADHD do not profoundly change from childhood and adolescence to adulthood, regardless of some changes in psychopathology (33).

Other meta-analytical studies on sMRI of ADHD children have identified right putamen/globus pallidus regional GM reduction indicating involvement offronto-striatal circuit (34, 35). Similar changes were not observed in the present study.

Conclusion

In conclusion the present study highlights the importance for VBM analysis of sMRI images clinically diagnosed with ADHD, as an advanced NeuroImaging analytical tool to detect any specific GM volume alterations of brain regions involved in attention and working memory function. Moreover, this study also reports detecting specific deficits in GM volume in the occipital region of adolescents with combined type ADHD, suggesting possible defects in visual processing and shorter attention span. However, to confirm this finding, additional detailed VBM analytic studies correlated with behavioral data need to be conducted on a larger cohort of sMRI from adolescents with combined type ADHD. Thus, VBM studies may be helpful in detecting specific focal gray matter (GM) volume deficits in regions of brain sMRI of ADHD, possibly aiding for appropriate treatment protocols.

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This VBM analytic study was carried out using downloaded datasets from the International NeuroImaging Data-sharing Initiative (INDI), NeuroImage webpage (http://fcon_1000.projects.nitrc.org/indi/adhd200). INDI allows contributing or downloading MRI datasets, which are permitted to be used for publication under Creative Commons License (Attribution Non-Commercial license). The authors gratefully acknowledge the permission given to use these downloaded MRI datasets for publication.

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PROTECTIVE EFFECT OF α-TOCOPHEROL AGAINST HEMATOTOXICITY, HEPATOTOXICITY AND NEPHROTOXICITY INDUCED BY NICKEL SULFATE IN MALE ALBINO RATS

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Abstract : Among the chemical hazards, heavy metal like nickel (Ni) is considered to be a serious one. It induces severe liver and kidney damage by altering several marker enzymes and ascorbate-cholesterol metabolism. The objective of the study was to investigate the possible protective role of α-tocopherol on $NiSO_4$ (Ni II) exposed alteration of hematological parameters, markers of liver and kidney functions, hepatic and renal antioxidant defense system in male albino rats. We have studied the effects of α-tocopherol supplementation on nickel sulfate induced alteration of body weight, hematology, liver and kidney toxicity markers (SGOT, SGPT, total protein, urea, creatinine) and hepatic and renal antioxidant defense system of male albino rats. Nickel toxicity results in decreased body weight gain and relative liver and kidney weight. Nickel treatment also resulted in alteration of hematological parameters along with increased liver and kidney toxicity markers. Nickel sulfate administration significantly increased the level of lipid peroxides and decreased antioxidant enzyme activities in hepatic and renal tissue. Simultaneous treatment with á-tocopherol exhibited a possible protective role on the toxic effect of nickel on body and organ weights, hematological parameters, SGPT activity and improved tissue antioxidant defense system. α-tocopherol, may partially prevent nickel induced alteration of hematological and biochemical parameters as well as have amelioratic effects on nickel induced alteration of antioxidant status of liver and kidney.

Key words : α-tocopherol antioxidant defense hematology nickel sulfate SGOT/SGPT

INTRODUCTION

Nickel is a naturally occurring element, which can be found, in all environmental media. Nickel is released into environment through the extraction, processing and use

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of nickel compounds. After entering into the body nickel penetrates all organs, accumulating primarily in bone, liver and kidney and excreted in bile and urine (1). If nickel enters and accumulates in the tissue faster than the rate at which the body's detoxification pathways can dispose it, a gradual build up of toxins may occur.

It is interesting to note that nickel partially facilitates iron absorption from GIT (directly as ferric form) and helps hematopoiesis particularly when iron status is low in blood. But animal studies have shown that high dietary nickel may adversely affect hematopoiesis when in conjunction with marginal iron status (2). Nickel implanted rats showed a significant decrease of red blood cells, hemoglobin and haematocrit at the time of morbidity, including possible nickel induced anemia (3). Nickel induced severe liver and kidney damage by altering several marker enzymes like SGOT, SGPT and ascorbate-cholesterol metabolism has been reported earlier (4). Nickel sulfate administration to male albino rat significantly increased the lipid peroxide (LPO) level and simultaneously decreases the antioxidant enzyme activities (5).

A number of oxygenated compounds are produced during the attack of free radicals against membrane lipoproteins and polyunsaturated fatty acids (PUFA). Malondialdehyde (MDA) is one of the aldehydes produced during this attack from PUFA. MDA may be an indicator of oxidative stress, as its concentration in tissues and plasma increases after the influence of intensified free-radical processes (6). The most plausible mechanism operating *in vivo* is the generation of reactive oxygen species (ROS), which may initiates lipid peroxidation, oxidative damage to macromolecules such as protein, DNA and cell damage and death (7). Liver the major site of detoxification is the primary target of environmental and occupational toxicity (8).

The body is, however, not defenseless when facing free radicals. The antioxidant system enables transformation of reactive forms of oxygen into inactive and harmless compounds or molecules. Natural antioxidant enzymes manufactured in the body provide an important defense against free radicals. Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) are the most important antioxidant enzymes.

Among the non-enzymatic antioxidants, vitamin E is listed; its activity has been studied to a reasonable extent. α-tocopherol (Vitamin E) is a natural component of membrane lipid bilayer and thus helps to maintain membrane stability. The molecular and cellular effects of vitamin E have been explained either by acting as an antioxidant preventing damage to membranes or proteins and regulating their activity by specifically scavenging reactive oxygen species or by interacting or regulating specific enzymes and influencing cellular structures (9).

It has been observed that nickel sulfate induces increase in concentrations of lipid peroxidation products with concomitant decrease in α-tocopherol concentration in the liver (10). α-tocopherol acting in conjugation with glutathione peroxide (GSH-Px) could directly reduce phospholipids hydroperoxides within the membrane and lipoproteins to inhibit lipid peroxidation (11).

Therefore the aim of the study was to evaluate the effect of nickel sulfate on body and organ weight, hematological parameters as well as its effects on liver and kidney tissues. Further to investigate whether the antioxidant vitamin E (α-tocopherol) has any protective effects against nickel induced toxicity.

MATERIAL AND METHODS

Adult (aged 60 to 70 d) laboratory-bred male Wister strain rats (160±5g) were fed with laboratory stock diet (consists of 70% carbohydrate, 7% fat, 18% protein, 4% salt mixture and 1% vitamin mixture) and water *ad libitum* for 7 days. The animals were kept in an air conditioned animal house maintained at 22°C to 24°C with ~70% relative humidity. The acclimatized animals were divided into four groups of six animals each. Group I served as untreated control. Group II rats were administered nickel sulfate ($NiSO_4$, $6H_2O$) (Sigma) in double–distilled water at a dose of 2.0 mg/100 g b.wt., (ip) on alternate days until the tenth dose (12). Group III rats were treated orally with á-tocopherol (13) at a dose of 10 mg/100 g. b.wt (im), and Group IV rats were given both nickel sulfate (2.0 mg/100 g. b.wt.; ip) and α-tocopherol simultaneously (10 mg/100 g.b.wt., im) on alternate days until the tenth dose. The dietary isocaloric status was maintained in each group by pair feeding technique. The entire animal experiments were performed according to the ethical guidelines suggested by ICMR and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Culture, Government of India. After treatment, the animals were sacrificed by cervical decapitation between 9:00 h and 11:00 h.

Measurement of body and organ weights:

All animals were weighed by automatic balance on day 1 of the nickel sulfate treatment and the day of sacrifice. After decapitation the animals were dissected, liver and kidney were excised and weighed after washing in ice-cold saline to the nearest of 0.1 mg in a single pan electronic balance. The percentage of body weight gain, hepatosomatic index and relative kidney weight were calculated. The weights of the tissues were expressed after correction for difference in body weight i.e. relative body weight (per 100 gm body weight).

Determination of hematological parameters:

About 2 ml of blood were collected in commercial tubes containing about 40 µl of potassium salt of EDTA as anticoagulant and analyzed within 24 h by fully automated hematological cell counter (Sysmax K-4500 of Transasia Ltd.) (14). The parameters measured were red blood cell (RBC) count, white blood cell (WBC) count, hemoglobin (Hb) concentration, packed cell volume (PCV%), mean cell volume (MCV), platelet count. The values of the mean corpusular hemoglobin (MCH) and MCHC were calculated. Each sample was run in duplicate.

Biochemical determination of markers:

Serum free of hemolysis separated from blood cells as soon as possible after collection. 2 to 3 ml of blood samples were centrifuged at 3000 rpm for 15 min and later the following parameters were analyzed.

Assay of serum aspartate aminotranseferase activity (SAST/SGOT): The serum AST or

SGOT activity has been measured by modified UV (IFCC), kinetic assay using a commercial kit (Span diagnostics Ltd, India) according to manufacturer's protocol (15).

Assay of serum alanine aminotranseferase activity (SALT/SGOT): The serum ALT or SGPT activity has been measured by modified UV (IFCC), kinetic assay using a commercial kit (Span diagnostics Ltd, India) according to manufacturer's protocol (16).

Estimation of serum total protein: Total serum protein has been measured by modified Biuret method with end-point colorimetry according to manufacturer's protocol using semi automated analyzer (17).

Estimation of serum urea: Serum urea has been measured by urease, Berthelot end point assay using a commercial kit (Span diagnostics Ltd, India) according to the manufacturer's protocol (18).

Estimation of serum creatinine: Serum creatinine was determined by modified Jaffe's reaction (initial rate assay) using a commercial kit (Span diagnostics Ltd, India) according to the manufacturer's protocol (19).

Biochemical determination of oxidant stress/ antioxidant status of tissues :

The tissues of each side (liver, kidney) of each group were separately dissected out, washed in ice-cold saline, and weighed. Approximately 100 to 150 mg tissue was homogenized in 0.05 M Tris-Cl/1 mM EDTA (pH 7.0) buffer by Polytron (setting 7, 20 sec). The homogenate was centrifuged for 20 min at 3,000 × g at 40°C, and the supernatant was used for assaying antioxidant status.

1. Concentration of TBARS level: Lipid peroxidation was estimated by measuring thiobarbituric acid reactive substances (TBARS) and was expressed in terms of malondialdehyde (MDA) content, according to the method of Ohkawa et al (20). Lipid peroxide level was expressed in terms of µmoles TBA reactants/g protein and were estimated in terms of 2-thiobarbituric acid reactants, using 1,1',3,3', tetramethoxypropane as standard.

2. The glutathione level was measured by the method of Moron et al. (21). In this method 5, 5-dithiobis 2-nitrobenzoic acid (DTNB) was reduced by -SH groups to form 1 mole of 2-nitro-5-mercaptobenzoic acid per mole of SH. The nitro mercaptobenzoic acid anion released has intense yellow color and can be used to measure -SH groups at 412 nm. The unit was expressed as µmoles/g tissue weight.

3. The activity of superoxide dismutase: Total (Cu-Zn and Mn) superoxide dismutase activity was determined according to the method of Misra and Fridovich (22). The ability of superoxide dismutase to inhibit the auto oxidation of epinephrine at pH 10.2 has been used as the basis of a convenient and sensitive assay for this enzyme. One unit of SOD activity was defined as the amount of enzyme that inhibited the oxidation of epinephrine by 50%. Activity was expressed as units/mg of protein.

4. The activity of glutathione peroxidase: GSH-Px activity was determined using a modification of the method of Flohé and Günzler (23). The millimolar extinction

coefficient of 6.22 mM/cm was used to determine the activity of GSH-Px. Activity was expressed as units/mg of protein.

5. The activity of catalase: Catalase activity was determined by the method of Aebi (24). The molar extinction coefficient of 43.6 mM/cm was used to determine CAT activity. CAT activity was expressed as units/mg of protein.

6. Protein was determined by the method of Lowry (25).

Statistical analysis :

The data, obtained from all the control and experimental samples, has been subjected to statistical analysis. Mean±SD values were calculated for each group. To determine the significance of inter-group differences, we analyzed each parameter separately. Analysis of data was done by one-way ANOVA and post-hoc by Tuky-Krammer test by using statistical software (Stat Pac for Windows, Version 11.0). The level of significance was fixed at $P<0.05$.

RESULTS

Evaluation of body and organ weights:

No death was observed in any of the experimental groups. Table I shows that nickel sulfate treatment in Group II produced a significant decrease in final body weight as compared to untreated control (Group I). But simultaneous administration of α-tocopherol with nickel sulfate in Group IV rats, the decrease in final body weight improved remarkably. There was no significant difference in final body weight of nickel + α-tocopherol rats with control rats. When compared to respective initial body weight, control, α-tocopherol and nickel +

TABLE I: Changes in body and organ weight of male rats after nickel sulfate treatment alone or in supplementation with α-tocopherol.

Body/ Tissue weight	*Untreated control*	*Nickel sulfate*	*α-tocopherol*	*Nickel sulfate + α-tocopherol*	*F-ratio & P value*
(i) Initial Body wt (g)	179.67±6.334	184.00±2.583	180.50±3.528	$188.67±3.422^{*}$	F=5.678 P=.0056
(ii) Final Body wt (g)	247.83±12.776	$167.67±4.154^{****}$	$247.33±5.110^{++++}$	$221.00±10.89^{***++++\$\$\$}$	F=104.784 P=0.0000
% of body wt gain [(i) vs. (ii)]	38.30±7.732	$–8.67±3.309^{****}$	$37.26±3.699^{++++}$	$16.98±4.790^{****++++\$\$\$\$}$	F=108.896 P=0.0000
Absolute liver wt (g)	6.90±0.095	$4.65±0.143^{****}$	$6.92±0.136^{++++}$	$6.07±0.109^{*+++\$}$	F=28.623 P=0.0000
Hepatosomatic index (g/100 g body wt)	2.75±0.051	2.77±0.049	2.796±0.078	2.79±0.180	F=0.204 P=0.8926
Relative Kidney wt (g/100 g body wt)	0.38±0.012	$0.57±0.024^{****}$	$0.37±0.017^{++++}$	$0.51±0.038^{****++\$\$\$\$}$	F=94.821 P=0.0000

Group I – Control, Group II – Nickel treated, Group III - α-tocopherol treated, Group IV – Nickel sulfate + α-tocopherol. Each value is mean±SD of six observations in each group. Analysis of data was done by one-way ANOVA and post-hoc by Tuky-Krammer test. The * depicts comparison with Group I (* P< 0.05, **P<0.01, ***P<0.001, ****P<0.0001); + depicts comparison with Group II ($^{+}$P<0.05, $^{++}$P<0.01, $^{+++}$P<0.001, $^{++++}$P<0.0001); $ depicts comparison with Group III ($^{\$}$P<0.05, $^{\$\$}$P<0.01, $^{\$\$\$}$P<0.001, $^{\$\$\$\$}$P<0.0001).

α-tocopherol rats showed a significant difference between initial and final body weight. A significant decrease in absolute liver weight was found in nickel treated rats in comparison with control groups although the hepatosomatic index of the nickel treated group was non-significant as compared with control. This may be indicative of more a proportional decrease in liver weight with decrease in respective body weight. Simultaneous treatment with α-tocopherol in Group IV rats had improved the absolute liver weight as well as hepatosomatic index up to the level of control group. There was also a significant increase in relative kidney weight in nickel treated group of rats compared to control group. In nickel + α-tocopherol treated rats, simultaneous α-tocopherol administration did not show any significant changes from Group II rats.

Changes in hematological parameters:

Table II shows that nickel sulfate administration resulted in significant decrease of RBC count, haematocrit value (PCV %) and Hb concentration in nickel treated group of rats when compared to untreated control. Nickel sulfate + α-tocopherol treated group also showed a significant decrease of RBC count, PCV % and Hb concentration in comparison with their control but when compared with nickel alone treated rats, a significant increase of

TABLE II : Changes in hematological parameters of male rats after nickel sulfate treatment alone or in supplementation with á-tocopherol.

Hematological parameters	*Untreated control*	*Nickel sulfate*	*α-tocopherol*	*Nickel sulfate + α-tocopherol*	*F-ratio & P value*
RBC (10^6 cell/μL)	8.46±0.39	5.59±0.39****	8.63±0.30^{++++}	7.03±0.34$^{****++++\$\$\$\$}$	F=94.886 P=0.0000
Hemoglobin (gm/dL)	18.07±0.27	12.39±0.64****	18.18±0.33^{++++}	14.99±0.49$^{****++++\$\$\$\$}$	F=221.83 P=0.0000
PCV (%)	49.38±0.96	41.74±0.86****	49.80±0.76^{++++}	45.96±0.44$^{****++++\$\$\$\$}$	F=137.914 P=0.0000
Clotting time (min)	4.21±1.14	7.61±0.28****	4.55±0.09^{++++}	7.23±0.27$^{****\$\$\$\$}$	F=51.387 P=0.0000
WBC (10^3 cell/ μL)	9.30±0.35	4.91±0.14****	9.31±0.22^{++++}	6.20±0.21$^{****++++\$\$\$\$}$	F=507.926 P=0.0000
Platelets (10^3 cells/μL)	866.75±24.87	523.33±27.89****	843.64±21.10^{++++}	566.67±21.86$^{****+\$\$\$\$}$	F=336.043 P=0.0000
MCV (fL)	58.93±2.79	76.52±5.50 ****	61.58±5.11 $^{+++}$	66.04±2.87 $^{*++}$	F=19.891 P=0.0000
MCH (pg)	21.64±1.26	22.37±0.87 ^{a}	21.18±0.66 ^{a}	21.19±1.10 ^{a}	F=1.882 P=0.1651
MCHC (g/dL)	36.65±0.75	32.55±2.32 ***	36.58±1.02 $^{+++}$	32.62±0.97 $^{***\$\$\$}$	F=16.032 P=0.0000

Group I – Control, Group II – Nickel treated, Group III – α-tocopherol treated, Group IV – Nickel sulfate + α-tocopherol. RBC, Red blood corpuscles; PCV, Packed cell volume; WBC, White blood corpuscle; MCV, Mean corpuscular volume; MCH, Mean corpuscular haemoglobin; MCHC, Mean corpuscular haemoglobin concentration. Each value is mean±SEM of six observations in each group. Each value is mean±SD of six observations in each group. Analysis of data was done by one-way ANOVA and post-hoc by Tuky-Krammer test. The * depicts comparison with Group I (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001); + depicts comparison with Group II ($^{+}$P<0.05, $^{++}$P<0.01, $^{+++}$P<0.001, $^{++++}$P<0.0001); $ depicts comparison with Group III($^{\$}$P<0.05, $^{\$\$}$P<0.01, $^{\$\$\$}$P<0.001, $^{\$\$\$\$}$P<0.0001).

all the above mentioned parameters were noticed. No significant alterations of any of those parameters were found in case of only α-tocopherol treated rats when compared with untreated control rats.

