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Indian J Physiol Pharmacol 2018; 62(2) : 159

Editorial

Pursuing original research in Medicine

Most of the research published by Indian physiologists is gradually shifting focus to 'application' domains. Such as effect of 'X' intervention on 'Y' disease or 'Z' state. Here the 'X' is a known intervention. Therefore, such research cannot be called as original. Even if 'X' and 'Y' or 'Z' are new to each other, I would hesitate myself to call this as original research.

Being in an important position in research domain, the physiologists occupy a unique position and therefore have both, the opportunity and the responsibility to indulge in original research. Original research means, the discovery of fundamental mechanisms that explain physiological phenomenon. For example Prof. A.S. Paintal, a celebrated Indian physiologist, discovered the mechanism of cardio-respiratory response to interstitial fluid pressure/volume in lungs of anaesthetized cats, resulting in the discovery of 'J' receptors. This, is original research in the truest of notions. He, later, characterized the 'J' receptors, resulting in the advancement of science. This brought laurels to Prof. A.S. Paintal, Patel Chest Institute, Indian physiology community and to our country. This is the concept of original research which each one of us needs to contemplate deep inside.

The time has come to put a system in place to encourage original research in physiology. Currently any physiology lab has the material potential to become better than what Prof. A.S Paintal created by himself, and the funding is much more than what existed in mid 60's in the country.

For original research, we need to nurture the curiosity in young scientists and help them in developing the art of scientific questioning. We need to encourage them to pursue goals to the limit. This is the very thought that brings originality in research.

The original research provides a significant impact to the development of medical sciences in the country and to the world at large. The funding of original research provides the ammunition for translational research. Without original research, the translational research will be futile. In a complex manner, innovation and original research are connected to the country's economy.

The original question may take birth in Physiology during ongoing research for it may be generated during interaction with clinical colleagues. Therefore, the department and senior colleagues need to create such opportunities for youngsters, who should be equally alert and receptive to catch them. To encourage original research, brainstorming sessions may be conducted by experts in the department or during conferences or public defense. Innovation through challenges is another active mode of original research.

On a happy note, Science and Engineering Research Board (SERB), Department of Science and Technology (DST), Council of Scientific and Industrial Research (CSIR) and *Department of Biotechnology* (*DBT*) have defined mandate to support fundamental and original research. Anybody who has a brilliant idea alongwith necessary research skills is surely on the path of success.

Dr. K. K Deepak

Executive Editor (IJPP)

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Original Article

Nonlinear Dynamics of Heart Rate During Slow Breathing Exercise

Andrey A. Porto[1], Bruna S. Tavares[2], Giovanna Vidigal[2], David. M. Garner[3], Rodrigo D. Raimundo[4], Luiz Carlos de Abreu[4], Danilo Bocalini[5] and Vitor E. Valenti[2]*

[1]Disciplina de Cardiologia, Departamento de Medicina, UNIFESP, São Paulo, SP, Brazil

[2]Centro de Estudos do Sistema Nervoso Autônomo (CESNA), Programa de Pós-Graduação em Fisioterapia, FaculdadeCiências e Tecnologia, UNESP PresidentePrudente, SP, Brazil

[3]Cardiorespiratory Research Group, Department of Biological and Medical Sciences, Faculty of Health and Life Sciences, Oxford Brookes University, Gipsy Lane, Oxford OX3 0BP, United Kingdom

[4]Design of Studies and Scientific Writing Laboratory at the ABC School of Medicine, Santo Andre, Sao Paulo, Brazil

[5]Translational Physiology Laboratory, Department of Physical Education and Aging Sciences, São Judas Tadeu University, São Paulo, SP, Brazil

Abstract

The acute effects of slow breathing exercises on the complex behaviour of heart rate regulation were investigated. We evaluated 21 healthy male volunteers aged between 18 and 30 years old. Heart rate variability was investigated 10 minutes during spontaneous breathing and five minutes during slower breathing exercises (6 cycles/min). The consequent nonlinear metrics of heart rate variability were applied: Symbolic analysis, Shannon Entropy, Rényi Entropy, Tsallis Entropy, Approximate Entropy, Sample Entropy and Detrended Fluctuation Analysis. The symbolic exhibited an increase in two like variation and decrease in two unlike variation. Detrended Fluctuation Analysis was significantly *higher* during slow breathing exercises (0.6454±0.201 vs. 0.3949±0.205; p=0.0003). Approximate entropy was significantly *lower* during slow breathing exercises (0.8620±0.121 vs. 0.7677±0.134; p=0.0221). No significant changes were detected for Shannon Entropy, Rényi Entropy, Tsallis Entropy and Sample Entropy. In conclusion, slow breathing exercises decrease nonlinear behaviour of heart rate dynamics in healthy young males followed by reduced vagal control of heart rate dynamics. We propose that the linear behaviour of respiratory patterns influences the complexity of HRV.

***Corresponding author :**

Vitor E. Valenti, UNESP, Av. Hygino Muzzi Filho, 737, Mirante. 17.525-900 – Marília, SP, Phone: +55 (14) 3402-1300; E-mail: vitor.valenti@marilia.unesp.br

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Introduction

Slow breathing has been considered as a behavioral intervention and is widely applied for psychiatric disorders, which includes stress-related disorders, anxiety and depressive syndromes (1).

Cardiovascular and respiratory techniques including the effects of slow breathing have been reported in the research literature. Slow breathing exercise was reported to improve cardiovascular disorders through beneficial effects on the autonomic nervous system (2).

So, autonomic regulation of heart rate may be assessed through heart rate variability (HRV). HRV evaluates the fluctuations of the intervals between consecutive heart beats (RR intervals) (3). HRV is widely assessed through linear time and frequency domain indices (4). Nevertheless, linear analysis is limiting since it provides only temporal and quantitative information about heart rate dynamics; whilst nonlinear methods provide qualitative analysis of the time series (5). In most cases, only some measurable quantities, which depend on the underlying and usually unidentified dynamics of the RR interval distribution are accessible, namely time and frequency domain HRV analysis (6). The qualitative analysis of nonlinear methods includes predictability of RR intervals with extraordinary sensitivity to initial conditions and do not consider only the sequence of the signal.

Nonlinear methods are related to complexity theory since they examine specific characteristics such as, sensitivity to initial conditions and system parameter variations. Moreover, chaotic systems have been reported to be involved in groups of problems in numerous areas of life, natural and engineering sciences (6), including heart rate modulation (7).

The literature has demonstrated that changes in complexity of heart rate dynamics are associated with alterations in vagal and sympathetic regulation of heart rate in postoperative complications in hip fracture patients (8), depression (9) and diabetes (10). Additionally, nonlinear HRV was also investigated in healthy subjects during physiological stimuli such as music, in response to postural maneuvers (11) and during recovery from exercise (12).

Some procedures have been undertaken such as Poincaré plot, Tsallis Entropy and Rényi Entropy, which could potentially generate more robust outcomes. More specifically, entropy relates to the probability density function of a variable, when the entropy decreases due to the sequence lengthening, the system is predictable and highly regular, indicating reduced complexity. In this way, high entropy corresponds to unpredictable RR intervals (13). Sample entropy and approximate entropy were reported to offer an alternative measurement of sympatho-vagal balance, since they both *decreased* during sympathetic activation induced by the head-up tilt test (14).

The analysis of nonlinear methods applied to HRV in response to different breathing patterns has been previously documented in exercise (15). It was found that Detrended Fluctuation Analysis (DFA), sample entropy and approximate entropy were significantly influenced by respiratory patterns. Yet, the singular effect of slow breathing exercise on different nonlinear HRV metrics such as symbolic analysis is unclear. Furthermore, an enhanced understanding of the nonlinear dynamics of heart rate during slow breathing would enable us to achieve novel mechanisms on this intervention in the regions of cardiovascular and behavioural impairment. In this sense, we theorized that controlled slow breathing would reduce the complexity of HRV, since it increases the linearity of the respiratory pattern. Accordingly, this study was commenced to evaluate the acute effects of slow breathing exercises on nonlinear heart rate dynamics.

Materials and Methods

Study population

The subjects participating in the study were 21 healthy male students - all non-smokers, aged 20.35±1 years old, height 1.78±0.3 m, mass 76.5±16 kg and body mass index (BMI) of 22.4±4 m/kg^2. All subjects were informed about the procedures and the objectives of the study and gave confidential

written informed consent. All study events were approved by the Ethics Committee in Research of our Institution (No. 2014-953), and were in accordance with Resolution 196/96 National Health 10/10/1996.

Exclusion criteria

We excluded subjects outside the following conditions: Body Mass Index (BMI) >35 kg/m^2; systolic blood pressure (SBP) >140 mmHg or diastolic blood pressure (DBP) >90 mmHg (at rest); cardiovascular, respiratory, endocrine, anxiety and reported neurological disorders that did not permit the subjects to perform the procedures. Subjects taking medication(s) that influenced the autonomic nervous system were excluded.

Initial evaluation

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

Slow breathing protocol

The experimental procedures were completed in the same soundproofed room for all subjects. The relative humidity ranged between 40% and 60% and temperature ranged between 21°C and 25°C. Subjects were instructed to have a decent sleep, with empty bladder and stomach, without ingesting caffeine, alcohol or other autonomic nervous system stimulants for 24 hours before the evaluation. Datasets were collected on an individual basis between 18:00 and 21:00 to standardize circadian influences (16). All procedures necessary for the data collection were explained to each subject individually, and the subjects were told to remain at rest and avoid conversation during the collection.

The slow breathing protocol was founded on research literature which emphasized cycles with 10 to 12 seconds duration, hence a breathing rate of 5 to 6 cycles per minute (17). During this *modus operandi* the subjects performed approximately 6 cycles per minute with a frequency of 0.1 Hz for five minutes. The investigator guided the volunteers' breathing patterns with a metronome. These volunteers were instructed to perform deep, but slow inspirations, and similar expirations with lung volumes ranging from the total lung volume to residual volume, which is the remaining volume after maximal expiration.