Table II also depicts significant increase of clotting time (CT) followed by decrease of platelet count and WBC count in nickel treated rats in comparison to untreated control. In case of nickel + α-tocopherol treated rats a significant increase in clotting time and decrease of platelets count and WBC count were noticed when compared with control rats but when it was compared with nickel alone treated rats a significant rise in WBC count were found. No significant changes were found in any of these parameters in only α-tocopherol supplemented rats when it was compared with untreated control. In nickel treated rats, MCV was increased significantly when compared to control group of rats. This alteration was not rectified even after simultaneous supplementation with α-tocopherol. MCH and MCHC values remained statistically unchanged in all the groups.

Evaluation of biochemical changes :

Table III shows a very significant increase in serum total protein level in nickel treated rats in comparison with untreated control. No significant difference of serum total protein level was noticed in nickel sulfate + α-tocopherol treated rats when it was compared to nickel alone treatment. Hepatotoxicity was monitored by quantitative analysis of SGOT and SGPT activities, which were used as biochemical markers of liver damages. The activity of both SGOT and SGPT in rats after nickel treatment had increased significantly from that of control group (Table III). In nickel + α-tocopherol treated rats, the SGOT activity did not show any significant improvement from that of nickel alone treated rats. Whereas SGPT activity improved significantly in nickel + α-tocopherol treated rats when compared to nickel treatment alone.

TABLE III : Changes in SGOT and SGPT activities, serum total protein, urea and creatinine value of male rats after nickel sulfate treatment alone or in supplementation with á-tocopherol.

Treatment group	*Untreated control*	*Nickel sulfate*	*α-tocopherol*	*Nickel sulfate + α-tocopherol*	*F-ratio & P value*
SGOT (U/L)	276.483 ± 14.208	$330.117 \pm 7.837^{****}$	$277.667 \pm 10.502^{++++}$	$316.800 \pm 9.045^{***\$\$\$}$	F=39.393 P=0.0000
SGPT (U/L)	41.02 ± 3.461	$85.542 \pm 4.384^{****}$	$36.767 \pm 2.147^{++++}$	$69.333 \pm 4.061^{****++++\$\$\$\$}$	F=248.866 P=0.0000
Total protein (mg/dL)	7.30 ± 0.245	$11.77 \pm 1.029^{****}$	$7.14 \pm 0.237^{++++}$	$10.19 \pm 0.302^{****\$\$\$\$}$	F=96.83 P=0.0000
Urea (mg/dL)	42.280 ± 1.049	$48.193 \pm 0.429^{****}$	$40.662 \pm 1.347^{*++++}$	$46.997 \pm 0.653^{****\$\$\$\$}$	F=89.487 P=0.0000
Creatinine (mg/dL)	0.643 ± 0.024	$1.515 \pm 0.131^{****}$	$0.632 \pm 0.045^{++++}$	$1.305 \pm 0.053^{****+++\$\$\$\$}$	F=219.351 P=0.0000

Group I – Control, Group II – Nickel treated, Group III – α-tocopherol treated, Group IV – Nickel sulfate + α-tocopherol. Each value is mean±SD of six observations in each group. Analysis of data was done by one-way ANOVA and post-hoc by Tuky-Krammer test. The * depicts comparison with Group I (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001); + depicts comparison with Group II ($^{+}$P<0.05, $^{++}$P<0.01, $^{+++}$P<0.001, $^{++++}$P<0.0001); $ depicts comparison with Group III ($^{\$}$P<0.05, $^{\$\$}$P<0.01, $^{\$\$\$}$P<0.001, $^{\$\$\$\$}$P<0.0001).

Table III also shows significant increases in serum total protein, urea and creatinine levels in nickel treated rats in comparison with untreated control. No significant difference was noticed in these three parameters in nickel sulfate + α-tocopherol supplemented rats when compared to nickel treatment alone.

Hepatic and renal oxidant stress/antioxidant status :

Table IV shows that nickel induced a significant increase in hepatic and renal lipid peroxide levels in comparison with their respective controls, whereas in nickel sulfate + α-tocopherol treated rats both hepatic and

TABLE IV : Changes in hepatic and renal antioxidant status of male rats after nickel sulphate treatment alone or in supplementation with á-tocopherol.

Tissue analyzed	*Treatment group*	*Untreated control*	*Nickel sulfate*	*α-tocopherol*	*Nickel sulfate + α-tocopherol*	*F-ratio & P value*
Liver	Lipid peroxide (µmoles TBA reactants/g protein)	4.02±0.16	7.28±0.33****	3.95±0.27^{++++}	4.27±0.25^{++++}	F=229.32 P=0.0000
	Glutathione (µmoles/g tissue weight)	5.51±0.30	3.52±0.17****	5.28±0.15^{++++}	4.57±0.43$^{***+++\$\$}$	F=58.848 P=0.0000
	GSH-Px (units/mg protein)	0.09±0.004	0.05±0.002****	0.09±0.003^{++++}	0.07±0.006$^{****++++\$\$\$\$}$	F=135.387 P=0.0000
	SOD (units/mg protein)	63.67±2.51	44.26±3.55****	63.00±2.97^{++++}	55.26±2.97$^{***+++\$\$}$	F=53.636 P=0.0000
	CAT (units/mg protein)	8.54±0.40	4.84±0.53****	8.51±0.48^{++++}	6.09±0.66$^{****++\$\$\$\$}$	F=73.322 P=0.0000
Kidney	Lipid peroxide (µmoles TBA reactants/g protein)	4.59±0.63	7.63±0.45****	4.31±0.43^{++++}	5.37±0.48$^{*++++\$\$}$	F=53.576 P=0.0000
	Glutathione (µmoles/g tissue weight)	5.09±0.41	3.86±0.35***	5.05±0.22^{+++}	4.41±0.54$^{*++\$}$	F=13.02 P=0.0001
	GSH-Px (units/mg protein)	0.097±0.007	0.038±0.009****	0.099±0.004^{++++}	0.061±0.010$^{****+++\$\$\$\$}$	F=87.162 P=0.0000
	SOD (units/mg protein)	120.89±9.01	73.66±2.08****	116.72±5.41^{++++}	95.30±8.84$^{***+++\$\$\$}$	F=58.942 P=0.0000
	CAT (units/mg protein)	16.07±2.77	8.83±1.05****	15.34±0.85^{+++}	11.28±1.38$^{***+\$}$	F=24.69 P=0.0000

Group I – Control, Group II – Nickel treated, Group III – α-tocopherol treated, Group IV – Nickel sulfate + α-tocopherol, SOD, superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase; TBA, 2-thiobarbituric acids. Each value is mean±SEM of six observations in each group. In each column, values with different superscripts (a, b, c) were significantly different from each other (P<0.05). Each value is mean±SD of six observations in each group. Analysis of data was done by one-way ANOVA and post-hoc by Tuky-Krammer test. The * depicts comparison with Group I (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001); + depicts comparison with Group II (+P<0.05, ++P<0.01, +++P<0.001, ++++P<0.0001); $ depicts comparison with Group III ($P<0.05, $$P<0.01, $$$P<0.001, $$$$P<0.0001).

renal lipid peroxide level decreased significantly when compared with nickel treatment alone. The result shows that after nickel treatment, both the liver and kidney SOD, CAT, and GSH-Px activities were significantly suppressed in the experimental nickel treated rats in comparison with the control. In nickel sulfate + α-tocopherol combined treated rats an improvement of all antioxidant enzyme activities of liver and kidney were noticed in comparison with only nickel treatment. In case of liver and kidney glutathione concentration, a significant reduction of GSH was observed after nickel treatment but simultaneous treatment with á-tocopherol the GSH concentrations were found to be improved significantly in both liver and kidney. Rats of only α-tocopherol treatment did not show any significant variation in the parameters studied above when compared with their respective controls.

DISCUSSION

Nickel induced growth retardation and consistent reduction in body weight is already reported as it is found in our studies (26). Simultaneous treatment with α-tocopherol had shown a beneficial effect on the percentage decrease in body weight after nickel treatment. α-tocopherol induced restoration of growth rate has been reported in earlier studies (27). In the current study, administration of nickel sulfate also induced a significant decrease in absolute liver weight with a non-significant change in hepatosomatic index. The decreased absolute liver weight may be indicative to the degenerative effect of nickel sulfate on hepatic tissue, as it is a major site of metabolism. A significant increase in relative kidney weight after nickel sulfate treatment may be due to renal inflammatory response.

The results obtained in our present study show that the treatment with nickel sulfate induces anemia (decrease RBC count, PCV% and Hb concentration) in rats. Nickel implanted rats showed a significant decrease of red blood cells, hemoglobin and haematocrit at the time of morbidity (5). In our study the decrease in RBCs count, PCV% and hemoglobin concentration may be due to non-regenerative anemia arising from nickel induced direct injury of hematopoietic stem cells resulting in decreased erythrocyte, leucocyte and platelet count. Effects on blood hemostatic mechanisms are assessed by both clotting time determination and platelet count. Decreased production or increased consumption of platelets may lead to fall of platelet count. The leucopenia after nickel treatment may be attributed to the inhibition of white blood cell maturation, their release from tissue reservoir or occurrence of leucopoenia by an organism as a response to a stress caused by toxic compounds associated with allergic reaction (5, 27). A decrease in hemoglobin concentration may be due to increased rate of destruction or reduction in rate of formation of RBC. In addition, reduction in the blood parameters (RBC count, Hb concentration, PCV %) may be attributed to hyperactivity of bone marrow leading to production of RBC with impaired integrity that easily destructed in the circulation (5). Simultaneous treatment of α-tocopherol decreased the toxic effects of nickel sulfate on hematological values and also showed a protective role in anemia and leucopenia. An inverse correlation between increased lipid peroxidation and α-tocopherol

levels have been found in bone marrow of nickel chloride treated rats as well as in blood (28). Our study supports the previous observations which show a significant increase in RBC count, PCV% and Hb concentration after α-tocopherol supplementation in athletes who are exposed to exercise induce oxidative stress (28). This could be attributed to the protective effect of α-tocopherol.

Increased activity of both SGOT and SGPT after nickel treatment may be due to leakage of enzymes from liver cytosol into the blood stream giving an indication on the hepatotoxic effect of nickel (27). Following cell damage, the membranes become permeable and enzyme activity is found in the extra cellular fluid and serum, so the highest activity of alanine amino transferase was recorded in the serum. Similar results are published by Sindhu et al (29). Activity of SGPT was increased significantly following nickel treatment to normal rats. The improvement of SGPT activity towards control value in the rats simultaneously treated with α-tocopherol proved the hepatoprotective effect of α-tocopherol due to its antioxidant properties.

In our study, serum protein level had increased significantly after nickel sulfate treatment. This is in agreement with Gopal et al. who recorded that the exposure of *Caprinus carpio* to heavy metal salts (Cu and Ni) at lethal and sublethal concentrations induced an increase in total protein (30). Although most of the earlier studies showed a decrease in the level of the serum protein after metal treatment but in this study the increase in level of serum protein after nickel exposure may be due to the liberation of synthesized proteins as a result of cytolysis and to other pathological changes manifested in the liver tissue associated with progression of the toxicity condition (27).

A significant rise in serum urea and creatinine with nickel treatment in our study indicates that, synthesized proteins were liberated as a result of cytolysis and other pathological changes. It has been reported that low-level of oral exposure to soluble nickel either induces changes of glomerular permeability or enhances the normal age-related glomerular nephritis lesions in ageing rats (27). The hyperactivity of renal tubules as well as altered glomerular filtration altogether may have increased the serum urea and creatinine level in our study. Administration of α-tocopherol could not decrease serum urea and creatinine level when given simultaneously with nickel. A study by El-Demerdash et al. realized similar results like ours (31). Although α-tocopherol, as antioxidant, acted as a preventive measure in heavy metal induced lipid peroxidation in renal tissues, but in this study vitamin supplementation could not show any improvement in rise in serum urea and creatinine level which may be due to non-interference of these antioxidant vitamins on protein catabolism pathway as such.

The increased lipid peroxidation in the liver of nickel-treated rats suggests an increase in phospholipase activity during peroxidic decomposition of different sub organelle and plasma membrane lipids. These changes may be attributed to the effect of

nickel on the hepatic cells. Following cell damage, the membranes become permeable and enzyme activity are found in the extracellular fluid and serum (8, 27). Injury from nickel exposure may also be due to activation of Kupffer cells and a cascade of events involving several types of hepatic cells and a large number of inflammatory and cytotoxic mediators. In the nickel-treated rats, the decreased activities of hepatic SOD, CAT, and GSH-Px, suggests an interaction between the accumulated free radicals and the active amino acids of these enzymes. Nickel-induced decrease in glutathione levels may be due to its increased use in protecting the –SH-containing proteins from lipid peroxides. In Group IV rats a significant improvement in the activity of these antioxidant enzymes compared to Group II could be due to the relative ability of α-tocopherol to scavenge reactive oxygen species within the lipid region of the membrane. The α-tocopherol acting in conjugation with GSH-Px could directly reduce phospholipids hydroperoxides within the membrane and lipoproteins to inhibit lipid peroxidation (32).

The increase in lipid peroxidation resulted from renal cell injury caused by the induction of the Fenton reaction, generating hydroxyl radicals. In the nickel-treated experimental group, the decreased activities of SOD, CAT, and GSH-Px suggests an interaction between the accumulated free radicals and the active amino acids of these enzymes (5). Like the other metabolic tissues, nickel-induced decrease in glutathione levels in kidney seen here may be due to its increased use in protecting –SH-containing proteins from lipid peroxides (6). Studies also revealed that pretreatment with α-tocopherol reduced significantly the lipid peroxidation of renal cells and renal dysfunction induced by renal ischemia-reperfusion in rats (27). It has been found that, higher doses of vitamins are effective to protect oxidative renal damage. The protection is mediated partially by preventing the decline of renal antioxidant status (33).

It may be concluded from present findings that nickel induces oxidative damage in erythrocytes and other major metabolic tissues like liver and kidney. This results in disruption of overall hematology and liver and kidney function and also disrupts tissue antioxidant defense system. But simultaneous treatment with á-tocopherol may protect against toxic influence on above stated hematological and biochemical parameters, and as well as protect from heavy metal induced lipid peroxidation in hepatic and renal tissues.

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IMPACT OF DIFFERENT STARTING DOSES OF ATORVASTATIN ON REACHING TARGET LOW DENSITY LIPOPROTEIN CHOLESTEROL LEVELS AND HEALTH RELATED QUALITY OF LIFE IN DYSLIPIDEMIC PATIENTS

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Abstract : Objectives : 1. To compare the percentage of patients that reach target LDL-C goals with 10 mg Vs. 20 mg of atorvastatin as a starting dose. 2. To compare Health Related Quality of Life (HRQOL) in patients on 10 mg Vs. 20 mg of atorvastatin.

Methods : A prospective, double blind, parallel groups, unicentric study of patients of dyslipidemia, randomized to receive atorvastatin 10 mg (n=75) or atorvastatin 20 mg (n=75) once daily for 12 weeks. Safety reporting of incidence of adverse events was done.

Results : Significantly more number of patients ($P<0.05$) reached target LDL-C levels at the end of 12 weeks in the 20 mg group (77.27% in the high risk group, 100% in moderately high risk group and 100% in the moderate risk group) when compared to 10 mg group (32% in the high risk group, 75% in moderately high risk group and 83.33% in the moderate risk group). Increase in HRQOL at the end of 12 weeks was also significantly greater ($P<0.001$) in 20 mg group (27.89%) vs. 10 mg group (19.26%).

Conclusions : Selecting the starting dose of atorvastatin according to the patients risk category (by using the Framingham's algorithm for calculating cardiovascular risk) and the percentage reduction in LDL required, will result in greater success in achieving LDL goals and better quality of life.

Key words : atorvastatin dyslipidemia LDL-C goal quality of life

INTRODUCTION

Dyslipidemia is a broad term that refers to a number of lipid disorders. Eighty percent of the lipid disorders are related to diet and lifestyle, whereas the other twenty percent are familial disorders (1). Statins are the most effective and best-tolerated agents for treating dyslipidemia. Statins are a class of cholesterol-lowering drugs that are competitive inhibitors of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-

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CoA) reductase, which catalyzes an early, rate-limiting step in cholesterol biosynthesis. Statins occupy a portion of the binding site in HMGCoA, thus blocking access to the active site (2). A number of trials have demonstrated the efficacy of statin treatment in reducing fatal and nonfatal coronary heart disease (CHD) events, strokes, and total mortality (3).

The effectiveness of statins in lowering low density lipoprotein cholesterol (LDL-C) varies per milligram dose. Hence, for each statin, the dose required to achieve a target LDL-C reduction varies. Achieving LDL-C targets can be a long and difficult process. Target LDL-C levels are frequently not achieved in patients who require large reductions in LDL-C. Several dose titrations, typically months apart, are often required and some patients still do not reach the target. A treatment strategy that helps patients quickly achieve their LDL-C target will therefore not only have a positive clinical impact, as illustrated by recent studies comparing aggressive atorvastatin treatment with usual care (4, 5), it may also increase adherence to treatment.

Current statistics show that many dyslipidemic patients are not even diagnosed with dyslipidemia (6, 7, 8). Of those patients that are diagnosed, only a few receive treatment for this condition (9, 10). Current management of dyslipidemia is sub-optimal. Treatment of dyslipidemia is likely to reduce preventive morbidity and premature mortality and decrease the health care costs of expensive cardiovascular and hence it is important to treat dyslipidemia aggressively (5, 6, 7).

Despite an increasing body of evidence on the benefit of lowering elevated levels of low-density lipoprotein cholesterol (LDL-C), there is still considerable concern that patients are not achieving target LDL-C levels (11). The INTERHEART study demonstrated that traditional cardiovascular disease risk factors play an important role in the prediction of myocardial infarction in populations around the world, including South Asians (12). Multiple studies of migrant South Asian populations have, however, confirmed a 3- to 5-foldincrease in the risk for myocardial infarction and cardiovascular death as compared with other ethnic groups (13, 14, 15). Hence it is extremely important to study the how many patients of dyslipidemia would reach their target LDL- goals in the Indian context. To our knowledge there is currently no such study to test the efficacy of atorvastatin to reach target LDL levels in statin naïve Indian patients.