HRV analysis

The Polar® RS800CX heart rate device comprised of an elastic band and two electrodes placed on the participants' chest, at the level of the xiphoid process and just below the pectoralis. HRV was analysed according to instructions from the Task Force guidelines (17). RR intervals were recorded via a digital telemetry system. A sampling rate of 1 kHz was enforced with prior validation and then downloaded to the Polar Precision Performance program (v.3.0, Polar Electro, Finland). This software enabled the visualization of heart rate and the extraction of RR interval. Consequent digital filtering was complemented with manual filtering for the elimination of premature ectopic beats and artefacts. Only series with sinus rhythm greater than 95% were included in the study.

Poincaré plot

For the visual analysis of the plot, an ellipse was fitted to the points of the chart, with the centre determined by the average RR interval.

The plot was qualitatively analysed by HRV analysis software based on the figures formed by its attractor (18, 19): figures in which an increase in the dispersion of RR intervals is observed with increased intervals, characteristic of a normal plot and small figures with beat-to-beat global dispersion without increased long-term dispersion of RR intervals.

Symbolic analysis

Symbolic analysis was performed by grouping the patterns with 3 symbols into four families as follows:

(a) no variation (0V: all the symbols are equal, i.e. 2,2,2 or 4,4,4); (b) one variation (1V: 2 consecutive symbols are equal and the remaining symbol is different, i.e. 4,2,2 or 4,4,3); (c) two like variations (2LV: the 3 symbols form an ascending or descending ramp, i.e. 5,4,2 or 1,3,4); and (d) two unlike variations (2UV: the three symbols form a peak or a valley, i.e. 4,1,2 or 3,5,3). The rate of occurrence for each pattern was defined as 0V%, 1V%, 2LV%, and 2ULV%. It has been observed that 0V% reflects only sympathetic modulation, 1V% reflects sympathetic and parasympathetic modulation, 2LV% and 2ULV% reflect, exclusively, vagal modulation (19).

Nonlinear analysis

Nonlinear analysis included Shannon Entropy, Rényi Entropy, Tsallis Entropy, Approximate Entropy, Sample Entropy and Detrended Fluctuation Analysis (DFA) (20).

Statistical analysis

Parametric statistics usually assume the data are normally distributed, hence the use of the mean as a measure of central tendancy. If we cannot normalise the data we should not compare means. To test our assumptions of normality we applied the Anderson-Darling and Ryan-Joiner tests. The Anderson–Darling test applied an empirical cumulative distribution function, whereas the Ryan-Joiner test is a correlation based test similar to Shapiro-Wilk test. Since the results were inconclusive we are unable to confirm the observations present a normal distribution. Consequently we have a probability plot of both normal and non-normal data and we apply both the one-way analysis of variance (ANOVA1) and the Kruskal-Wallis - the parametric and non-paramertric tests of significance respectively.

Principal Component Analysis (PCA) is a multivariate statistical procedure where the random observations are transformed into a smaller set of uncorrelated variables called Principal Components.

Effect size

In order to quantify the magnitude of differences between spontaneous and during slow breathing we used Cohen's guidelines of small (0.25), medium (0.5), and large (0.9) effects.

Results

Figs. 1 and 2 illustrate symbolic analysis of HRV at spontaneous breathing and during slow breathing exercise. We observed reduced 2LV and increased 2UV during slow breathing exercise in both absolute units and percentage values.

The results illustrate that there is a wide variation in both the mean values for both normal breathing and slow breathing (Table I). The p-values calculated are the ANOVA1 and Kruskal-Wallis parameters. The algorithm calculates a significant statistical result for two of the six combinations with the probability of a type I error was less than 5% ($p<0.05$). For ApEn the slow breathing exhibited a *decrease* in the output whereas with DFA there was an *increase*. This is to be expected since DFA responds in the

TABLE I: The table below shows the mean values for the five entropic measures for control and slow breathing subjects RR intervals. The number of RR intervals is 256. ANOVA1 and Kruskal-Wallis tests of significance was applied to results. Notice here the DFA is included with the five measures of entropy as a benchmark.

Entropy Type & DFA	*Mean±SD Normal Breathing (n=21)*	*Mean±SD Slow Breathing (n=21)*	*ANOVA1 (p-value)*	*Kruskal-Wallis (p-value)*	*Cohen's*	*Effect size*
Approximate	0.8620±0.121	0.7677±0.134	0.0216	0.0221	0.73	Medium
Sample	0.7235±0.141	0.7426±0.144	0.6672	0.5973	0.13	Small
DFA	0.3949±0.205	0.6454±0.201	0.0003	0.0003	1.23	Large
Shannon	0.7742±0.126	0.7542±0.123	0.6044	0.6327	0.16	Small
Renyi (α=0.25)	0.9919±0.005	0.9910±0.005	0.5665	0.6507	0.18	Small
Tsallis (q=0.25)	0.7981±0.113	0.7797±0.111	0.5973	0.6507	0.16	Small

(DFA) Detrended Fluctuation Analysis; (SD) Standard Deviation.

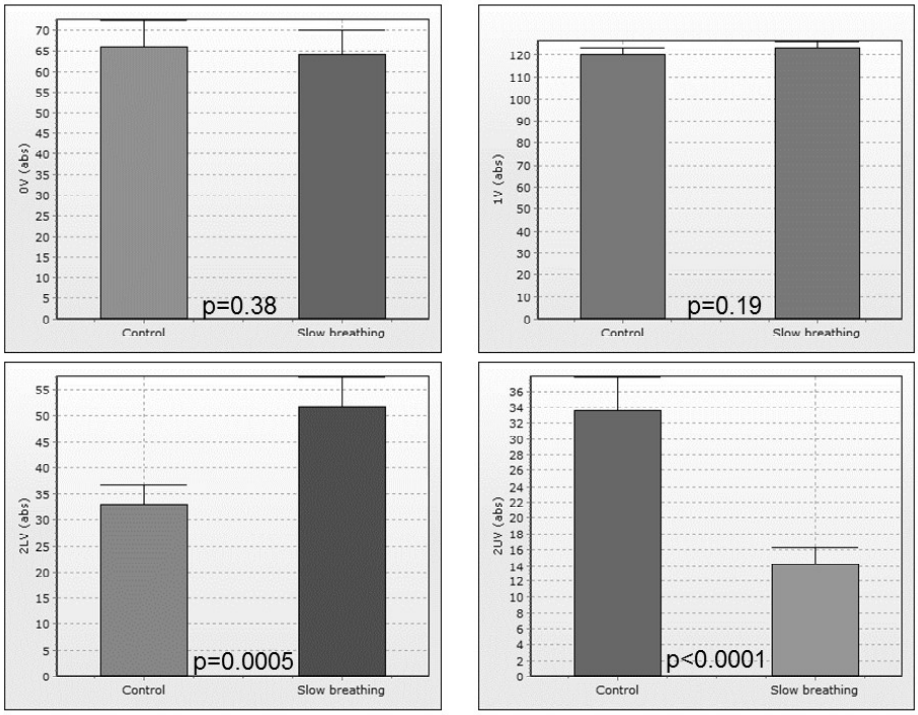

Fig. 1 : Symbolic analysis of HRV at spontaneous breathing and during slow breathing exercise in number of occurrences. (0V, Cohen's: 0.07; small effect size) three identical symbols; (1V, Cohen's: 0.294, small effect size) two identical one dissimilar symbols; (2LV, Cohen's: 0.842, medium effect size) three dissimilar symbols varying monotonically; (2UV, Cohen's: 1.33, large effect size) three dissimilar symbols varying non monotonically; (abs) number of occurrences.

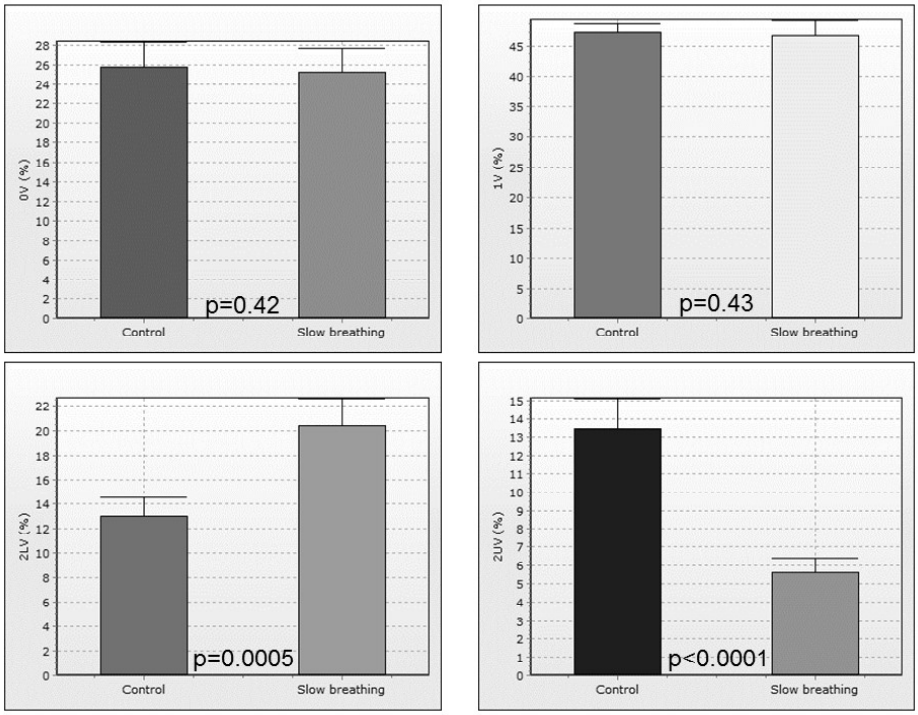

Fig. 2 : Symbolic analysis of HRV at spontaneous breathing and during slow breathing exercise in percentage. (0V, Cohen's: 0, small effect size) three identical symbols; (1V, Cohen's: 0.12, small effect size) two identical one dissimilar symbols; (2LV, Cohen's: 0.91, large effect size) three dissimilar symbols varying monotonically; (2UV, Cohen's: 1.48, large effect size) three dissimilar symbols varying non monotonically; (%) percentage.

contradictory way to entropies. Regarding DFA an increased parametric response is generated by a *decrease* in chaotic response. It is usual to subtract the value from unity and make the statistics analogous, hence (1-DFA). This is also the case with *spectral* Detrended Fluctuation Analysis (sDFA).