It is currently unknown whether aggressive lipid-lowering with a higher atorvastatin dose is indicated in Indian patients of dyslipidemia who have not had an acute coronary event (for e.g. myocardial infarction or stroke) in the past. Hence it would be worthwhile to compare the dose related effects of atorvastatin on lowering of lipid levels in patients of dyslipidemia who have not suffered from history or clinical evidence of myocardial infarction, unstable angina, stable angina, coronary artery procedures (angioplasty or bypass surgery), or clinically significant myocardial ischemia to determine the number of patients reaching the target LDL goals. It was also interesting to see the effect on the quality of life in patients receiving treatment at a

higher dose as compared to patients receiving treatment at a lower dose. The benefits of a higher dose therapy should be weighed carefully against the risk of adverse events. Hence, we also proposed to monitor the patients for the incidence of adverse events while on therapy with a higher dose and a lower dose of statin.

Hence the objectives of the above study were to compare the percentage of patients who reach LDL-C goals with 10 mg vs. 20 mg of atorvastatin as starting dose and to compare the incidence of adverse events in patients with 10 mg vs. 20 mg of atorvastatin in patients who had not suffered from an acute coronary event in the past; and to compare the improvement in Quality of life in patients on 10 mg vs. 20 mg of atorvastatin by calculating the Health Related Quality of life (HRQOL) score.

METHODS

The project was completed in Department of Pharmacology, Jawaharlal Nehru Medical College (J.N.M.C.) and Department of Medicine, Acharya Vinoba Bhave Rural Hospital (A.V.B.R.H.), Sawangi (Meghe), Wardha, Maharashtra, India.

The study was initiated after permission from the institutional ethics committee and written, informed consent from all participating subjects. The study was carried out according to the Indian Council of Medical Research (ICMR) guidelines for Biomedical Research in Humans (2006); and in compliance with the ICH/GCP Guidelines.

This study was comparative, randomized, double blind, parallel groups, single centre study. The patients of dyslipidemia attending outpatient department (O.P.D.) of Medicine were enrolled into the present study.

Inclusion criteria

1. Patients ≥18 years of age of either sex.

2. Patients who are willing to give written informed consent.

3. Dyslipidemic patients eligible for lipid lowering therapy at baseline, as determined by NCEP ATP III guidelines

   - ≥2 NCEP risk factors that confer a 10-year risk for CHD of >20%, LDL-C ≥100 mg/dL (2.6 mmol/L). - High risk group

   - Or ≥2 NCEP risk factors that confer a 10-year risk for CHD of 10% to 20%, LDL-C ≥130 mg/dL (3.4 mmol/L)- Moderately high risk group.

   - Or ≥2 NCEP risk factors that confer a 10-year risk for CHD of <10%, LDL-C ≥160 mg/dL (4.1 mmol/L)- Moderate risk group

4. Patients willing to adhere to a low fat diet as advised by the study investigator.

Exclusion criteria

1. Patients with history or clinical evidence of myocardial infarction, unstable angina, stable angina, coronary artery procedures (angioplasty or bypass surgery), or evidence of clinically significant myocardial ischemia.

2. Patients with history or clinical evidence

of non-coronary forms of atherosclerotic disease (peripheral arterial disease, abdominal aortic aneurysm, carotid artery disease, transient ischemic attacks or stroke of carotid origin or 50% obstruction of a carotid artery).

3. Patients with uncontrolled diabetes (i.e. fasting blood sugar > 140 mg/dl).

4. Impaired hepatic function (aspartate aminotransferase [AST] or alanine aminotransferase [ALT] ≥2 × the upper limit of normal [ULN]).

5. Uncontrolled hypertension (diastolic blood pressure >95 mm Hg).

6. Evidence of gastrointestinal disease limiting drug absorption or partial ileal bypass.

7. Secondary causes of hyperlipoproteinemia, defined as uncontrolled primary hypothyroidism (thyroid-stimulating hormone ≥1.5 × ULN).

8. Individuals with decompensated kidney function blood urea nitrogen ≥30 mg/dL, creatinine ≥1.2 mg/dL, or creatine kinase (CK) ≥3 × ULN.

9. Any decompensated metabolic/hormonal disorders.

10. Patients with clinically significant respiratory or haematological disorder or any other severe concurrent illness, or acute infection, gram negative sepsis or cancer within past 5 years.

11. Patients with current use of lipid lowering drugs (example- bile acid sequestrant, fibrate, nicotinic acid, fish oil), CYP 3A4 inhibitor (including cyclosporine, itraconazole, ketoconazole, erythromycin, clarithromycin, macrolide antibiotics) or oral corticosteroid.

12. Intolerance to statins at any time in the past.

13. Recent major surgery/illness/tissue injury/muscle injury/extensive burns.

14. Family history or past history of muscular disorders.

15. Excessive physical exercise of any form.

16. Patients who have experienced any unexplained muscle pain, tenderness or weakness in the past.

17. Participation in any clinical trial in the past six months.

18. Pregnant/Lactating mothers and women of childbearing potential not using medically accepted methods of contraception.

19. Active alcohol intake.

20. Drug or medication abuse within the last 6 months or any condition that would indicate the likelihood of poor subject compliance.

21. Subjects not willing to comply with the procedures described in this protocol.

22. Any serious or unstable medical or psychological condition that in the opinion of the investigator would compromise the patients safety or successful participation in the trial for e.g. physical examination, laboratory test, ECG.

Study procedure

At baseline, a thorough clinical examination and ECG was done which was followed by the baseline investigations. Patients were evaluated for lipid profile, liver function tests (LFT), fasting and post prandial blood sugar level and serum creatinine. Blood samples were tested in the Central Research Laboratory, A.V.B.R. Hospital. The patients were advised a low fat diet and anthropometry measurements were taken. For each patient Health Related Quality of life (HRQOL) score was evaluated using SF 36-Item Health Survey questionnaire. Patients were classified into their risk category by using the online version of the 10-year risk calculator made available by The National Heart, Lung and Blood Institute based on the Framingham algorithm. [(hin.nhlbi.nih.gov/atpiii/calculator.asp)/ (hin.nhlbi.nih.gov/atpiii/riskcalc.htm)].

A total of one hundred and fifty (n=150) patients were enrolled in the study. They were randomly allocated into two groups of seventy five each, using a computer generated randomization chart. Drugs were in the form of tablets to be taken orally once a day, for a period of 12 weeks. Both 10 mg and 20 mg atorvastatin tablets for 12 weeks were packed in identical boxes. Allocation concealment was obtained by number coding the drug boxes containing 10 mg and 20 mg atorvastatin. Patients were given a unique alpha-numeric code consisting of their initials and the code of the drug box that they were given and a key to the code was prepared to identify the group to which the patients belonged after completion of the study. This key was not disclosed to the investigator during the conduction of the study. The drugs were given for 15 days to every patient by the clinical assistant. After every 15 days all patients were asked to come for follow up, refilling of medications and monitoring of adverse drug events. At the end of 4 weeks lipid profile estimation was done to ensure compliance to the medication and anthropometry measurement was done to ensure that the patient was following a low fat diet. At the end of study intervention (12 weeks) all the baseline investigations (lipid profile, liver function tests (LFT), fasting and post prandial blood sugar level, serum creatinine), anthropometry measurements , Health Related Quality of life (HRQOL) score evaluation using 36-Item Health Survey questionnaire were done (16). Patient counselling regarding diet and medication were done at the end of the study.

Testing for myalgia:

In order to test if the patients were suffering from myalgia; the power in the proximal muscles was tested by the investigator during each visit. A visual analogue scale was provided to determine the degree of myalgia experienced by the patients. Any patient experiencing myalgia was withdrawn from the study.

Outcome measures:

1. The percentages of subjects who reach LDL-C goals as per NCEP ATP III guidelines. [Time Frame: After 12 weeks of study drug]
2. Health Related Quality of life (HRQOL) score evaluation using SF 36-Item Health Survey questionnaire. [Time Frame: *Before* and at the *end* of the study after 12 weeks of study drug]

Sample size calculation :

A sample size of 120 subjects was considered adequate for the study statistical power (17). Assuming a dropout rate of 15 to 25% over a 12 weeks period, 150 patients were randomized to drug treatment to detect, with 90% power, a significant difference of 15% (P≤0.05) between each treatment arm.

Statistical analysis : Statistical analysis was carried out by using Student's unpaired t-test for comparing quantitative data between the 10 mg and 20 mg study groups after therapy. Comparison of categorical (qualitative) data between the study groups was done using Fischer's test.

RESULTS

In all, two hundred (200) patients were screened and after meeting the inclusion and exclusion criteria, a total of one hundred and fifty (150) patients were enrolled in the study. Of the 150 participants randomized to treatment, 119 completed the treatment phase successfully. The data of only these 119 patients was considered for statistical analysis. Out of the 31 patients who did not complete the study, 9 patients were lost to follow up. 22 patients were discontinued during the double-blind period, primarily as a consequence of treatment-emergent adverse event of myalgia (n=7); noncompliance to diet (n=10) and withdrawal of informed consent (n=5) (Fig. 1).

Baseline characteristics :

Both the groups were similar in demographic profile and baseline CHD risk factors at the start of the study (Table I).

TABLE I : Demographic Characteristics; Baseline CHD risk factors and baseline lipid profile before study.

Parameters	*Atorvastatin 10 mg (Mean±SD) n=61*	*Atorvastatin 20 mg (Mean±SD) n=58*	*P value*
Age (years)	55.33±11.67	53.83±11.91	0.4891
Males	36 (59.02%)	32 (55.17%)	0.8117
Female	25 (40.98%)	26 (44.83%)	0.8117
Male ≥45 years	26 (42.62%)	19 (32.76%)	0.3575
Female ≥55 years	12 (19.67%)	09 (15.52%)	0.7235
Cigarette smoking	25 (40.98%)	27 (46.55%)	0.6692
Diabetics	13 (21.31%)	15 (25.86%)	0.7123
Hypertensive	23 (37.70%)	18 (31.03%)	0.5671
TC (mg/dL)	242.60±28.47	245.07±31.15	0.6533
TG (mg/dL)	201.97±34.14	208.53±29.65	0.2658
LDL (mg/dL)	167.46±32.30	167.83±32.65	0.9507
HDL (mg/dL)	34.75±3.21	35.53±3.11	0.1814
VLDL (mg/dL)	40.39±6.83	41.71±5.93	0.2658
Non HDL (mg/dL)	207.85±29.48	209.53±31.45	0.7638

TC - Total cholesterol, TG - triglycerides, LDL-C- Low Density Lipoprotein Cholesterol, HDL-C- High Density Lipoprotein Cholesterol, VLDL-C- Very low Density Lipoprotein Cholesterol, Non HDL-C = TC - HDL-C/LDL+VLDL.

Baseline laboratory parameters :

The baseline laboratory parameters between the two groups were similar at the start of the study. After treatment for 12 weeks, there were no important changes in haematology or biochemical laboratory values.

There were no significant differences in the mean baseline levels of lipid parameters TC, TG, LDL-C, VLDL-C and HDL-C at the start of study (Table I).

Efficacy : Changes in Serum Lipids :

Both doses of atorvastatin reduced the levels of TC, TG, LDL-C, VLDL-C, and Non-HDL-C from baseline to week 4 to week 12. (Table II) At both doses of 10 mg and 20 mg

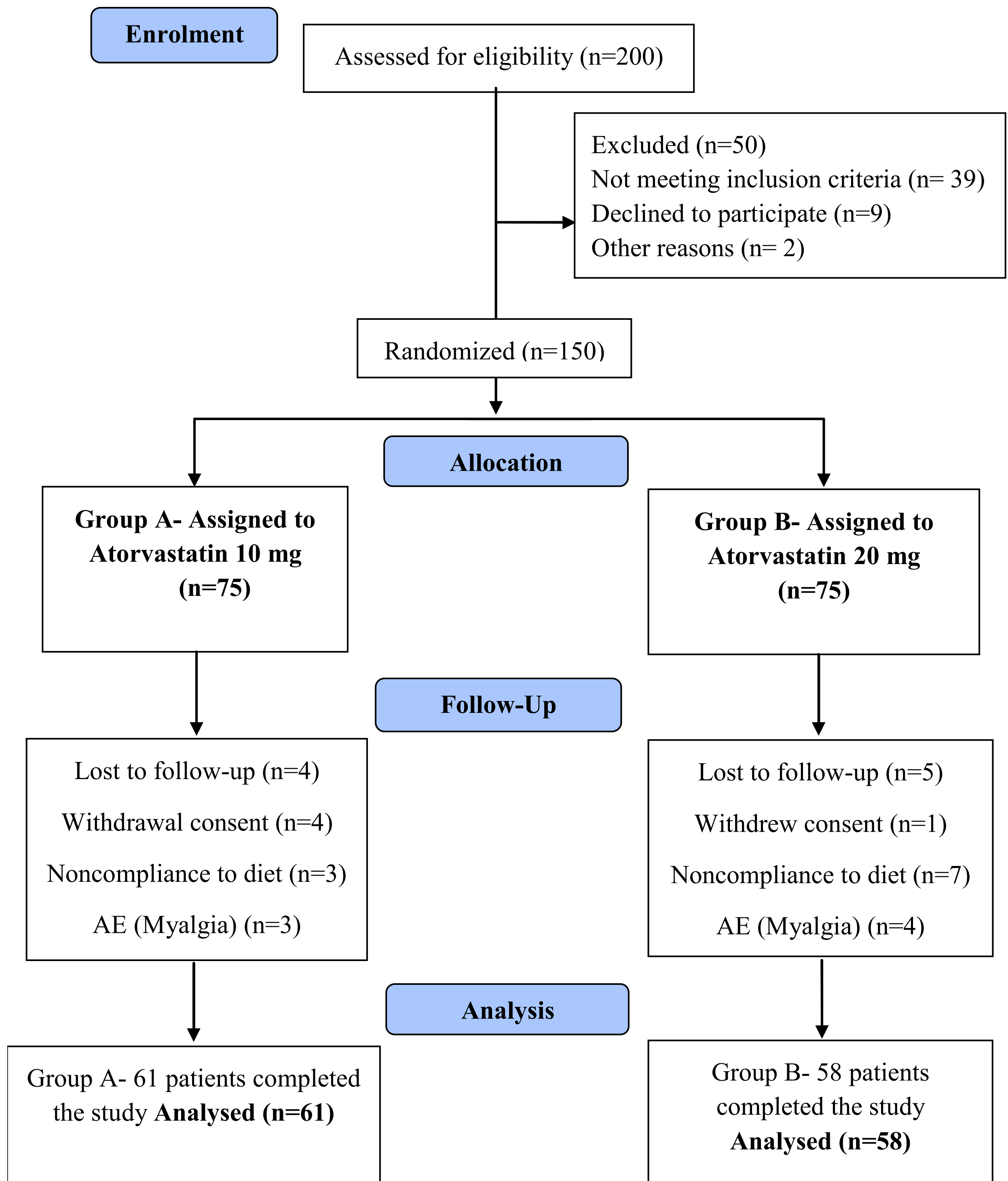

Fig. 1 : Study flow diagram.

TABLE II : Percent reduction in lipid profile with atorvastatin 10 mg and 20 mg doses at 4 and 12 weeks (values are mean±SD).

Lipid profile	*Atorvastatin 10 mg (n=61)*				*Atorvastatin 20 mg (n=58)*			
	At baseline	*At 4 weeks*	*Mean change*	*Percent change (%)*	*At baseline*	*At 4 weeks*	*Mean change*	*Percent change (%)*
TC mg/dL	242.60±28.47	201.59±20.61	41.02±13.96	16.91	245.07±31.15	189.85±22.58	55.22±14.05**	22.53
TG mg/dL	201.97±34.14	187.54±32.83	14.43±13.14	7.14	208.53±29.65	186.72±28.34	21.81±19.21*	10.46
LDL mg/dL	167.46±32.30	128.36±23.25	39.10±14.31	23.35	167.83±32.65	115.90±21.14	51.93±15.39**	30.94
HDL mg/dL	34.75±3.21	35.71±3.30	0.967±0.26	2.78†	35.53±3.11	36.60±3.2	1.07±0.41†	3.01
VLDL mg/dL	40.39±6.83	37.5±6.57	2.89±2.63	7.16	41.71±5.93	37.35±5.67	4.36±3.84*	10.45
Non HDL mg/dL	207.85±29.48	165.87±21.44	41.98±13.96	20.20	209.53±31.45	153.24±22.77	56.29±13.98**	26.86
Lipid profile	*At baseline*	*At 12 weeks*	*Mean change*	*Percent change*	*At baseline*	*At 12 weeks*	*Mean change*	*Percent change*
TC mg/dL	242.60±28.47	169.25±17.48	73.35±16.85	30.23	245.07±31.15	156.04±19.54	89.03±16.99**	36.32
TG mg/dL	201.97±34.14	134.67±23.21	67.30±20.59	33.32	208.53±29.65	133.06±21.01	74.87±32.14	35.90
LDL mg/dL	167.46±32.30	105.38±18.94	62.08±18.66	37.07	167.83±32.65	91.16±18.8	76.67±17.87**	45.68
HDL mg/dL	34.75±3.21	36.96±3.42	2.20±0.57	6.36†	35.53±3.11	38.15±3.42	2.61±0.51†,**	7.35
VLDL mg/dL	40.39±6.83	26.97±6.64	13.46±4.11	33.33	41.71±5.93	26.74±4.20	14.97±6.43	35.89
Non HDL mg/dL	207.85±29.48	131.70±17.86	76.15±16.67	36.64	209.53±31.45	117.89±19.79	91.65±16.9**	43.74

†-percent increase for HDL, *P<0.0154, **P<0.0001 when mean change of atorvastatin 10 mg and 20 mg doses were compared.

atorvastatin, a large percentage of the total decrease in LDL-C was evident by week 4, and this decrease was sustained at week 12. A dose-dependent response was apparent, with greater decreases achieved by patients receiving the 20 mg as compared to 10 mg of atorvastatin. On comparison of the changes in the lipid levels at week 4 brought about by 10 mg of atorvastatin vs. 20 mg of atorvastatin, there was a highly significant decrease in the levels of TC, LDL-C, VLDL-C, and Non-HDL-C (P<0.001); significant decrease in the levels of VLDL-C (P<0.05) while changes in the levels of HDL were not significant. However at week 12, on comparison of 20 mg vs.10 mg of atorvastatin; there was a highly significant decrease (P<0.001) in the levels of TC, LDL-C, and Non-HDL-C and there was also a highly significant increase (P<0.001) in the levels of HDL-C.

Efficacy : NCEP ATP III LDL-C Goals :

Patients were classified according to their level of CHD risk based on the Framingham's risk algorithm to determine the percentage of patients that attained their NCEP ATP III LDL-C goal by the end of the study (Table III).

High risk category :

There was a statistically significant

TABLE III : Number of patients achieving NCEP-ATP III LDL goal at end of 12 weeks.

Risk category	*Risk of developing CHD in the next 10 years*	*Target LDL Level*	*Atorvastatin 10 mgn=61*			*Atorvastatin 20 mgn=58*		
			Total number of patients	*Number reaching target*	*Percentage of patients reaching target (%)*	*Total number of patients*	*Number reaching target*	*Percentag of patients reaching target (%)*
High Risk (CHD or CHD risk equivalents)	10-year risk >20%	Target LDL-100 mg/dL	25	8	32	22	17	77.27*
		Optional target LDL - <70 mg/dL	25	0	0	22	8	36**
Moderately High Risk (2+ risk factors)	10-year risk >10%–20%	Target LDL-130 mg/dL	24	18	75	26	26	100*
		Optional target LDL - <100 mg/dL	24	12	50	26	21	80.77*
Moderate Risk (2+ risk factors)	10-year risk <10%	Target LDL-130 mg/dL	12	10	83.33	10	10	100

*P<0.05, **P<0.001 (Fischer's test).

(P<0.05) higher percentage of patients with CHD or CHD risk equivalents i.e. high risk group (n=25 in 10 mg group and n=22 in 20 mg group) to reach goal LDL of 100 mg/dL at higher starting dose of 20 mg than at lower starting dose of 10 mg (32% at the 10-mg dose, 72.77% at the 20-mg dose). In high-risk persons, the recommended LDL-C goal is < 100 mg/dL. An LDL-C goal of < 70 mg/dL is a therapeutic option on the basis of available clinical trial evidence, especially for patients at very high risk. There was statistically highly significant difference (P<0.001) in achieving the optional goal of < 70 mg/dL (n=25; 0% patients attaining the goal at the 10-mg dose, n=22; 36.36% patients attaining the goal at the 20-mg dose.

Moderately high risk category :

Similar trends of statistically significant difference between the two groups (P<0.05) were also apparent for individuals at moderately high risk for CHD (≤2 CHD risk factors) (n=24; 75% patients attaining the goal at the 10-mg dose, n=26; 100% patients attaining the goal at the 20-mg dose).The optional LDL-C goal for the moderately high risk category is < 100 mg/dl. There was statistically significant difference (P<0.05) in achieving the optional goal of < 100 mg/dL between the two groups (n=24; 50% patients attaining the goal at the 10-mg dose, n=26; 80.77% patients attaining the goal at the 20-mg dose).

Moderate risk category :

For patients at moderate risk for CHD (<2 risk factors) (n=12, 83.33% patients attaining the goal at the 10-mg dose, n=10; 100% patients attaining the goal at the 20-mg dose); the difference was not statistically significant, but it is noteworthy that 100% of patients attained their goal LDL-C when they were on 20 mg dose.

Health Related Quality of Life Score (HRQOL score) :

As shown in Table IV, there was no statistically significant difference in the HRQOL score before treatment. The mean increase in HRQOL score at the end of 12 weeks was significantly greater (P<0.001) in patients receiving 20 mg dose as compared to patients on 10 mg dose.

Adverse events :

There were 24 incidences of adverse events in the 10 mg group and 26 incidences of adverse events in the 20 mg group. There were 7 cases of myalgia; with 3 cases of myalgia in the 10 mg Group and 4 cases of myalgia in the 20 mg Group (Table V). The most common adverse event in the both the groups was paresthesias with 4 patients in the 10 mg group and 6 patients in the 20 mg group suffering from paresthesias. The other adverse events noted in the 10 mg group were headache, asthenia, constipation, diarrhoea, dyspepsia, nausea, flatulence and dizziness. In the 20 mg group all the above adverse events were seen except for constipation. One patient in the 20 mg group also suffered from depression. Overall all the adverse events were mild or moderate in nature and no incidence of death or serious adverse events were observed during the study period.

TABLE IV : Mean increase in 'Health related quality of life' (HRQOL) score.

HRQOL Score	*Atorvastatin 10 mg (Mean±SD) n=61*	*Atorvastatin 20 mg (Mean±SD) n=58*
At baseline	65.44±11.05	65.69±8.56
Mean increase after 12 weeks	12.84±4.94	18.32±5.81*

*P<0.0001 (unpaired t test).

TABLE V : Incidence of Adverse events (AE).

Incidence of AE	*Atorvastatin 10 mg n=61*	*Atorvastatin 20 mg n=58*
Headache	2	3
Asthenia	2	3
Digestive system		
Constipation	1	0
Diarrhoea	2	1
Dyspepsia	3	2
Nausea	2	1
Flatulence	3	4
Nervous system		
Dizziness	2	1
Paresthesia	4	6
Depression	0	1
Musculoskeletal system		
Myalgia	3	4
Total	**24**	**26**

DISCUSSION

Atorvastatin has a long $t_{1/2}$ (14 hours), which allows administration of this statin at any time of the day. Rosuvastatin and pravastatin also have long $t_{1/2}$ of 19 hours and 22 hours respectively; however rosuvastatin is a very expensive drug and pravastatin had been implicated for serious

adverse drug events (e.g.,breast cancer) in the past; hence, we selected atorvastatin as our study medication.

LDL-C target goals :

At 4th week, atorvastatin 10 mg reduced LDL levels by 23.35% and atorvastatin 20 mg reduced LDL levels by 30.94%. Similarly, at 12 weeks atorvastatin 10 mg reduced LDL levels by 37.07 % and atorvastatin 20 mg reduced LDL levels by 45.68%. The reductions in LDL reported in this study are consistent with previously published values (18). In 2004 modifications were recommended to the NCEP ATP III guidelines which proposed reduced LDL target levels in patients at risk for CHD – in high-risk subjects – LDL target of less than 100 mg/dL and optional LDL target of < 70 mg/dL; in moderately high risk subjects – LDL target of less than 130 mg/dL and optional LDL target of < 100 mg/dL; in moderate risk subjects – LDL target of less than 130 mg/dL (19). In our study, across all the risk categories, in total 40.98% of patients did not reach the goal LDL levels at the 10 mg dose (i.e. 25 out of 61 patients did not achieve their goals). Compared to this, only 8.62% of the patients did not reach the goal LDL levels at the 20 mg dose (i.e. 5 out of 58 patients did not achieve their goals).

Health related quality of life score :

Many researchers looking at cardiac populations have used the generic SF-36 as their measurement tool of choice when assessing the Health Related Quality Of Life (HRQOL) (20, 21, 22, 23). The SF 36 questionnaire measures eight concepts: Physical Functioning, Role limitations due to physical health, Bodily pain, General health perceptions, Vitality, Social functioning, Role limitations due to emotional problems, and General mental health. The reliability of the eight scales has been estimated using both internal consistency (reliability co-efficient greater than 0.75 for all dimensions except social functioning which is 0.74) and test–retest methods (24). SF-36 has content validity in the field of heart disease because it covers the areas of energy/vitality and bodily pain. Besides SF 36 is very user friendly and requires approximately ten minutes for completion (25). The 10 mg group displayed an increase of 19.62% and the 20 mg group displayed an increase of 27.89% in the Health related Quality of Life score. The mean increase in the Health related Quality of Life score between the two groups was statistically significant (P<0.0001).

Adverse events :

It has been observed from various randomized clinical trials (26, 27, 28, 29, 30, 31) that statin therapy reduces the relative risk of major cardiovascular events or death in relation to the absolute magnitude of LDL reduction and across wide range of cholesterol levels irrespective of history of any coronary artery disease. But, to use high dose statins to reach LDL goals physicians require robust safety data in addition to the efficacy data. Statins are well tolerated in majority of patients and the benefits far outweigh the potential risks. Patient differences in drug pharmacokinetics could make certain patients more vulnerable to the adverse effects of statin therapy (32, 33). Seven patients in our study suffered from myalgia. In a 5 year trial, involving 20,000

subjects, randomized to statin or placebo, it was found that one-third of patients complained of myalgia at least once, whether taking statins or placebo (24). Serum creatinine kinase (CK) levels need not be monitored routinely in patients taking statins, as an elevated CK in the absence of symptoms does not predict the development of any myopathy and also does not necessarily suggest the need for discontinuing the drug. Hence, we clinically evaluated our patients for myalgia, by checking the power in their proximal muscles and assessing if they were having myalgia, using a visual analogue scale.

Use of risk algorithm for prescribing the correct dose of statins by physicians:

Risk for CHD is calculated using the Framingham Study criteria and incorporates age, gender, total cholesterol, HDL, systolic blood pressure, hypertension treatment, and cigarette smoking (34, 19, 35). Despite the availability of guidelines for calculating risk, the use of the Framingham's risk algorithm has lagged in primary care. The number and complexity of the guidelines impede their implementation. Lack of time for physicians to act on guidelines presents an additional hurdle (36). The education regarding prescribing according to guidelines and the publication of guidelines is not adequate (36, 37). Hence many doctors do not use risk charts to assess a patient's risk for CHD, thereby underestimating many asymptomatic patients susceptibility for CHD events, and consequently under-treating these patients. Physicians tend to take the decision to initiate statin therapy based on high cholesterol levels. However the decision to initiate statin therapy should be based on the patients overall cardiovascular risk profile rather than abnormal lipids. A patient having relatively lower level of LDL, may still fall in the high risk category because of his other risk factors such as diabetes, smoking, etc. It would become necessary for such a patient to keep his LDL below target levels for his risk category to prevent cardiovascular morbidity and mortality. Thus, benefit from high dose statin therapy is possible, and is a viable therapeutic option for those patients who are at risk for CHD and/or have high LDL levels. Increasing the level of LDL target attainment and thus reducing the risk for the occurrence of cardiovascular events are likely to have significant economic benefits both at the level of the individual and society as a whole. In addition to the economic benefits of reducing CHD mortality and morbidity, mainly apparent as a decreased requirement for expensive revascularization procedures, treating patients to goals more efficiently will be associated with the use of fewer medical resources including laboratory tests, medication, and hospital visits. Indirect costs such as patient travel time, waiting time, or time to undergo examinations and tests are also likely to be reduced (38).

Limitations:

The present study has certain limitations. In this study short-term end points were studied, and thus the long-term rates of development of AEs could not be assessed. Also compliance with the higher dose of the drug over a long period of time could not be studied. Rather, the trial was designed to examine the efficacy of a treatment schedule-based assignment of drug dose according to patient's cardiovascular

risk rather than the traditional approach of starting at a low dose and gradually titrating the dose upwards. Such a trial design has been adopted by researchers in the past (39, 40).

Conclusions :

A significantly greater number of patients are not achieving the ATP III guideline specified LDL goal levels with lower dose of statin. More patients are attaining goal LDL levels with higher dose therapy.Selecting the starting dose of atorvastatin according to the patients risk category (by using the Framingham's algorithm for calculating cardiovascular risk) and the percentage reduction in LDL required, will result in greater success in achieving LDL goals and may result in better quality of life. Patients in the high risk category should be considered for a higher dose of atorvastatin, without compromising safety.

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EFFECT OF *CYP2C9* AND *VKORC1* GENETIC POLYMORPHISMS ON WARFARIN DOSE REQUIREMENT IN SOUTH INDIAN POPULATION

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Abstract : Cytochrome P450 2C9 *(CYP2C9)* and vitamin K epoxide reductase complex subunit 1 (*VKORC1)* genetic polymorphisms were strongly associated with warfarin dose requirement in Caucasians, African Americans and other populations. Our aim was to evaluate the effect of *CYP2C9* and *VKORC1* genetic polymorphisms on warfarin dose requirement in south Indian population. A total of 150 patients on warfarin with stable INR (2-3.5) for the past 3 months were recruited. The genotypes of *CYP2C9*2* and **3* and *VKORC1 -1639G>A* were compared with mean daily warfarin dose (MDWD). The variant allele frequency of *VKORC1 -1639G>A* was found to be 10.4% and *CYP2C9**2 and *CYP2C9*3* were found to be 4.5% and 6.6%, respectively. Our study showed that the mean daily warfarin dose is higher in patients with wild type genotypes of *CYP2C9* and *VKORC1* compared to those with variant genotypes. Multivariate regression analysis revealed that age, body mass index (BMI), duration of therapy and genetic polymorphisms of *CYP2C9* and *VKORC1* together contribute to 36.1% variability in MDWD in south Indian population.

Key words : CYP2C9 VKORC1 genetic polymorphism warfarin south Indian

INTRODUCTION

Warfarin, an oral anticoagulant, is a racemic mixture of R and S- warfarin. S-warfarin is more potent than R-warfarin and it contributes to 70% of the anticoagulant effect of warfarin (1). It has been in clinical use for more than 60 years. It is indicated for both the prophylaxis and treatment of thromboembolic conditions like deep vein thrombosis, pulmonary embolism, atrial fibrillation, prosthetic heart valves and also

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for the reduction of death risk in myocardial infarction (2, 3). Warfarin is a drug with narrow therapeutic index, small change in dose can lead to bleeding risk or lack of therapeutic effect and it requires careful monitoring. The anticoagulation profile is monitored by International Normalized Ratio (INR) which is an index of its therapeutic effect and safety (4). Besides this limitation of being a narrow therapeutic index drug, large interindividual variability exists attributable to both genetic and non-genetic factors (5, 6, 7).

The cytochrome P450 enzymes are involved in the metabolism of warfarin. S-warfarin is metabolised predominantly by CYP2C9 and R-warfarin is metabolised by multiple enzymes such as CYP3A4, CYP1A2 and CYP1A1 (8). Since the greater proportion of anticoagulant effect is contributed by S-warfarin, abnormal function of CYP2C9 enzyme leads to altered dose-response to warfarin (9). CYP2C9 enzyme is encoded by the gene *CYP2C9* which is polymorphically expressed in different ethnic populations (10, 11, 12, 13). Genetic polymorphisms in *CYP2C9* affect the drugs metabolised by CYP2C9 like phenytoin, warfarin, NSAIDs etc. (14, 15, 16). *CYP2C9*2* and *CYP2C9*3* are found to be important variants in warfarin metabolism (17). The individuals with these variants have reduced capacity to metabolise warfarin which is clinically implicated in the requirement of lower dose and increased risk of bleeding (5, 18). The frequency of these genetic polymorphisms differs between various ethnic populations (10, 11, 12, 13). Studies have shown the variation in dose and risk of over anticoagulation correlating with the variant allele in different population (9, 19, 20, 21, 22, 23).

On the other hand, vitamin K epoxide reductase complex subunit 1 (VKORC1) is the target site for warfarin, which it inhibits for its anticoagulation effect. This enzyme is encoded by the gene *VKORC1* (24). Number of single nucleotide polymorphisms in *VKORC1* has been identified. Of these promoter region polymorphism (*VKORC1 – 1639G>A*) appears to be the most important. Polymorphism at this region leads to abnormal expression of the enzyme which correlates well with the dose required by those individuals with this variant (25, 26). Several studies have shown that the gene *VKORC1* as the single biggest predictor of warfarin dose requirement (6, 27, 28, 29). The *VKORC1 -1639 GG* (wild) genotype requires high dose and *VKORC1 -1639 AA* (homomutant) genotype lower while the *VKORC1 -1639 GA* (heteromutant) genotype requires intermediate dose (25, 26). The frequency of these genotypes varies in different ethnic groups (25, 27, 30, 31, 32, 33). Studies have shown that the Caucasians require higher dose compared to Asians which correlates well with their higher *VKORC1 -1639 GG* and *VKORC1 -1639 AA* genotype frequency respectively in these populations (20, 21, 22, 26, 33).

The frequency of *CYP2C9* polymorphism has been established in south Indian population (34, 35). Limited information is available on *VKORC1* polymorphism in south Indian population and the influence of these polymorphisms on warfarin dose requirements (36). The present study was designed to find out the effect of *CYP2C9* and *VKORC1* genetic polymorphisms on warfarin dose requirement in south Indian population.

MATERIALS AND METHODS

Study settings

The study was done in the Department of Pharmacology, Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Puducherry in collaboration with the Department of Cardiology and Department of Medicine, JIPMER. This study was conducted in patients on warfarin therapy visiting Cardiology and Medicine out-patients department and also in-patients of these departments during the period from March 2010 to July 2011.The study protocol was approved by the Institute Ethics Committee (IEC) prior to commencement of the study.

Sample size

The sample size calculations were undertaken before the study. The minimum important difference (difference in mean dose) between the groups was taken to be 0.8% with a standard deviation of ± 1.2 with ratio of subjects between wild normal and variant allele groups 4:1. Using the Power and Sample Size (PS) Calculations software version 3.0 (Vanderbilt University, Nashville, Tennessee, USA) for a power of 80% and a significance level of 5%, the sample size was calculated to be a total of 110 for the study.

Study subjects

Patients receiving warfarin maintenance therapy for cardiovascular disorders, cerebrovascular disorders and deep vein thrombosis with an INR within the range of 2.0 to 3.5 for the past 3 months, age ranged 18-65 years, either gender and of south Indian origin were included. The nativity was assessed by family history of three generations living in Tamil Nadu, Pondicherry, Kerala, Karnataka, Andhra Pradesh, and speaking any of the south Indian languages as their native language. Patients with liver or renal dysfunction, taking CYP2C9 inducers and inhibitors (eg. barbiturates, carbamazepine, rifampicin, amiodarone, ketoconazole etc.), pregnant and lactating women, smokers and alcoholics were excluded. Written informed consent was obtained from patients participated in this study. Mean daily warfarin maintenance dose (MDWD) [the mean of two recent doses over a period when two consecutive stable INR values achieved 2.0 to 3.5] was calculated.

Genotyping for *CYP2C9* and *VKORC1 -1639G>A*

Five millilitre of venous blood was collected from the study participants for genotyping. DNA was extracted by using phenol-chloroform extraction method (37). Genotyping for *CYP2C9*2* and *CYP2C9*3* polymorphisms and *VKORC1 -1639G>A* were done by Real Time Thermocycler (ABI Prism 7300, Foster City). TaqMan drug metabolism genotyping assays were obtained from Applied Biosystems, Foster City for *CYP2C9* alleles [*CYP2C9*2 (rs1057910*) (Assay by design), *CYP2C9*3* (Assay ID: C_27104892_10)] and *VKORC1 -1639G>A* (C_30996661_30). The qRT-PCR reaction was carried out using a final volume of 15 µl (7.5 µl of Taqman Universal master mix (2X), 0.375 µl of 20X working stock of genotyping assay, 3.375 µl of deionized water and 3.750 µl of genomic DNA (50 ng/µl) diluted in DNAase free water). The allelic discrimination was performed using 7300 SDS software (version 1.3.1).

Data analysis

Statistical analysis was performed using SPSS version 16.0 and GraphPad Instat version 3.06 software packages. Unpaired t-test, Chi-square and Fischer's exact test were used appropriately to compare between the baseline parameters. Comparison of MDWD in *CYP2C9* and *VKORC1* genotype was done by using Mann-Whitney U test and in combined *CYP2C9* and *VKORC1* genotypes was done by Kruskal-Wallis test. Multiple linear regression analysis was done to study the association of independent variables known to influence MDWD. P value less than 0.05 was considered statistically significant.