We had the values of five groups (all entropies except DFA) for 21 subjects who are slow breathing subjects, hence a grid of 5-by-21 to be evaluated. The First Principal Component (PC1) had a variance (eigenvalue) of 3.8159 and accounted for 76.3% of the total variance. The Second Principal Component (PC2) had an eigenvalue of 0.8842 and summed with PC1 accounted for 94.0% of total variance. PC2 had a proportion of influence of 17.7%. The Third Principal Component (PC3) had an eigenvalue of 0.2960 and summed with PC1 and PC2 accounted for 99.9% of total variance. PC3 had a proportion of influence of 5.9%. Therefore we assumed that most variance was attained in the first three principal components, so a slightly steep scree plot.

In view of the principal components we observe that the Shannon, Rényi (α=0.25) and Tsallis (q=0.25) entropies have very similar PC1, PC2 and PC3. Whereas, the ApEn and Sample entropy are correspondingly grouped with similar PC1, PC2 and PC3. Most of the variance is attained within the first three components and so we need not deliberate fourth (PC4) or fifth (PC5) principal components cited in Table II. We then represent the HRV data using the first three principal components corresponding to the most significant eigenvectors.

Fig. 3 displays the boxplots of nonlinear HRV analysis, indicating entropies and DFA during slow breathing.

Fig. 4 displays an example of the Poincaré plot patterns from one subject during spontaneous breathing and during slow breathing. We detected no visual difference between the two conditions.

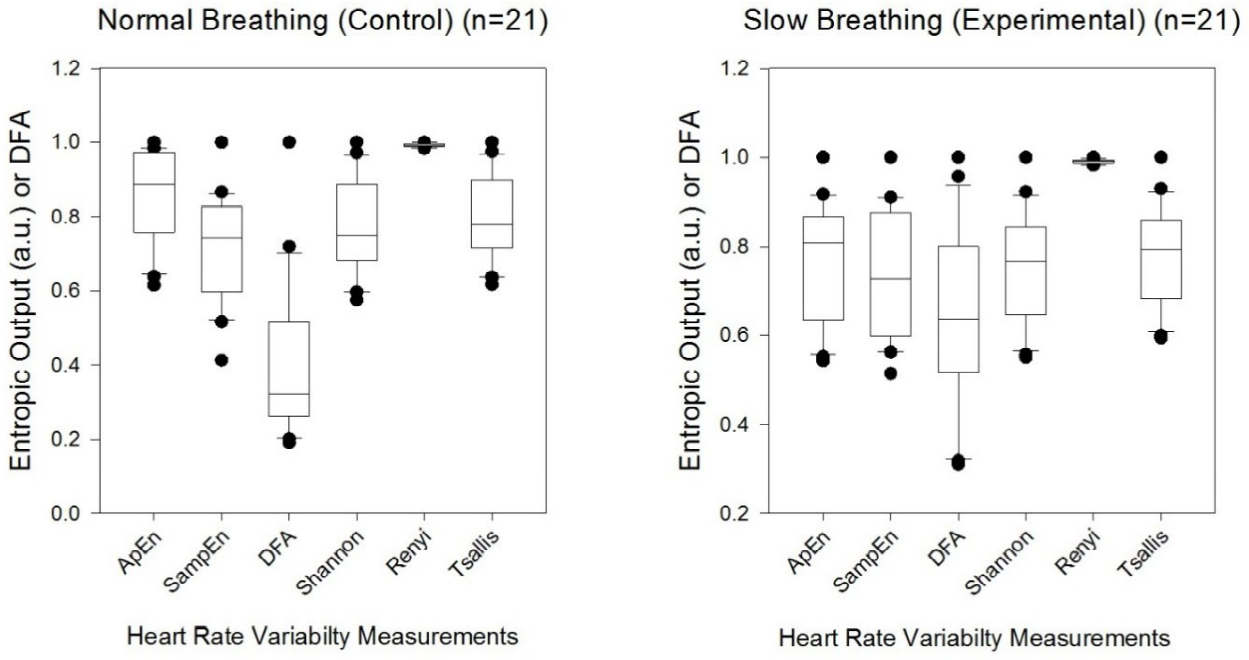

Fig. 3 : The box plots illustrate six HRV measurements for the 256 RR intervals of 21 normal breathing subjects (left) and 21 slow breathing subjects (right). The point closest to zero is the minimum and the point farthest away is the maximum. The point second closest to the zero is the 5th percentile and the point second farthest away is the 95th percentile. The boundary of the box closest to zero indicates the 25th percentile, a line within the box marks the median (not the mean), and the boundary of the box farthest from zero indicates the 75th percentile. The distance between the outer edges of the boxes represents the interquartile ranges. Whiskers (or error bars) above and below the box indicate the 90th and 10th percentiles.