RESULTS

Study participants

A total of 150 patients were recruited depending on the inclusion and exclusion criteria. Among them 6 patients were excluded for the data analysis due to the poor quality or missing of the DNA samples for the analysis. The patient characteristics are given in Table I. The baseline characteristics in patients with wild and variant genotype for *CYP2C9* and *VKORC1 -1639G>A* were compared. The baseline characteristics were not significantly different in *CYP2C9* genotype and in *VKORC1* genotype groups. The baseline characteristics across combined genotype of *CYP2C9* and *VKORC1 -1639G>A* were found to be statistically non-significant.

***CYP2C9, VKORC1 -1639G>A* allele and genotype frequencies of study participants**

The allele and genotype frequencies of *CYP2C9* and *VKORC1* are given in the Table II. The genotype frequencies of *CYP2C9* and

TABLE I: Subject characteristics.

Parameters	*Overall (N=144)*	*VKORC1*		*CYP2C9*	
		GG (N=115)	*Non GG (N=29)*	**1*1 (N=114)*	*Non *1*1 (N=30)*
Age (years)#	43.4±11.6	42.5±11.2	46.7±12.5	43.1±11.9	44.5±10.5
Height (cm)#	156.6±6.9	156.8±6.9	155.8±6.9	156.4±7.1	157.7±6.2
Weight (kg)#	56.11±8.2	56.3±8.5	55.5±6.7	56.6±8.1	54.4±8.1
Body mass index (kg/m^2)#	22.8±2.9	22.8±2.9	22.9±2.6	23.1±2.8	21.9±3.1
Duration (months)#	10.8±7.8	10.7±7.8	10.4±7.7	11.6±8.2	8.8±5.4
Hypertensive (N)(%)	17(11.8)	12(10.4)	5(17.2)	12(10.5)	5(16.7)
Diabetic (N)(%)	9(6.2)	8(7)	1(3.4)	8(7)	1(3.3)
Indications (N)(%)					
Valvular heart disease	104 (72.2)	80(73)	20(68.9)	80(70.2)	24(80)
Prosthetic valves	3(2.1)	2(1.7)	1(3.4)	2(1.8)	1(3.3)
Atrial fibrillation	11(13.7)	10(8.7)	1(3.4)	9(7.9)	2(6.7)
Deep vein thrombosis	8(5.6)	6(5.2)	2(6.9)	7(6.1)	1(3.3)
Others	18(12.5)	15(13)	3(10)	16(14)	2(6.7)
Concomitant drugs (N)(%)					
Phenoxymethylpenicillin	73(62.9)	59(51.3)	14(48.3)	57(50)	16(53.3)
Furosemide	89(76.7)	71(61.7)	18(62.1)	73(64)	16(53.3)
Digoxin	54(46.6)	40(34.8)	14(48.3)	42(36.8)	12(40)
Atenolol	41(54)	32(27.8)	9(31)	33(28.9)	8(26.7)

(#-Values in mean±SD; N=Number of subjects).

TABLE II : Genotype and allele frequency of *CYP2C9* and *VKORC1 -1639G>A* (n=number of subjects).

Gene	*Nucleotide position*	*n*	*Genotype frequency (%)*				*n*	*Allele frequency (%)*		
CYP2C9	CYP2C9*2 (430 C>T) CYP2C9*3 (1075 A>C)	144	*1*1 114(79.2)	*1*2 11(7.6)	*1*3 17(11.8)	*2*3 2(1.4)	288	*1 256(88.9)	*2 13(4.5)	*3 19(6.6)
VKORC1	-1639G>A	144	GG 115(79.9)	GA 28(19.4)	AA 1(0.7)		288	G 258(89.6)	A 30(10.4)	

VKORC1 -1639G>A in the present study was found to be in Hardy–Weinberg equilibrium. *CYP2C9* homozygous variants *(*2/*2 or *3/*3)* were not reported in the patients.

Comparison of MDWD in *CYP2C9* and *VKORC1 -1639G>A* genotype

MDWD was compared between wild and variant genotypes of *CYP2C9* and *VKORC1 -1639 G>A* and also the effect of combined genotypes of both the genes were studied (Table III). There was significant difference observed when the MDWD was compared between the *CYP2C9* genotype wild and *CYP2C9* variant genotype (4.8±1.6 mg vs. 2.9±1.1 mg, P<0.0001). Similarly significant difference was observed between *VKORC1*

TABLE III : Comparison of MDWD in *CYP2C9*, *VKORC1 -1639 G>A* and combined *CYP2C9* and *VKORC1 -1639 G>A* genotypes.

CYP2C9		*N=144 (%)*	*MDWD (mg) (95% CI)*	*P value*
*1/*1		114(79.17)	4.8±1.6 (4.47–5.07)	P<0.0001#
non*1/*1		30(20.83)	2.9±1.1 (2.46–3.35)	
VKORC1 -1639G>A		***N=144 (%)***	***MDWD (mg)***	***P value***
GG		115(79.9)	4.6±1.6 (4.32–4.97)	P<0.001#
nonGG		29(20.1)	3.4±1.1 (2.96–3.84)	
VKORC1 -1639G>A	***CYP2C9***	***N=144(%)***	***MDWD(mg)***	***P value***
GG	*1/*1	95(66)	4.97±1.5 (4.64–5.30)	P<0.0001$
nonGG	non*1/*1	10(7)	2.6±0.4 (2.29–2.93)	
nonGG	*1/*1	19(13.2)	3.8±1.1 (3.23–4.37)	
GG	non*1/*1	20(13.8)	3.0±1.3 (2.39–3.72)	

Values in mean±SD; P<0.05 is significant; N=Number of subjects.
- Mann-Whitney U test was used.
$ - Kruskal-Wallis test was used.

TABLE IV : Multiple linear stepwise regression analysis for factors determining MDWD.

S. No.	*Independent variable*	*Unstandardized regression coefficient*	*Standard error*	*Standardized regression coefficient*	*P value*
1.	Constant	3.05			
2.	Age	–0.036	0.010	–0.255	<0.0001
3.	BMI	0.129	0.041	0.226	<0.05
4.	Duration of therapy	0.038	0.015	0.178	<0.05
5.	CYP2C9 Genotype	–0.691	0.159	–0.308	<0.0001
6.	VKORC1 Genotype	–0.859	0.276	–0.221	<0.05

Predictors in the Model: (Constant), Age, BMI, duration of therapy, *CYP2C9* genotypes, *VKORC1* genotypes, R=0.621; R^2=0.386; Adjusted R^2=0 .361; P=<0.0001.

Independent variables: Age, BMI, duration of therapy, *VKORC1 -1639G>A* genotype and *CYP2C9* genotype were considered.

wild and variant genotype (4.6±1.6 mg vs. 3.4±1.1 mg, P<0.001). When comparing combined wild genotypes with combined variant genotypes a strong significant difference were observed.

Multiple linear regression for factors determining MDWD

Multiple linear regression analysis was carried out for the factors which can determine the MDWD. Age, body mass index (BMI), duration of therapy, *CYP2C9* and *VKORC1 -1639G>A* genotypes were the independent variable considered. Age, BMI, duration of therapy and genotypes of *CYP2C9* and *VKORC1 -1639G>A* were found to be significantly associated with the MDWD in this study (Table IV). All these factors together contributed to 36.1% variability in MDWD (adjusted R^2 = 0.361).

DISCUSSION

The frequency of *CYP2C9* in the current study did not differ from the previously established frequency in our population (Table II) (38). The frequency of *VKORC1* genotypes was found to be similar to few studies done in Indian population and was quite different from other world population particularly the Chinese and Japanese population (Table II) (22, 25, 30, 32, 39, 40). The frequency distribution of variant alleles differs in various ethnic groups and so the mean dose required. Studies have shown that frequency of AA genotype is higher in Asians when compared to the Caucasians and thus Asians require lower maintenance dose to maintain a stable INR (25, 27, 30, 31, 32, 33). In our study the patients carrying the wild genotype (*CYP2C9*1/*1*) required higher MDWD (4.8±1.6 mg) when compared to the variant genotypes (*CYP2C9 non*1/*1*) (2.9±1.1 mg). These findings are in concordance with findings obtained in the studies conducted previously in various population (Table III) (19, 22, 23, 41,42, 43, 44).

In the present study, an association between the *VKORC1* genotype and MDWD was observed. MDWD was significantly higher in the wild genotype compared the variant genotype (4.6±1.6 mg vs. 3.4±1.1 mg, P<0.001) (Table III). A study done in 104 Chinese patients showed that AA genotype required lower dose when compared to

combined GA and GG genotype (2.61±1.10 mg/ day vs 3.81±1.24 mg/day, P<0.0001). This study also assessed the enzymatic activity in wild and variant allele which showed that *VKORC1* promoter with the G allele had a 44% increase in luciferase activity when compared with the A allele. This increase in enzymatic activity could result in increase in dose requirement in those carrying G allele (25).

In our study we studied the promoter region polymorphism alone in *VKORC1*. Studies have demonstrated that this polymorphism is in strong linkage disequilibrium with six other SNPs (rs7196161, rs2884737, rs9934438, rs8050894, rs2359612 and rs7294) and this polymorphism alone to some extent explains the pharmacologic variation to oral anticoagulants (45). Studies have demonstrated that the promoter region polymorphism *VKORC1 -1639G>A*, is the most important predictor of warfarin initiation dose (6, 27, 28, 46). The current study findings are in agreement with the studies done elsewhere and also few studies done in Indian population (7, 22, 27, 30, 31, 32, 33, 41, 47). MDWD was significantly higher in the combined wild genotype of *CYP2C9* and *VKORC1* when compared with the combined variant genotypes (Table III). This finding is similar to the previous study done in Caucasian population (39). The major finding of this study was that MDWD was lower in patients carrying variant combined genotypes *CYP2C9* and *VKORC1* when compared to the wild genotypes. Both these polymorphisms along with age, BMI and duration of therapy contributed to 36.1% variability in MDWD (Table IV). In a study done in Andhra Pradesh population genetic and clinical factors contributed up to 61% variability in warfarin dose requirement. They have explained that age, gender, BMI, vitamin K intake, *CYP2C9 (*2,*3* and **8)* and *VKORC1 (*3, *4* and *-1639 G>A), CYP4F2 V433M, GGCX G8016A* and thyroid status contributed the variability (36). Previous studies have shown the effect of age, weight and height on warfarin maintenance dose requirements (6, 39, 48, 49). In our study we found that age and BMI significantly influenced the MDWD (Table IV).

Many of the drugs which can potentially interact with warfarin were excluded as part of the exclusion criteria (eg. barbiturates, rifampicin, carbamazepine, fluconazole, cimetidine etc.). Other concurrent medications which were essential as a part of the treatment required by the patients were not excluded and they were not found to be contributing to the MDWD variation since they were equally distributed among different genotype groups.

Diet, particularly the amount of vitamin K intake can also affect anticoagulant response to warfarin (50). In this study although we did not consider dietary consumption of vitamin K, we assumed that all patients had relatively stable vitamin K consumption, given that they had stable INR. Patient compliance is another factor which may affect the anticoagulation response to warfarin (51). The patients had relatively good compliance as their INR was stable in our study. They may be the patients who were counseled well when starting the anticoagulation therapy by the clinicians. In addition to *CYP2C9* and *VKORC1*, other genetic polymorphisms in gamma glutamyl carboxylase (*GGCX*), *CYP4F2*, microsomal epoxide hydrolase 1(*EPHX1*), calumenin

(*CALU*) and apolipoprotein E (*APOE*) are minor contributors for warfarin dose requirement (52, 53, 54, 55, 56). In the present study we have not focussed on these genes. The strengths of our study were that we followed strict exclusion criteria to exclude the co-morbid conditions, smoking status and other concomitant medications that may potentially interact with warfarin. This implies that the effect of *CYP2C9* and *VKORC1* polymorphism on warfarin dose requirement found in our study is more reliable. The major limitation of our study was that the contribution by other genetic factors like gamma glutamyl carboxylase, epoxide hydrolase and *CYP4F2* polymorphisms and also other polymorphisms in *VKORC1* were not addressed.

CONCLUSION

MDWD is higher in patients with wild genotypes of *CYP2C9* and *VKORC1* compared to those with variant genotypes and along with age, BMI and duration of therapy they contribute to 36.1% variability in MDWD. In future replicate study with large sample size with additional genetic factors taken into consideration needs to be carried out to develop an algorithm for determining warfarin dose before introduction into routine clinical practice. The present study provides the basic information for larger studies in our population and contributes to establish the pharmacogenetic based warfarin therapy in south Indian population.

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DIAGNOSTIC VALUE OF BIOCHEMICAL PREDICTIVE INDEX IN OVARIAN MALIGNANCY

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Abstract : The aim of the study was to determine if diagnostic performance of CA-125 in ovarian malignancy can be improved by considering age of the patient. The study was a retrospective analysis of the medical records of 306 patients who underwent a CA-125 measurement in our Institute. Of the 306 patients, 31% had malignant ovarian diseases, 45% had benign ovarian diseases, 6% had non-ovarian gynaecological diseases and 18% had non-gynaecological diseases. A positive association was found between age and CA-125 levels in patients with ovarian diseases. Patients were divided into three age groups of 18–37 yrs, 38–56 yrs and 57–74 yrs and were given age scores as 1, 2 and 3 respectively. Biochemical predictive index (BPI) was calculated by multiplying age score and CA-125 value. Among the age groups ranging 38–56 yrs and 57–74 yrs, the CA-125 and BPI values were significantly higher in malignant ovarian diseases compared to benign ovarian diseases. ROC curve analysis revealed a cut-off of 86 for the BPI with the sensitivity, specificity, positive predictive value and negative predictive value as 58%, 78%, 56% and 80% respectively. BPI had better specificity and negative predictive value compared to CA-125, can be used in the screening of ovarian pathology.

Key words : biochemical predictive index; CA-125; age score; ovarian diseases; screening

INTRODUCTION

Ovarian cancer has become one of the commonest malignancy affecting Indian women. A steady increase has been observed in the incidence of the ovarian cancer in several registries (1). Ovarian cancer is the most frequent cause of death from gynecological cancer and the fourth most frequent cause of death from cancer in women in developed countries (2).

Almost 90% of patients are diagnosed with metastatic disease in the pelvis or abdomen, for whom the 5-year survival rates are less than 30%. But, detection at stage I

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ovarian cancer confined to the ovaries have a 5-year survival rate in excess of 90%. Ovarian cancer is characterised by late presentation, poor response to current treatment and a poor prognosis. But, the availability of ultrasonography and CA-125 has made the screening for early diagnosis of ovarian cancer quite effective (3). In recent studies, algorithm based on combination of serum biomarkers has been carried for risk assessment of Ovarian Malignancy. The algorithm included CA125 and human epididymal protein 4 and were found to predict the malignancy with higher sensitivity (4, 5).

CA-125 is a high molecular weight glycoprotein, which is expressed by a most of the epithelial ovarian cancers (6). Its detection using murine OC125 monoclonal antibody was developed by Bast *et al* in 1981. Immunofluorescence was used to detect the presence of CA-125, which had no reaction with non-malignant tissues but reacted with few non-ovarian neoplasms. CA-125 can assist in differentiating the different types of ovarian tumors, as it is increased mainly in the epithelial type (7). CA-125 is also helpful as a prognostic marker. It aids in monitoring response to treatment in patients with epithelial ovarian cancer (8).

In an effort to increase the specificity, combinations of CA-125 with various markers and ultrasonography have been evaluated (2). The best individual performance is found in CA-125 levels at a cut-off of 35 U/ml, (sensitivity of 78% and specificity of 75%), followed by ultrasound score (sensitivity of 75% and specificity of 73%) and menopausal status (sensitivity of 73% and specificity of 69%) (9). Three criteria were combined in a risk of malignancy index (RMI) which was calculated from serum CA 125 level, the ultrasound scan and the menopausal status. RMI is found to be more effective in delineating the malignant and benign ovarian diseases, as it combines the various markers or risk factors (10).

The aim of the study was to determine whether by adding age of the patient as a factor along with CA-125 to improve its diagnostic performance. The objectives were to investigate the benign and malignant cases of ovarian disease who underwent CA-125 measurements and to determine the sensitivity, specificity and predictive values of Biochemical Predictive Index, calculated by multiplying age score and CA-125, in the diagnosis of the above said patients.

MATERIALS AND METHODS

The study was a retrospective analysis of the medical records of 306 patients who underwent a CA-125 measurement between March 2010 to July 2011 in our Institute, which was approved by the Institute Scientific Committee and the Ethics Committee. The patients were categorized based on the histopathlogical findings. Patients with ovarian diseases who underwent CA-125 measurements were categorized into three groups - ovarian diseases, non-ovarian gynaecological diseases and non-gynaecological diseases. The CA-125 assays performed in the clinical chemistry laboratory were considered, from which an abnormal result was taken to be 35 U/litre or above. CA-125 assay was done by two-site sandwich immunoassay in chemiluminescence system, Advia Centaur CP, Japan. The assay

uses two monoclonal mouse antibodies specific for CA 125. The first antibody is directed toward the M11 antigenic domain, and is labeled with acridinium ester. The second antibody is directedtoward the OC 125 antigenic domain and is labeled with fluorescein. The immunocomplex formed with CA 125 is captured with monoclonal mouse anti-fluorescein antibody coupled to paramagnetic particles in the Solid Phase. A direct relationship exists between the amount of CA 125 present in the patient sample and the amount of relative light units (RLUs) detected by the system. The sensitivity is 93.5% and CV% is 2.3.

Statistical analysis

Statistical analysis was done using SPSS software. Kolmogrov Smirnov test was used to analyze the normality of distribution. One way ANOVA with post hoc Tukey analysis was done for comparison between groups. Mann Whitney test was used to compare the levels of CA-125 and Biochemical Predictive Index (BPI) in malignant and benign ovarian diseases. Receiver operating characteristic (ROC) curve analysis was done to find the diagnostic significance of CA-125 and BPI. Sensitivity was defined as the proportion of patients with ovarian cancer correctly identified by CA-125 or BPI, and specificity as the proportion of patients without ovarian cancer correctly identified by CA-125 or BPI. Youden index was used as the summary measure of ROC curve. It helped to measure the effectiveness of CA-125 / BPI and enabled the selection of an optimal threshold value for the marker.