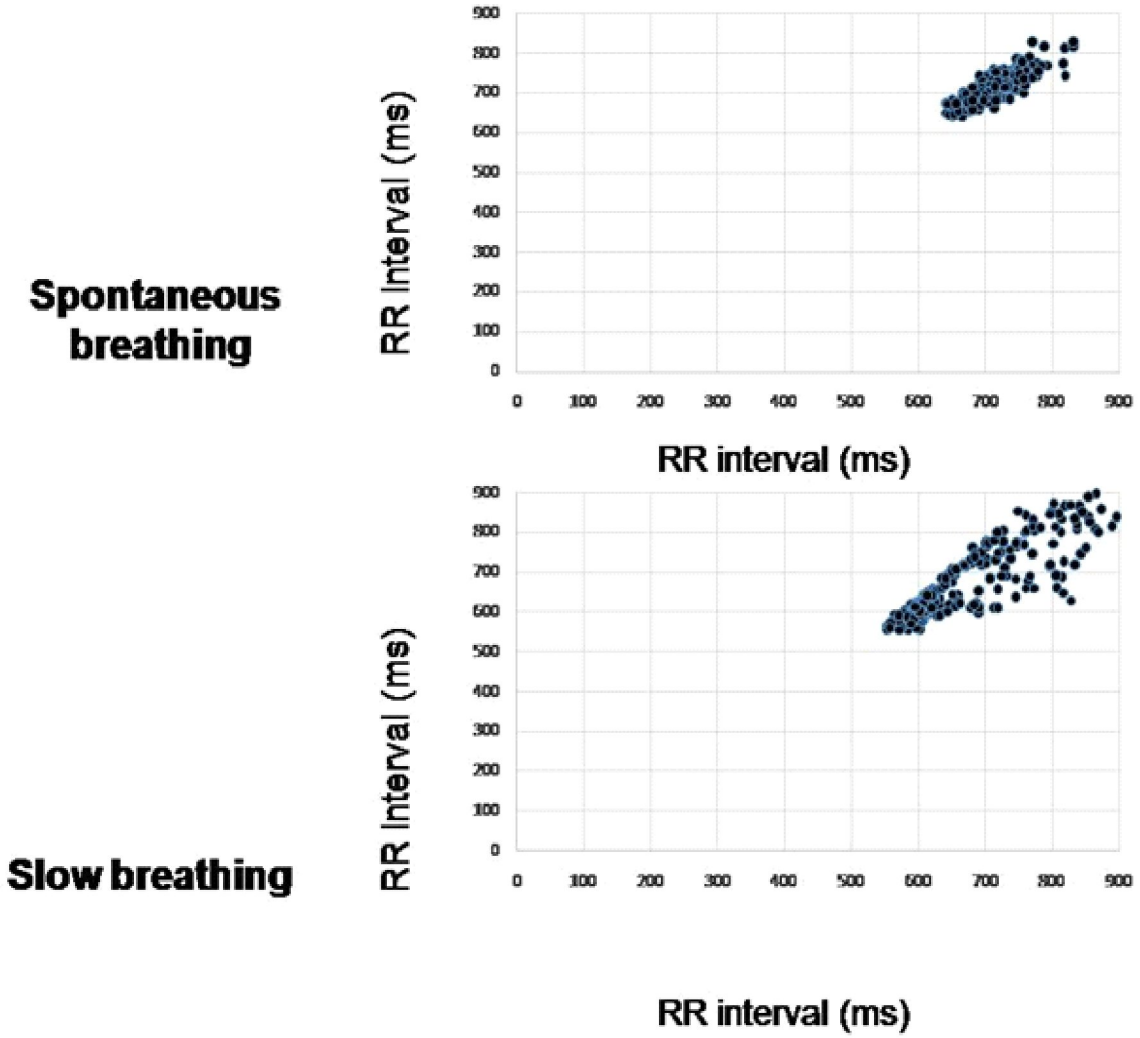

Fig. 4 : Visual pattern of the Poincaré plot observed in one subject during spontaneous breathing and during slow breathing.

TABLE II : The table below is the Principal Component Analysis for five groups of entropy for 21 subjects who are slow breathing subjects (experimental dataset with n=21).

Entropic parameters	*PC1*	*PC2*	*PC3*	*PC4*	*PC5*
Approximate	0.335	–0.725	–0.602	–0.012	<0.001
Sample	0.405	–0.466	0.787	–0.010	–0.001
Shannon	0.490	0.301	–0.080	–0.478	–0.659
Rényi α=0.25	0.493	0.279	–0.078	0.816	–0.088
Tsallis q=0.25	0.491	0.298	–0.079	–0.325	0.747

PC1 represents the First Principal Component, PC2 the Second; until the fifth component PC5. For Rényi and Tsallis entropy the values of entropic order (α=0.25) and entropic index (q=0.25). For Approximate entropy and Sample entropy (m=2; r=0.2 of Standard Deviation). Notice we do not include DFA in the PCA since we are only comparing entropies which respond in the same way akin to increasing chaos; by increasing response.

Discussion

We aimed to investigate the complex behaviour of heart rate autonomic regulation during slow breathing exercise in healthy young men. We detected decreased complex behaviour of HRV through symbolic analysis, entropies and DFA during slow breathing.

Different methods have been established to identify the nonlinear dynamics of heart rate, each approach is facilitated in specific ways. The Poincaré plot considers consecutive RR intervals and provides a graphic with dispersion of the points indicating whether it is more or less linear (19). The symbolic

analysis divides RR intervals into symbols and evaluates the repetition of those symbols (15). The entropies perform mathematical calculations to evaluate the predictability of the RR intervals repetition (6). DFA assesses the self-similarity of RR intervals distribution (21).

All approaches stated above provide balancing information for the traditional HRV methods, including time and frequency domain indices. The linear methods have quantitative characteristics indicating increase or decrease in the parasympathetic or sympathetic regulation of heart rate whilst the nonlinear methods indicate the self-similarity, predictability and repetition rate of RR intervals (14).

So, symbolic analysis of HRV indicated that sympathetic and vagal influence on heart beat represented by 2LV was higher and that the parasympathetic component of heart rate control represented by 2UV (21) was decreased during slow breathing exercise. This response is explained by the activation of the both sympathetic and parasympathetic subdivisions (23).

Formerly, slow breathing associated with HRV biofeedback was reported to reduce arousal induced by traumatic situations and decrease anxiety levels (24). Traditional analysis of linear indices of HRV in the frequency domain indicated that musicians who performed a single session of slow breathing presented an increase in the parasympathetic component of heart rate modulation while it decreased the sympathetic component, signifying higher levels of parasympathetic influence on heart rate under stress. The parasympathetic activation through slow breathing was suggested to allow subjects to better modulate physiological arousal before music presentation and to improve their performance (25).

The effects of slow breathing on the autonomic nervous system is due to influences on mechanosensitive sensory nerve endings in the walls of the carotid sinuses. Baroreceptors are deactivated when arterial pressure increases and compress the carotid wall, sending afferent nerve impulses into the central nervous system that reflexively increase parasympathetic outflow and decrease sympathetic outflow, leading to bradycardia. In reverse, the baroreceptors cessation of firing after blood pressure falls, inducing tachycardic reflex (26).

According to our discoveries, ApEn was significantly *reduced* during slow breathing. It can be observed as an approximation of the differential entropy rate of a process. ApEn estimates the entropic rate of RR intervals, this component gradually decreased during activation of the sympathetic activity through head-up tilt test. Consequently, ApEn is associated with sympatho-vagal balance (27). Together, the behaviour of ApEn in our study implies a reduced complex behaviour of HRV during slow breathing exercise. Yet, sample entropy was not significantly altered during slow breathing. Sample entropy was originally established to improve ApEn.

We reported that DFA was significantly greater during slow breathing exercise, indicating *decreased* complex responses of heart rate dynamics.

The physiological interpretation of nonlinear approach to analysis HRV is evidenced in previous studies (27, 28). Turianikova et al (27) evaluated the complexity of RR intervals during orthostatic challenge in 28 healthy subjects (mean age: 20.4 years old). The authors observed that reduction of the parasympathetic regulation of heart rate was trailed by decrease in the complexity of heart beat signals fluctuations. The well-designed study by Tulppo et al (21) theorized that decreased complex organization of heart rate dynamics is associated with sympathetic and vagal activation induced by cold face immersion in healthy subjects. As a key result they stated that reduced nonlinearity of short term HRV was noted during coactivation of sympathetic and vagal outflow. In this case, we suggest that this response was attributable to sympathetic activation during respiratory sinus arrhythmia (25).

A recent study performed by Silva et al (28) facilitated a better understanding of the physiological interpretation of DFA. It was reported that when β-receptors were blocked the RR intervals tended to be randomised whereas when muscarinic receptors were blocked to inhibit parasympathetic activity the

correlation property of RR intervals ceased to be associated by a power law.

Earlier, studies investigated the effects of different breathing patterns on HRV. Slow breathing was reported to be authoritative in heart rate dynamical fluctuations similar to respiratory sinus arrhythmia during meditation (29).

Another study examined breathing patterns and compared the effect of light exercise on nonlinear HRV (14). Male subjects were assessed during voluntary breathing, and metronomic guided breathing at 0.1 Hz (6 cycles/min), 0.2 Hz (12 cycles/min) and 0.4 Hz (24 cycles/min), undergoing light intensity cycling. While the significant effects of slow breathing on heart rate were not observed, DFA was strongly *elevated* and ApEn and sample entropy were *lowered* during slow breathing. This was supported by our data in this study.

Thus, the breathing pattern is a vital point to be addressed when investigating slow breathing effects on heart rate regulation. Volterra-Wiener series method was applied to RR intervals and it was conveyed that paced breathing reduced the non-linear behaviour of HRV compared to spontaneous breathing at a rate of 10 cycles/min (around 0.17 Hz), a breathing pattern greater than the one used in this study. It is imperative to realise that higher respiratory rates decreases linear behaviour of HRV (30), which enforces the difference between the mentioned study and our conclusions.

The aforesaid results and our findings here suggest that heart rate dynamics are more predictable during slow breathing due to linearity of respiratory pattern.

Although the quantitative analysis of nonlinear heart rate dynamics indicated that its complex behaviour decreases during slow breathing, the Poincaré plot did not support the quantitative analysis. The Poincaré plot is a simple technique used as a geometrical analysis by fitting an ellipse to the shape of the Poincaré plot in order to calculate HRV indices. In 2001, Brennan et al (30) performed techniques in order to investigate the nonlinear property of the Poincaré analysis. The authors converted a two-dimensional plot into several one-dimensional views, and the fitting of an ellipse to the plot shape and measuring the correlation coefficient of the plot. The study demonstrated that this method was insensitive to the nonlinearity of the intervals. In this sense, we believe that the Poincaré plot was not sensitive to detect changes which the DFA, symbolic analysis and entropy identified in HRV during slow breathing.