RESULTS

Of the 306 patients, 31% had malignant ovarian diseases, 45% had benign ovarian diseases, 6% had non-ovarian gynaecological diseases and 18% had non-gynaecological diseases. The age of patients with malignant ovarian diseases was significantly higher than that of benign ovarian diseases ($P<0.001$). The CA – 125 values were significantly higher in malignant ovarian diseases compared to the benign ovarian diseases ($P<0.001$) and non-ovarian gynaecological diseases ($P<0.001$) (Table I).

A positive association was found between age and CA-125 levels in patients with ovarian diseases.($r = 0.19$, $p = 0.005$) (Fig. 1) Based on this observation, patients were divided into three age groups of 18–37 yrs, 38–56 yrs and 57–74 yrs. Age score was given based on the age groups, 1 for 18–37 yrs, 2

TABLE I : Values of CA-125 and biochemical predictive in the four groups.

Type of disease	*% (N)*	*Age (years)*	*CA-125 (U/ml)*	*Biochemical predictive index*
Malignant ovarian diseases	31 (95)	47±13	205±242	451±559
Benign ovarian diseases	45 (138)	41±13**	59±129**	102±248**
Non-ovarian gynaecological diseases	6 (19)	45±13	67±126**	118±247**
Non-gynaecological diseases	18 (54)	47±12	125±221	278±532

N represents the number of patients in each group. Data is expressed as Mean ± S.D.**p < 0.001. p values represent comparison with the malignant ovarian diseases group. Analysis was done by One way ANOVA with post-hoc Tukey analysis.

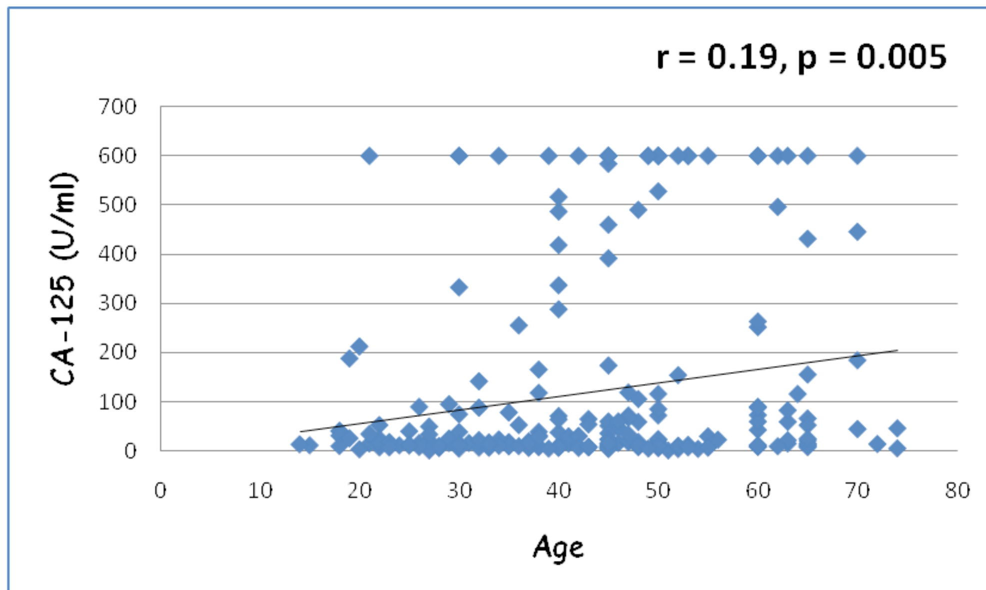

Fig. 1: Correlation between Age and CA-125 in patients with ovarian diseases.

for 38–56 yrs and 3 for 57–74 yrs. Biochemical predictive index was defined as age score multiplied by CA-125 value. The biochemical predictive index values were significantly higher in malignant ovarian diseases compared to the benign ovarian diseases (P<0.001) and non- ovarian gynaecological diseases (P<0.001), similar to CA-125. (Table I) Among the age groups ranging 38 – 56 yrs and 57–74 yrs, the CA-125 and BPI values were significantly higher in malignant ovarian diseases compared to benign ovarian diseases (Table II).

At a cut-off of 35 U/ml of CA-125, the sensitivity, specificity, positive predictive value and negative predictive value were 62%, 72%, 60% and 73% respectively. ROC curve analysis showed a cut-off of 38 U/ml for CA-125, which has sensitivity, specificity, positive predictive value and negative predictive value of 63%, 74%, 62% and 74% respectively. When the cut-off is raised from 35 U/ml to 38 U/ml, there is an increase in sensitivity, specificity and predictive values. ROC curve analysis revealed a cut-off of 86 for the Biochemical Predictive Index with

TABLE II: CA-125 values in different age groups.

Age group	*Age score*	*Malignant Ovarian Diseases*			*Benign Ovarian Diseases*		
		No	*CA-125*	*BPI*	*No*	*CA-125*	*BPI*
18–37	1	19	100±183	100±183	53	58±126	58±126
38–56	2	52	230±257**	459±514**	66	59±129	117±257
57–74	3	25	233±233*	700±699*	16	62±146	187±437

Data is expressed as Mean±S.D. **P<0.001, *P<0.01. Analysis was done by Mann Whitney U test.

TABLE III: Statistical differences of BPI and CA-125 using ROC analysis for differentiating benign and malignant ovarian disease.

	Cut-off	*Sensitivity*	*Specificity*	*PPV*	*NPV*	*Youden index*	*AUC±SEM*	*95% CI*	*P*
BPI	85.50	58	78	56	80	0.37	0.695±0.035	0.627–0.764	<0.001
CA-125	37.85	63	74	62	74	0.37	0.678±0.036	0.609–0.748	<0.001

AUC±SEM : Area under the curve±Standard Error of Mean
CI:Confidence interval
PPV:Positive predictive value
NPV:Negative predictive value

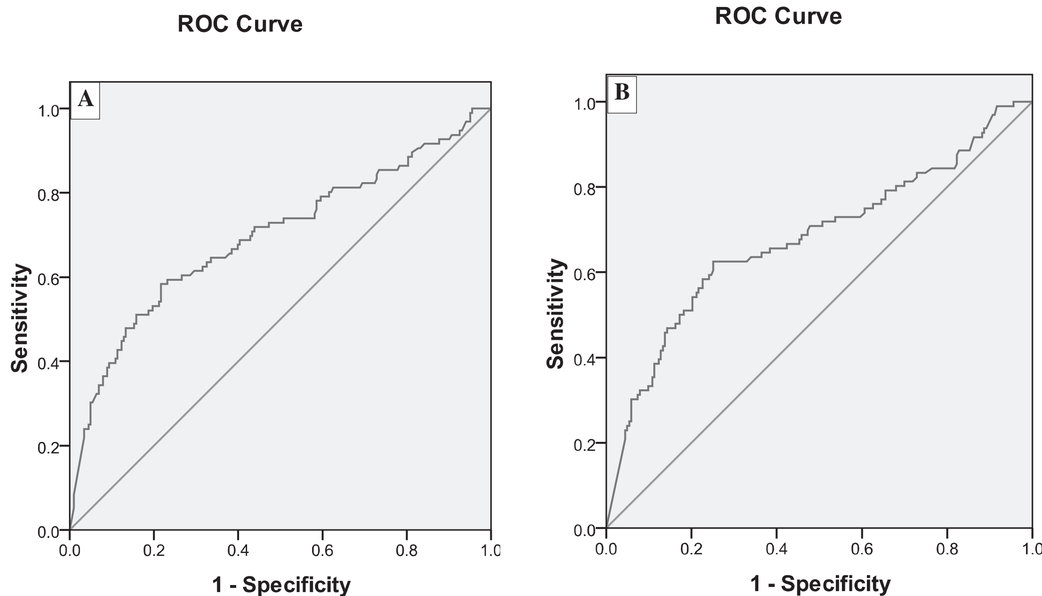

Fig. 2: ROC curve characteristics of (A) Biochemical Predictive Index and (B) CA-125 for malignant ovarian disease.

the sensitivity, specificity, positive predictive value and negative predictive value as 58%, 78%, 56% and 80% respectively. Biochemical Predictive Index has better specificity and negative predictive value compared to CA-125. (Table III; Fig. 2).

DISCUSSION

Though CA-125 is a very good marker for epithelial ovarian cancer, it has a high false positivity as it is increased in non-ovarian neoplasms like carcinoma of pancreas, liver, etc and also in benign diseases like benign ovarian cysts, ectopic pregnancy etc. Since ovarian cancer is predominantly a disease occurring in the older age, considering the age as in 'risk of malignancy index' is useful (2). Therefore, specificity of CA-125 is poor, which may be due to the common cut-off of 35 U/ml which is used in all age groups. Even in the 'risk

of malignancy index', age is not directly taken into consideration. Instead only the menopausal status has been incorporated. Maybe it is time for us to change this concept and study deeply about the variations in CA-125 values in different age groups. We have made an effort in this direction, which led us to framing a new index incorporating the CA-125 value and age.

In the present study, compared to the benign ovarian diseases and non-ovarian gynaecological diseases, malignant ovarian diseases have a significantly higher CA-125 levels. This reinstates the role of CA-125 as a marker of malignant ovarian diseases, inspite of its high false-positive rate.

Age has a role in the ovarian cancer, with post menopausal women having more predispositions (2). Two-third of the women with ovarian cancer are found to be above 55 yrs (11). Though the results of this study didn't exactly follow this distribution, we found a significant positive correlation between age and CA-125, which led us to investigate the CA-125 levels in different age groups. We defined a Biochemical Predictive Index, which can be obtained by multiplying the age score with the CA-125 value.

There wasn't any significant difference in CA-125 and BPI levels between benign and malignant ovarian diseases in the age group 18–37 yrs. Maybe this can be attributed to an improper matching of sample size in this age group, unlike the other two age groups. But, one notable fact is that the sample size is comparable in the other two age groups.

The CA-125 and BPI levels are significantly higher in the malignant ovarian diseases than in the benign ovarian diseases in the age groups 38–56 yrs and 57–74 yrs. Among the two age groups, the 38–56 age group shows more significant difference. Maybe the CA-125 and BPI levels can help in differentiating the benign and malignant ovarian diseases in age group 38–56 yrs better than in other age groups.

Most ovarian cancers occur after menopause when the ovaries have no physiological role. Since abnormal ovarian function causes no symptoms after menopause and due to the anatomical location of the ovaries deep in the pelvis, ovarian cancers become symptomatic only after they reach a large size or after metastasis (3).

The initiation of epithelial carcinogenesis due to ovulation may start from 20-30 yrs of age, tumor promotion may occur after age 30 either due to ovulation or due to the high levels of hormones associated with it. These events may culminate in the development of a tumor which may become clinically apparent after 30 (12). A relationship has been found between ovulation and the mutant p53 overexpression in epithelial ovarian cancer (13).

ROC analysis revealed 86 as cut-off for BPI, which has a better specificity and negative predicitive value than CA-125 alone. This index will be helpful in differentiating benign and malignant ovarian diseases, as it has more specificity. Even the marginal improvement in specificity is crucial, as it can eliminate the false positivity which might be quite alarming for the patient.

The limitation of our study was unavailability of data on ultrasound and menopausal state of the study subjects in their case records. These details would have helped us in calculating RMI score and to compute its association with Biochemical Predictive Index. We consider this as major limitation of our study.

Hence we conclude that Biochemical Predictive Index, which has a better specificity and negative predictive value than CA-125, can be used in the screening of ovarian pathology. Further large-scale studies on this regard will provide a cost-effective biochemical screening strategy.

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HEALING EFFECT OF *TERMINALIA CHEBULA* FRUIT EXTRACT ON TRINITROBENZENE SULFONIC ACID INDUCED COLITIS IN RAT

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Abstract : The present study aims to evaluate healing effect of 50% ethanolic extract of *Terminalia chebula* fruit pulp (TCE) on trinitrobenzene sulfonic acid (TNBS, intra-colonic route) induced colitis in rats. TCE (600 mg/kg, oral) was studied in TNBS-induced colitis for its effects on fecal output, food and water intake and body weight changes, histology, antibacterial activity and levels of free radicals (nitric oxide and lipid peroxidation), antioxidants (superoxide dismutase; catalase and reduced glutathione) and acute inflammatory marker (myeloperoxidase) in colonic tissue. TNBS administration increased colonic mucosal damage and inflammation (macroscopic and microscopic) and stool output but decreased body weight which was reversed by TCE treatment. TCE showed significant antibacterial activity and enhanced the antioxidants but decreased free radicals and myeloperoxidase activities affected in TNBS colitis. Thus, Terminalia chebula dried fruit pulp extract healed colitis by promoting antioxidant status and decreasing intestinal bacterial load, free radicals and myeloperoxidase responsible for tissue damage and delayed healing.

Key words : *terminalia chebula*, colitis, free radicals, antioxidants, myeloperoxidase, antimicrobial activity

INTRODUCTION

Ulcerative colitis (UC) is a major form of inflammatory bowel disease (IBD) which affects the colon and rectum and typically involves only the innermost lining or mucosa, manifesting as continuous areas of inflammation and ulceration, with no segments of normal tissue (1). Although the etiology remains largely unknown, it has been suggested that a combination of genetic susceptibility factors and the activation of the mucosal immune system or non-specific inflammatory reactions in response to luminal bacterial antigens along with persistent pathologic cytokine production

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contributes to the initiation and chronification of UC (2). Chronic inflammation due to chemical irritation, infection, or immune imbalance increases production of reactive oxygen species (ROS) and impairs antioxidant defenses, resulting in oxidative-stress (3). Activated neutrophils and macrophages are responsible for ROS or reactive nitrogen species (NOS) generation and the levels of ROS/NOS can be correlated with the severity of inflammatory changes in the colonic mucosa (4). Recently, we reported the healing effect of 50% ethanolic extract of dried fruit pulp of *Terminalia chebula* against acetic acid-induced colitis in rats (5).

Terminalia chebula (TC) is distributed in India, Nepal, China, Sri Lanka, Malaysia and Vietnam. It is called Haritaki in Hindi. It has been advocated in indigenous systems of medicine to treat many diseases such as parasitic infections, digestive diseases, irregular fevers, urinary diseases, flatulence, constipation, ulcers, vomiting and colic pain. It is reported to have antimicrobial, anti-inflammatory, antioxidant, immunomodulatory and adaptogenic properties (5). Acetic acid induces colitis by its direct necrotizing effect associated with acute inflammation and activation of arachidonic acid pathway while, TNBS (a hapten) induces a delayed-type hypersensitivity which proceed to develop colitis by chronic immunological inflammation followed by liberation of inflammatory markers like cytokines and arachidonic acid metabolites leading to oxidative stress, tissue damage and delayed healing (6). The present work evaluates the healing effect of TCE on another inducible model of intra colonic trinitrobenzene sulfonic acid (TNBS)-induced colitis in rats.

MATERIALS AND METHODS

Collection of plant material and extraction

Fruits of TC were collected in the months of October to February from Ayurvedic Garden, Banaras Hindu University (BHU), Varanasi. The fruit pulps were cut into small pieces and dried at room temperature and powdered. The plants and their parts were identified with the standard sample preserved in the department of Dravyaguna, Institute of Medical Sciences, BHU, Varanasi. Fifty percent (50%) ethanol extract of TC (TCE) was prepared by adding 500 ml each of ethanol and distilled water in 200 g of dried fine powder of TC. The mixture is shaken at intervals and the extract was filtered after two days. The procedure was repeated twice at an interval of two days. The ethanol containing extract so obtained each time was mixed and later dried at 40°C in incubator. The yield was 28.3% (w/w). TCE was stored at –20°C until further use.

Animals

Inbred Charles-Foster strain albino rats (180-210 g) and Swiss albino mice (25-30 g) of either sex were obtained from the Central animal house of Institute of Medical Sciences, Banaras Hindu University, Varanasi. They were kept in the departmental animal house at 26°±2°C, 44-56% relative humidity and 10:14 h light and dark cycle for 1 week before and during the experiments. Animals were provided with standard rodent pellet diet (Pashu Aahar, Ramnagar, Varanasi) and water was given *ad libitum*. 'Principles of laboratory animal care' (NIH publication no. 82-23, revised 1985) guidelines were followed.

Approval from the Institutional Animal Ethical Committee was taken prior to the experimental work.

Chemicals

Sulfasalazine (Sazo, Wallace, Mumbai, India; SS) and 2,4,6-trinitrobenzene sulfonic acid (Sigma-Aldrich St. Louis, MO; USA) and other chemicals used in the study were purchased from standard companies. Muller-Hinton agar and broth (Hi-media, Mumbai, India), was used for antibacterial activity.

Induction of colitis

Colitis was induced by intra-colonic administration of TNBS to 24 hr fasted rat (6). Rats were either given intracolonic normal saline (NS, 0.4 mL/rat, negative control) or TNBS alone (40 mg/0.4 mL of 40% ethanol/rat, control).

Treatment protocol

TCE and standard UC protective drug, sulfasalazine (SS) were suspended in 0.5% carboxy methyl cellulose (CMC) in distilled water and were given orally, once daily in the volume of 1 ml/100 g body weight. Each group in the study contains 6 rats. First dose of CMC/TCE/SS was given 4 h after the induction of UC with TNBS and then the treatment was further continued for a period of 14 days. The rats were put on fast on 14th day, after the oral administration of last dose of CMC (control), TCE (test extract) or SS (positive control) in TNBS induced colitis groups in rats while, rats with oral CMC plus intracolonic NS acted as negative control. TCE (600 mg/kg) and standard UC protective drug, SS (100 mg/kg) doses against TNBS-induced colonic mucosal damage score, weight and adhesions were selected on the basis of our earlier reported study on acetic acid induced colitis (5).

Assessment of diarrhoea

Diarrhoea was observed in intracolonic NS-treated (without colitis) and TNBS-induced colitis and after administration of oral CMC/TCE/SS. The effects were seen on the 7th and 14th day of the experiment. The result of TNBS was compared with NS while that of TCE/SS treated groups were compared with TNBS group.

Assessment of changes in body weight, food intake and water intake.

The above parameters were measured on the 7th and 14th day of the experiment. Each rat was individually weighed using standard rat weighing machine. Similarly a measured weight of enough food and water was given to each rat housed individually in the iron cages (8×11×7 cubic inches) at a fixed time of day and next day the amount of food and water left was calculated for individual rat.

Assessment of colonic damage and inflammation

All scorings of damage and excision of tissue samples were performed by an observer unaware of the treatment group. The rats in the various treatment groups were randomized before being sacrificed. The rats were weighed and sacrificed by an over dose of ether and proximal 8 cm of colon was removed. The colon was opened by a longitudinal incision, rinsed with tap water and pinned out on a wax block. Macroscopically visible damage was scored

on a 0-10 scale using the scoring system as described by Morris and associates (7), which takes into consideration these verity and number of ulcers in terms of tissue damage score, thickening and adhesions (signs of inflammation). Subsequently 8 cm of colon were taken for measurement of weight. The weight was expressed as mg/cm length of individual rat.

Histopathology of Colon

Histopathology of the colon was done in all the groups on 15th day of experiment to know the status of healing. Approximately 0.5 cm × 0.5 cm of colon was taken and fixed in 10% buffered formalin and paraffin embedded. 4-6 µm thick sections were stained with hemotoxylin and eosin stain for histological evaluation and examined under microscope at × 100 magnification.

Biochemical analysis

On 15th day of experiment the animals were sacrificed and colon of each rat was taken out and washed with cold normal saline. Antioxidants i.e. superoxide dismutase, SOD (8), catalase, CAT (9) and reduced glutathione, GSH (10); free radicals i.e. lipid peroxidase, LPO (11) and nitric oxide, NO (12); acute inflammatory marker, myeloperoxidase, MPO (13) and protein (14) were estimated in colonic mucosal homogenates following the standard procedures. LPO levels were estimated in terms of malondialdehyde (MDA) released during lipid peroxidation. Nitrites and nitrates are formed as end products of reactive nitrogen products during NO formation which are measured by using Griess reagent. SOD activity was estimated by its ability to inhibit reduction of nitro blue tetrazolium to blue coloured formazan in presence of phenazinemethasulphate (PMS) and NADH. One unit (U) of enzyme activity is defined as enzyme concentration required to inhibit the chromogen conversion by 50%. CAT measurement was done based on the ability of catalase to oxidize hydrogen peroxide. One unit (U) of catalase is the enzyme, which decomposes one mM of hydrogen peroxide per min at 25°C. GSH activity in the homogenate was estimated by the ability of GSH to reduce DTNB within 5 min of its addition against a reagent blank with no homogenate. MPO activity was determined as an indicator of polymorphous nuclear leucocyte accumulation. MPO activity was estimated by its ability to inhibit reduction of nitro blue tetrazolium to blue coloured formazan in presence of phenazinemethasulphate (PMS) and NADH. One unit (U) of enzyme activity is defined as enzyme concentration required inhibiting the chromogen conversion by 50%.

Antimicrobial activity

In vitro antibacterial susceptibility test of TCE was done using serial concentrations of 50, 100, 150 and 200 mg/ml following the approved standards of the National Committee for Clinical Laboratory Standards (15) against various intestinal pathogens i.e. *Escherichia coli* ATCC 25922, *Shigellaboydii*, *Shigellasonnei* and *Shigellaflexneri* obtained from the American Type Culture Collection (ATCC) and clinical strain preserved at Department of Microbiology, Institute of Medical Sciences, BHU, Varanasi, India following the disk diffusion method while, minimum inhibitory concentration (MIC) was performed by micro dilution method (16).

Acute toxicity study in mice

Six adult Swiss albino mice of either sex (3 males and 3 females), weighing between 25 to 30 g fasted overnight, were used for acute toxicity study as per OECD guideline. Suspension of TCE was orally administered at 3 g/kg stat dose (5 times of the optimal effective dose of 600 mg/kg) to mice. Subsequent to TCE administration, animals were observed closely for first four hours, for any toxicity manifestation, like increased motor activity, salivation, convulsion, coma and death. Subsequently observations were made at regular intervals for 24 h. The animals were under further investigation up to a period of two weeks (17).

Statistical analysis

The statistical analysis was carried out by using unpaired t-test and one way analysis of variance followed by Dunnett's test for multiple comparisons. The values are represented as mean±SEM. P<0.05 was considered significant.

RESULTS

Effects on diarrhea

TNBS rats showed increase in faecal output from 2.33±0.13 g/100 g body weight at day 0 to 3.48±0.27 g/100 g body weight (49.4% increase) and 3.67±0.31 g/100 g body weight (57.5% increase, P<0.01) at day 7 and 14 respectively. TCE treated rats showed increase in faecal output from 2.46±0.21 g/100 g body weight at day 0 to 3.06±0.25 g/100 g body weight (24.3% increase) and 3.08±0.23 g/100 g body weight (25.1% increase) thus leading to decrease in fecal output from 49.4% to 24.3% (25.1% decrease) and 57.5% to 25.1% (32.4% decrease) from TNBS group at day 7 and 14 respectively. SS treated rats showed increase in faecal output from 2.16±0.19 g/100 g body weight at day 0 to 2.97±0.32 g/100 g body weight (37.4% increase) and 2.50±0.12 g/100 g body weight (15.8% increase), a decrease by 12.0% and 41.7% from TNBS group at day 7 and 14 respectively (Figure 1a).

Effects on body weight, food and water intake

TNBS-treated rats showed decrease in body weight from 199.4±2.19 g at day 0 to 182.8±3.77 g (8.3% decrease) (P<0.01) and 171.1±2.71 g (14.2% decrease) (P<0.001) at day 7 and 14 respectively. TCE treated rats on the other hand, showed increase in body weight from 191.3±5.13 g at day 0 to 213.9±4.19 g (11.8% increase) and 220.4±6.79 g (15.2% increase) (P<0.01) while, SS treated rats showed increase in body weight from 187.4±2.79 g at day 0 to 207.3±2.31 g (10.6% increase) and 218.1±3.76 g (16.4% increase) (P<0.001) at day 7 and 14 respectively (Figure 1b). However, little change was observed on food and water intake between the TNBS-treated and TCE and SS treated animals from 0 day to 14th day of study treatments.

Effects on colonic damage, inflammation and adhesions

Normal saline did not show any colonic mucosal damage, thickening or adhesion and the colonic weight was 158.3±6.37 mg/cm. TNBS increased colonic mucosal damage score (5.17±0.31, P<0.001), adhesions (5/6 rats, 83.3%) and weight to 248.8±6.67 mg/cm (57.2% increase, P<0.001) compared with normal saline group. TCE (600 mg/kg)

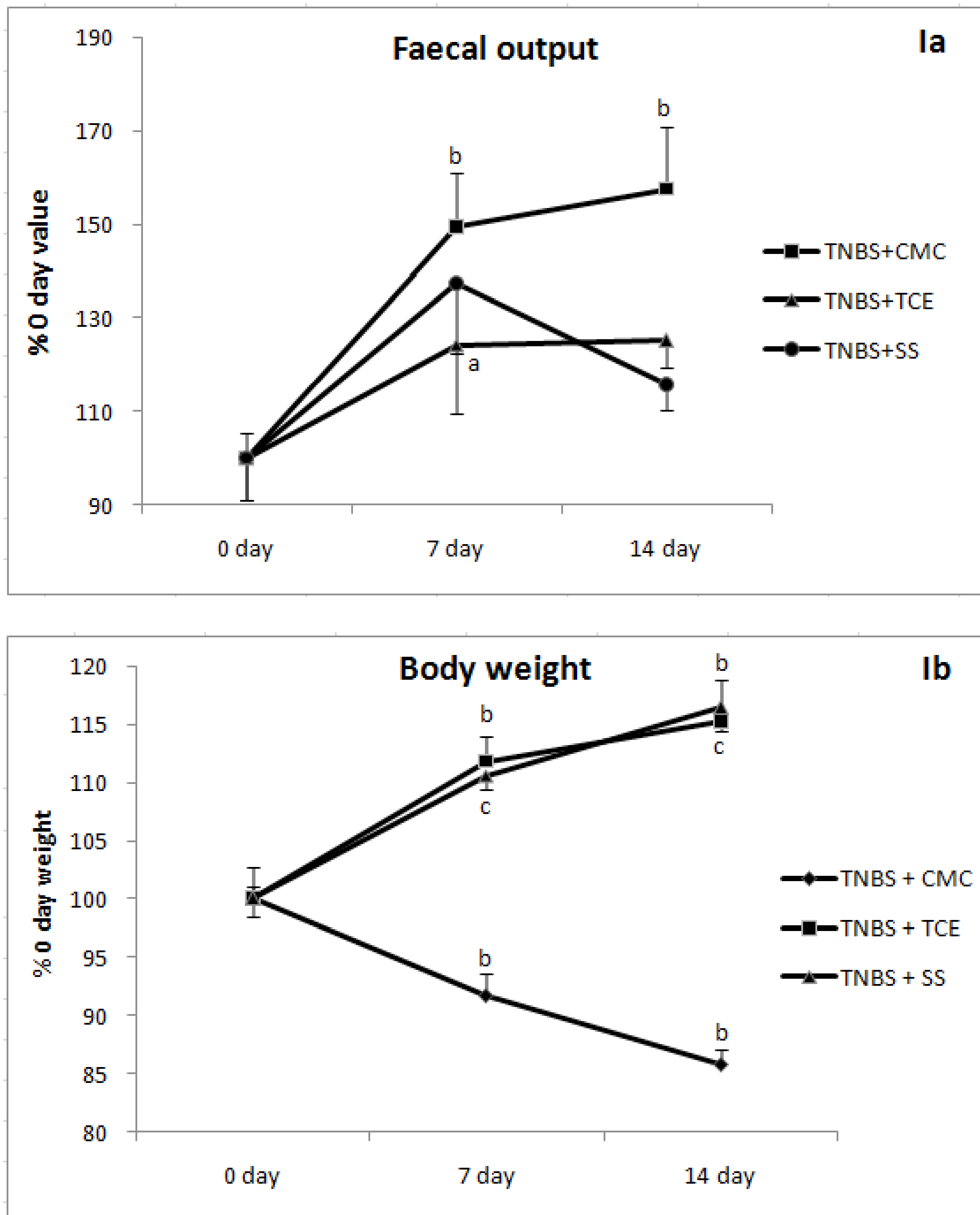

Fig. 1: Effects of TCE on TNBS-induced rat fecal output and body weight. Results are percent of mean±SEM of respective 0 day group value (n=6). [a]P<0.05, [b]P<0.01and [c]P<0.001 compared to respective 0 day group value % TNBS control.

reduced TNBS-induced colonic mucosal damage score by 61.3% (P<0.001), colonic weight by 27.0% (P<0.001) and adhesion by 60%. SS treated rats showed decrease in colonic damage score, colonic weight and adhesions by 77.4% (P<0.001), 33.5% (P<0.001) and 80.0% (1/6 rat) respectively compared with TNBS group (Figure 2).

Histopathology

Histology of colon of NS rats showed normal and clear structure with intact mucosa, submucosa and muscularis externa. TNBS colitis rats showed eroded mucosa, crypt destruction with severe cryptitis, lympho-plasmacytic infiltrate and transmural inflammation while, TNBS-induced colitis rats treated with TCE or SS showed improvement in the structures with near intact lamina propria with mild lympho-plasmacytic infiltrate and submucosa with mild lymphomononuclear aggregate (Figure 3a-d).

Effects on free radicals

TNBS enhanced both LPO and NO expressed as nmol/mg protein compared to NS rats. TCE and SS showed reversal of levels of both LPO and NO near to the NS level (Table I).

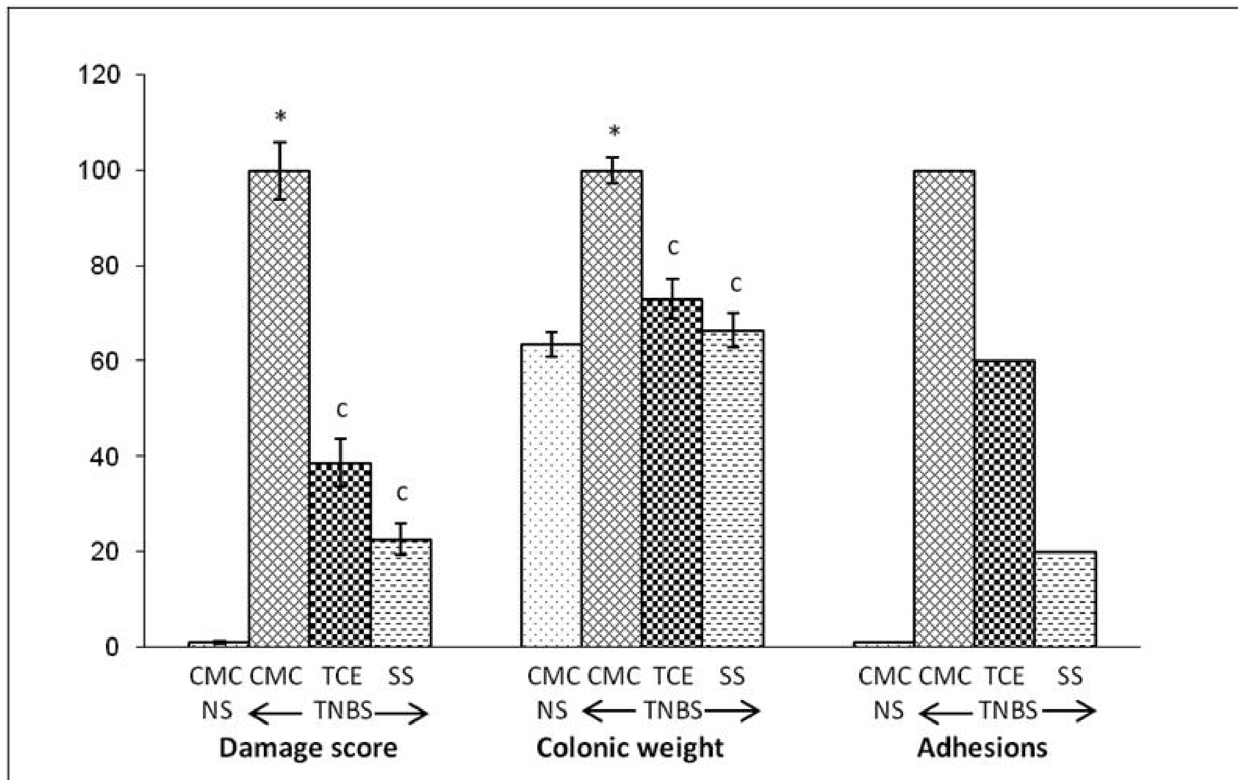

Fig. 2 : Effects of TCE on TNBS-induced rat colonic mucosal damage score, weightand adhesions. Results are percent of mean±SEM of respective TNBS control values (n=6). *P<0.001 compared to NS group (unpaired 't' test),and [c]P<0.001compared to respective TNBS group (one way analysis of variance followed by Dunnett's test).

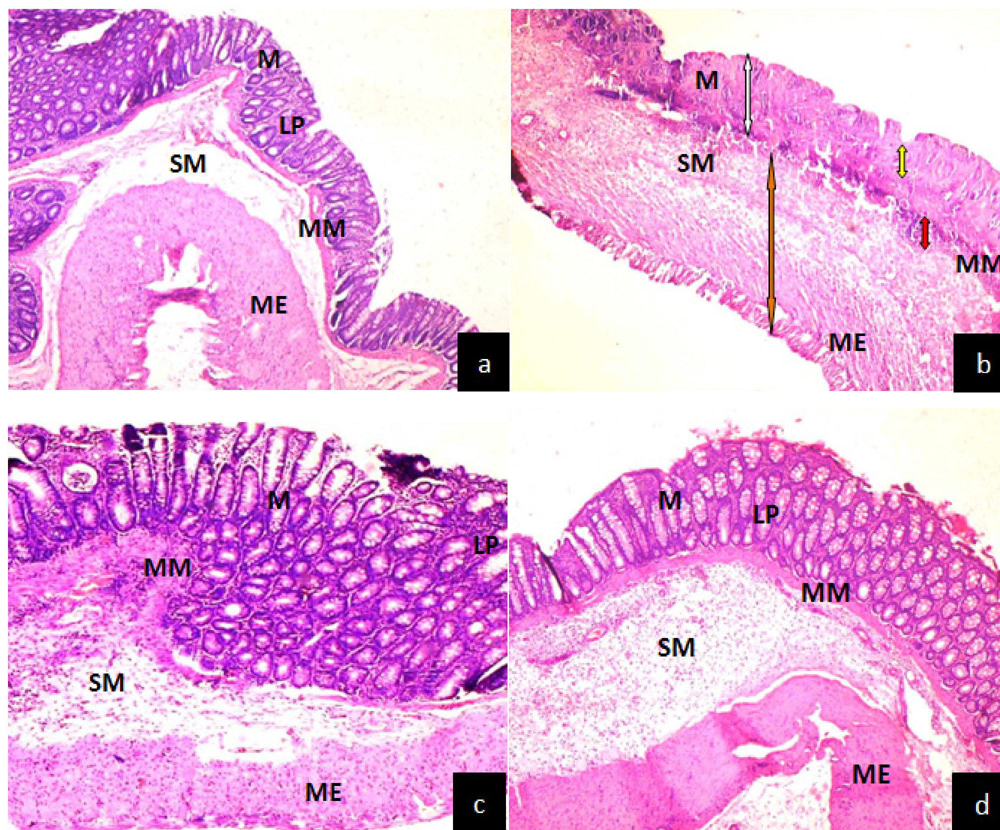

Fig. 3 : Histolgical section of rat colon stained with H & E stain (x100). **(a)** NS+CMC showing normal structure and clear with intact mucosa and sub mucosa. **(b)** TNBS+CMC showing ulcerated and eroded mucosa shown by white arrow, crypt destruction with severe cryptitis shown by yellow arrow, lymphoplasmacytic infiltrate shown by red arrow and transmural inflammation (predominantly-lymphocytes and plasma cells) shown by brown arrow. **(c)** TNBS+ TCE showing regenerative mucosa with mild crypt distortion and mild lympho-plasmacytic infiltrate in the lamina propria with oedematoussubmucosa and **(d)** TNBS+ SS showing intact mucosa with minimal lymphoplasmacytic infiltrate in the laminapropria. [M: Mucosa; SM: Submucosa; LP: Lamina propria; MM: Muscularis mucosa; ME: Muscularis externa].

Effect on antioxidants

TNBS treated animals showed significant decrease in SOD, CAT and GSH levels in the colonic mucosal incubates when expressed as mU (SOD and CAT) or nmol (GSH) per mg protein compared to NS group. TCE and SS treatments reversed the above changes in SOD, CAT and GSH levels in TNBS-induced colitis near to normal NS group (Table I).

Effect on myeloperoxidase

TNBS treated animals showed significant increase in MPO level in the colonic mucosal incubates when expressed as mU/ mg protein compared to normal NS rats. TCE and SS reversed the above changes in MPO level near to normal NS group (Table I).

Antimicrobial susceptibility and MIC

In-vitro antimicrobial susceptibility test

TABLE I : Effect of TCE and SS on TNBS-induced changes in free radicals (lipid peroxidation, LPO and nitric oxide, NO), antioxidants (superoxide dismutase, SOD; catalase, CAT and glutathione, GSH) and myeloperoxidase (MPO) in rat colonic mucosa.

Oral treatment (mg/kg, od × 14 days)	*Free radicals*		*Anti-oxidants*		*Myeloperoxidase*	
	LPO (nmol/mg protein)	*NO (nmol/mg protein)*	*SOD (mU/mg protein)*	*CAT (mU/mg protein)*	*GSH (nmol/mg protein)*	*MPO mU/mg protein*
NS+CMC	3.81±0.22	4.77±0.25	191.2±20.1	3.38±0.21	6.47±0.48	6.83±0.37
TNBS+CMC	10.50±0.62*	10.10±1.25*	443.7±10.5*	1.28±0.06*	3.61±0.27*	60.88±1.42*
TNBS+TCE 600	7.00±0.24[b]	7.09±0.49[c]	390.3±30.2[c]	3.30±0.18[c]	7.58±0.47[c]	20.03±1.23[c]
TNBS+SS 100	4.78±0.64[c]	5.57±0.39[c]	232.4±10.6[c]	3.19±0.22[c]	5.35±0.21[c]	16.96±2.19[c]

Results are mean±SEM (n=6). *$P<0.001$ compared to respective NS+CMC group (unpaired 't' test) and [b]$P<0.01$, [c]$P<0.001$ compared to respective TNBS group (one way ANOVA followed by Dunnett's test).

TABLE II : *In-vitro* antibacterial activity and minimum inhibitory concentration (MIC) of TCE.

Name of organism	*TCE Antibacterial activity (Zone of inhibition in mm)*				*MIC (mg/ml)*
	(50 mg/ml)	*(100 mg/ml)*	*(150 mg/ml)*	*(200 mg/ml)*	
E. coli ATCC 25922	7.1±0.57	8.3±0.34	9.0±0.43	10.1±0.92	6.25
Shigellasonnie	8.3±0.94	9.2±0.81	9.8±0.45	11.3±0.81	1.57
S. boydii	8.0±0.47	9.4±0.63	10.1±0.41	11.5±0.65	1.57
S. flexneri	8.7±1.41	9.8±0.35	10.6±0.62	12.1±0.87	0.79

Values are mean±SEM of 3 experiments in each group.

against gram negative intestinal bacteria like *E. coli*, *S. boydii*, *S. sonnie* and *S. flexneri* with TCE (200 mg/ml) as indicated by zone of inhibition >10 mm. However, the MIC value against *E. coli, S. sonnie, S. boydii* and *S. flexneri was* 6.25, 1.57, 1.57 and 0.79 mg/ml respectively indicating more susceptibility of *S. sonnie* and *S. boydii* to lower concentration of TCE (Table II).

Acute toxicity study

TCE did not show any acute toxicity manifestation like increased motor activity, salivation, colonic convulsion, coma and death in mice, observed up to a period of two week.

DISCUSSION

TNBS-induced colitis led to loss of body weight and increase in diarrhea/faecal output, which could be due to alterations in epithelial function produced, either directly or indirectly by products released from activated mast cells (18). Our present study, further indicated significant increase in colonic mucosal damage score, adhesions and colonic weight and crypt destruction with cryptitis, eroded mucosa, lymphoplasmacytic infiltrate and transmural inflammation, due t o TNBS-induced immunological inflammatory changes. TCE-treated rats showed reversal of above effects thus, indicated healing effect in TNBS-induced colitis. These effects may

be attributed to the anti-inflammatory, immunomodulatory and antiulcer properties of TC as reported earlier (19-21).

Neutrophils infiltration is one of the most prominent histological features in the inflamed colonic mucosa of colitis and source of myeloperoxidase (22). We found a several fold increase of myeloperoxidase in TNBS-induced colitis and reduction of MPO activity can be interpreted as a manifestation of the anti-inflammatory effect of TCE. Activated neutrophils produce ROS/NOS within intestinal mucosa inducing oxidative stress, which plays a significant role in the pathogenesis of UC/IBD (23-26). These chemicals attack the cell membranes and produce damaging fatty acid radicals and lipid hydroperoxides. Therefore, elimination of ROS could be an important strategy in healing of UC and antioxidants hasten it by destroying free radicals (27). TCE possessed significant antioxidant activity which helped in preventing oxidative damage and promoted healing. The above effects of TCE could be attributed to various active constituents namely phenolic compounds, triterpinoids, tannins, and flavonoids which are commonly known for their antioxidant activity (28-31).

Extensive immune activation and breakdown of the intestinal barrier provides bacteria access to the gut mucosal immune system, resulting in uncontrolled inflammation and dysbiosis (32). TCE exhibited considerable level of inhibition against the intestinal organisms as reported earlier for herbal products (33) and this could be due presence of certain phytochemical including flavonoids in TCE. The antibacterial effect could be contributory factors in helping healing of colitis induced by TNBS. TCE was also found to have no acute toxicity even with five times of the optimal effective dose administered to mice indicating its safety on use.

The results of the present study with 50% ethanol extract of fruit pulp of *Terminalia chebula* do indicate promising healing effects in TNBS-induced colitis. It further, substantiates our earlier reported work on its healing effect against acetic acid induced colitis in rats.

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SHORT COMMUNICATION

ANTHROPOMETRIC CHANGES PRECEDE THE CHANGES IN LIPID PROFILE AMONG THE HEALTHY YOUNG INDIVIDUALS WITH FAMILY HISTORY OF TYPE 2 DIABETES MELLITUS

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Abstract : Increase in diabetes mellitus (DM) is a major health concern. Offspring's of subjects with diabetes are known to express various trait characteristics. In the present study, anthropometric and metabolic parameters among healthy offspring's with (cases, n=50) and without (control, n=50) family history of type 2 DM is compared. Anthropometric measurements, fasting blood sugar and lipid profile were estimated. Cases showed significant increase in their anthropometric measurements than controls and they also demonstrated significant increase in total cholesterol, LDL and decreased HDL and HDL/LDL ratio. Cases were further categorized into two subgroups based on BMI (group 1, BMI=21.55±1.7 kg/m^2, group 2, BMI=29.03±4.3 kg/m^2). Groups 2, inspite of showing significant increase in their anthropometric measurements than group1 the lipid profiles were comparable. Thus, demonstrating a temporal dissociation between anthropometric and lipid changes, former preceding the later. Therefore, in younger age group, anthropometric measures could be used for risk stratification and as a metric to evaluate the efficacy of preventive intervention.

Key words : family history type 2 DM
anthropometric variables lipid profile

INTRODUCTION

Type 2 Diabetes Mellitus (DM) is an inherited disorder where the lifestyle and environmental factors play an important role in its pathogenesis (1). The incidence of type 2 DM is emerging as a major global health problem, India being major contributor especially from southern states where 47% population has family history of diabetes (2, 3). The life time risk of developing DM is about 3-5 times in offspring's with single parent and 6 times with both the parents having type 2 DM (4).

Offspring's with family history are known

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to be more obese, have increased Body Mass Index (BMI) (5) and the risk of developing type 2 DM increases fourfold with early onset DM (6). However, Indians are known to exhibit a unique 'Asian Indian Phenotype' characteristic with higher central obesity but with lower BMI (2). There is also clustering of cardiovascular risk factors like, dyslipidemia in the offspring with family history of type 2 DM (7). Lipoprotein abnormalities with insulin resistance are commonly reported in this risky group and these lipid abnormalities are demonstrated even before the development of type 2 DM (8).

Most of the studies evaluating the risk among offspring with family history DM were in the age range of 35-40 years and are from the western part of the globe (1, 9) and a very few studies are conducted among Indian young healthy adults (8). But, diabetic scenario in India is being unique as mentioned above, the present study attempts to evaluate the risk stratification among healthy young subjects between 18-25 years age group with family history of diabetes. Therefore, anthropometric, blood sugar and lipid profile in young offspring's with family history of type 2 DM is evaluated and compared with their counterparts without family history of type 2 DM.

MATERIALS AND METHODS

A case control study (n=100) of healthy non smokers and non alcoholics subjects (18-25 years) comprising of both genders. Cases (n=50, male=28. females=22) were with family history of type 2 DM (individual sharing at least 50% of genetic relation with their probands) and controls (n=50, male=24, female=26) were without family history of type 2 DM. This population based study was conducted in 2010, in Sri Siddhartha medical college, Tumkur, Karnataka, after Institutional Ethical Committee approval. The protocol was explained to the subjects and written informed consent was obtained. Subjects were screened clinically for any acute or chronic medical, psychiatric conditions and on any medications. All the female subjects had normal menstrual cycle. If subjects were found to be suffering from any disease condition on any medications they were excluded.

Subjects reported with 12 hrs fasting, anthropometric measurements were obtained. Weight (kg) was measured using standard calibrated balance scale (sensitivity ≈ 0.1 kg). Height (cm) was obtained using stadiometer and BMI was calculated. Waist circumference (WC) was measured (cm) at the level of umbilicus after normal expiration in standing position with feet together and arms by the side of the body. Hip circumference (HC) was measured (cm) as the maximum girth around the hip. Waist to hip ratio (WHR) and Waist to height ratio (WHtR) were calculated. Venous blood was drawn in sitting posture, centrifuged (5000 rpm) and serum was separated. Fasting blood sugar (FBS) by glucose oxidase method, Total cholesterol (TC), triglyceride (TG) and high density cholesterol (HDL) by enzymatic calorimetric test using ERBA diagnostic Mannheim Gmbh kit was estimated. Low Density Lipoprotein cholesterol (LDL) was calculated using friedwalds formula LDL = (Total cholesterol – HDL) – TG/5, approximate Very Low Density Lipoprotein (VLDL) concentration was derived using TG/5.

Descriptive statistics is given in mean and SD. Comparison of variables between groups was done using ANOVA by SPSS version 15. P<0.05 is considered as level of significance.

RESULTS

The mean and SD of anthropometric, FBS and lipid variables and their comparison between both the groups are depicted in Table. No. I. Subjects were age matched, but, cases weighed more than controls. BMI, WC and WHtR was significantly more among cases than controls (P<0.001). Whereas, WHR did not showed any significant difference between the two groups.

Fasting blood sugar did not show any significant difference between two groups. Total cholesterol and LDL were significantly (P<0.001) more and interestingly, HDL was significantly less in cases when compared to controls (p=0.009). Thus, HDL/LDL ratio was significantly less in cases than control (p=0.001). Whereas, triglycerides and VLDL were comparable between two groups.

BMI among cases ranged from 18 kg/m^2 to 37.20 kg/m^2 encompassing normal to obese range, with average being 24.39±4.7 kg/m^2, which is quite near to overweight value. Therefore cases were further classified into two subgroups based on BMI (median spilt 24.9 kg/m2) and their anthropometric and lipid profile were compared. Group.1 (n=31) average BMI was 21.55±1.7 kg/m^2 which is in normal range and Group 2 (n=19) average BMI was 29.03±4.3 kg/m^2 are in overweight range. The difference in their BMI was statistically significant (P<0.001). Group 2 weighed (75.94±16.09 kg) more than group 1 (60.83±8.5kg) P<0.001. Similarly, WC of group 2 (89.94±11.8) was significantly (P<0.001) more than group 1 (75.83±8.8 cm). Therefore, WHR also followed the same (group 1 = 0.816±0.05, group 2 = 0.865±0.05, p=0.003). The WHtR among group 1 (0.45±0.04) and group 2 (0.53±0.06) was significantly different (P<0.001). However,

TABLE I: Comparison of anthropometric, FBS and lipid parameters between controls and cases.

Variable	*Controls (n=50)*	*Cases (n=50)*	*F value*	*P value*
Age (years)	20.58±1.61	20.94±1.90	1.04	0.309
Weight (kgs)	56.4±9.56	66±13.9	18.17	0.000**
Height (cm)	165.4±8.2	167.72±7.64	1.17	0.281
Body Mass Index (BMI) Kg/m^2	20.6±2.29	24.39±4.71	25.31	0.000**
Waist Circumference (cm)	74.32±8.47	81.2±12.14	10.79	0.001**
Waist Hip Ratio	0.81±0.066	0.83±0.058	2.55	0.113
Waist Height Ratio	0.44±0.04	0.48±0.06	11.63	0.001**
Fasting blood sugar (mg/dl)	84.42±4.33	85.6±3.67	2.086	0.152
Total cholesterol (mg/dl)	152.95±17.88	169.3±25.75	13.62	0.000**
Triglycerides (mg/dl)	117.63±13.24	120.12±16.7	0.676	0.413
HDL (mg/dl)	38.22±2.03	37.22±1.7	7.077	0.009**
LDL (mg/dl)	92.84±16.5	108±24.06	13.618	0.000**
VLDL (mg/dl)	23.55±2.62	24.05±3.47	0.659	0.419
HDL/LDL ratio	0.41±0.066	0.36±0.063	12.012	0.001**

the most surprising and interesting was that the lipid profile which was comparable between both the groups.

In summary, our results have demonstrated that the offspring's with family history of type 2 DM are endowed with more BMI, WC, WHtR, total cholesterol, LDL and less HDL and HDL/LDL ratio when compared to offspring's without family history of type 2 DM. Cases who were overweight (i.e. Group 2) demonstrated comparable lipid profile than their counterparts with normal weight (i.e. Group 1). Thus, demonstrating that anthropometric changes precedes the alteration in lipid profile among youngsters of high risk group.

DISCUSSION

Family history of type 2 DM and obesity are the risk factors for development of type 2 DM (6). In contrast to WHO criteria, the BMI > 23 kg/m^2 in Indians is considered to be the high risk factor for developing DM - 'unique Asian phenotype (2, 10). Accordingly, the cases in the present study could be considered to be at higher risk to develop DM. In addition, indicators of visceral obesity like WC, WHtR, the predictors of glucose intolerance and insulin resistance (2, 11) were significantly higher among cases. Thus, the cases in the present study are known to be endowed with high risk physical characteristics that favour to develop type 2 DM. Increased BMI and WC are known to be one of the major causative factor to alter lipid profile (12). Further, insulin resistance which may be a genetically inherited trait (1) is also known to enhance lipolytic activity increasing fatty acid levels thus bringing about these altered changes in lipid profile and can also cause dyslipidemia in individuals with normal glucose tolerance (12). However, not estimating the insulin resistance in the present study is the limitation to directly correlate with the observed changes. Yet, the present study offers an important observation that the young healthy individuals with positive family history of diabetes who are euglycemic has shown difference in anthropometric and lipid profile favoring higher risk to develop DM. These measures could be used as the risk stratification tools and also the yard to assess the effectiveness of any preventive intervention.

In the process of evaluating the results, it was observed that BMI had a very wide range among the cases ranging between 18 kg/m^2 to 37.20 kg/m^2 (median BMI 24.9 kg/m^2) from normal BMI to obesity range, which could be one of the major confounding factors for the observed changes in the lipid profile. Therefore, based on BMI cases were further divided into two subgroups (group 1 = 21.55 kg/m^2, group 2 = 29.03 kg/m^2). Therefore, in accordance with cutoff for Asian Indians phenotype i.e. BMI > 23 kg/m^2 group 2 are at higher risk of developing type 2 DM. Anthropometric variables (WC, WHR and WHtR) in group 2 were significantly higher than group 1. However, the most interesting and intriguing finding was that the lipid profiles were comparable between these two groups. This is the unexpected but a unique observation of the present study. This observation offers an insight that the changes in anthropometric variables may not be aligned with the changes in lipid profiles. Our observation demonstrates that anthropometric changes could precede the alteration in lipid profile among healthy youngsters of high risk group to develop type 2 DM. There is a report demonstrating similar findings (13) in

children (10-14 years) with family history of type 2 DM where increase in BMI was not temporally linked to alterations in insulin levels. In similar lines, the present study has also demonstrated such a temporal dissociation between BMI and lipid profile among adolescents. To the best of our knowledge this is the second study other than earlier (14) wherein similar finding was reported among mixed age group in both children and adolescents. Thus, demonstrating that changes in anthropometric variables and lipid profile may not be temporally linked among healthy young adolescent with family history of type 2 DM. However, long term follow up study is warranted to understand the sequential changes.

Therefore, this study gives a glimpse that anthropometric changes precede far earlier than the onset of changes in lipid profile. Inspite of limitations like not evaluating insulin levels, the present preliminary study, can potentially put forth that the anthropometric variables would be a better measures for risk stratification for diabetes among high risk euglycemic younger age group with family history of type 2 DM.

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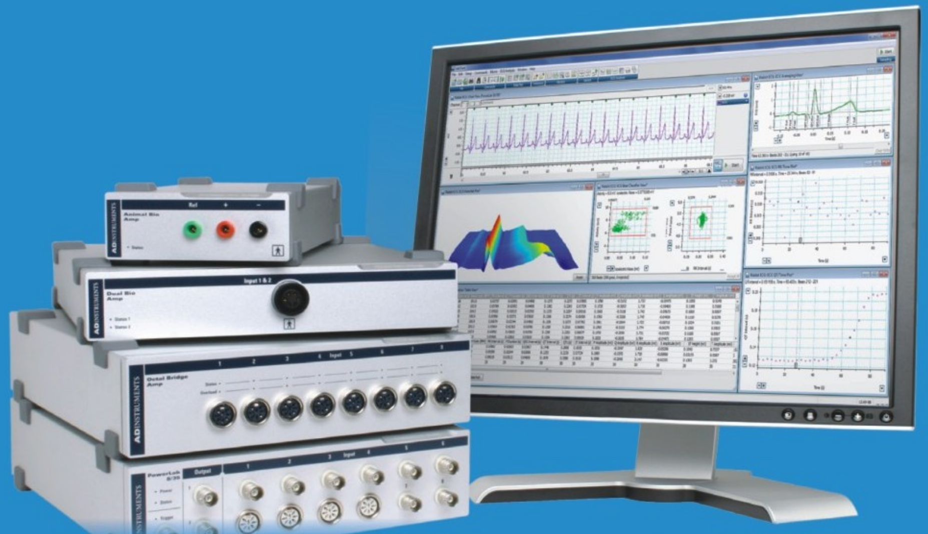

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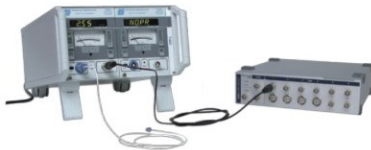

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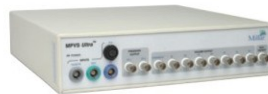

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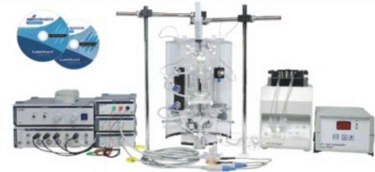

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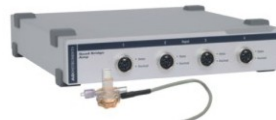

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