The foremost conclusion deduced from this study was that slow breathing decreased the chaotic behaviour of heart rate dynamics. Under these circumstances, the most relevant features of a chaotic system includes its deterministic profile, directing its behaviour and exhibiting high sensitivity to initial conditions. For example, a modest variation in the starting points may lead to significantly different outcomes, which are not random. Chaotic systems present a sense of order and pattern, which are unrepeatable (22).

The complex behaviour of HRV has received much attention. It was recently discussed in the review from the European Society of Cardiology together with the European Heart Rhythm Association and co-endorsed by the Asia Pacific Heart Rhythm Society (14).

Thus, we highlight nonlinear analysis of HRV as important for providing information with respect to the complex dynamics of RR intervals variability. This is appropriate to better understand physiological heart rate control mechanisms. Moreover, a benefit of nonlinear analysis includes its *qualitative*, correlation and scaling properties in the evaluation of the RR intervals. Likewise, the traditional linear indices of HRV in the time and frequency domains provide *quantitative* analysis of HRV (5).

Conclusion

Slow breathing exercises acutely *decreases* nonlinear behaviour of heart rate dynamics in healthy young men analysed through DFA and various entropies. We suggest that the linear respiratory pattern influences the complexity of HRV through increasing its predictability.

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Original Article

Blindness Enhances Texture Perception: Role of Haptic Sense

B.G. Bhirud[1] and L.M. Chandan[2]*

[1]Department of Physiology,
NKP Salve Institute of Medical Sciences & Research Centre,
Nagpur.

[2]Department of Physiology,
Seth G.S. Medical College & KEM Hospital,
Mumbai

Abstract

In congenitally blind subjects, visual experience and visual imaginary is lacking which in turn forces them to be dependent on haptic system for tactile discriminative task. Many studies suggest that visual experience facilitate the haptic processing of surrounding information making sighted and late blind more efficient in haptic perception compared to congenitally blinds. When it comes to texture identification, the role of visual imaginary is not clear. In the view of this,the present study want to investigate the role of visual experience in haptic processing in congenitally blinds, when they are compared with blindfolded sighted subjects for texture discriminative task. Method: This study was done on 30 congenitally blind Braille subjects & 30 age & gender matched sighted subjects who were blindfolded during the texture discriminative task. Participants were instructed to arrange the sandpapers of different grit size from rough to smooth grading and performance was evaluated in terms of speed & accuracy. Result: Congenitally blind subjects outperformed blindfolded sighted subjects, both in terms of speed & accuracy. Conclusion: Congenitally blind subjects appear to get the benefit of their haptic sense in texture discriminative task resulting into faster & more accurate perception.

Introduction

While extracting information from external world, both visual and somatosensory information is necessary for which touch emphasize on material properties and vision emphasize on spatial or geometric properties (1). During haptic exploration, blindfolded sighted individuals may get the benefit of past visual experience while congenitally blinds have to rely on their haptic sense.

Lederman et al. suggested that visual imaginary and visual experience is needed for interpretation of 2-dimensional patterns while haptic sense is important for perception of solid forms of substances like texture, hardness and thermal perception (2). Very few studies have been done on haptic texture perception and there is no Indian data published on it so far. In the view of this, present study has attempted to explore the role of haptic experience in texture discriminative task in congenitally blind

***Corresponding author :**

Dr. L.M. Chandan, B-201, Raj Amber CHS, Vijay Home Complex, Uttan Road, Bhyandar, West Thane – 401 101, Contact no. 9967834535; Email id: drlchandan@gmail.com

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individuals in whom visual experience and visual imaginary is lacking.

Methodology

The study was conducted at residential blind training centre for girls. Approval from ethical committee of the institute was obtained prior to study. Study participants were 30 congenitally blind girls of age group 18-27 years and 30 normally sighted female subjects of same age group. All participants were randomly selected for the study.

Participants with known sensorimotor deficit of hands like leprosy, neuropathy or neuritis were excluded from the study. An informed consent was obtained from each study participant. Visual acuity of all sighted subjects was checked and it was assured that they had normal vision. The sighted subjects were blindfolded for all tests for texture recognition to exclude vision as a source of information.

Test procedure was explained to all subjects in detail and one practice session was given to each of them to make them familiarize with the study procedure. The practice session used different set of sand papers from the ones used for actual testing of the subjects.

The study protocol was based on the procedure described by Heller et al. (3). Aluminum oxide abrasive sandpapers of different grit size were used. The grit value designates number of sharp particles per square inch of sandpaper. Sandpapers were mounted on square shaped cardboard of size 8 cm x 8 cm and divided into 2 sets. Set I contains coarse sandpapers while set II contains smoother sandpapers. Each set consists of random arrangement of sandpapers.

Set I grades 60, 80, 100, 120, 140

Set II grades 200, 240, 360, 400, 600

Test procedure

Each subject was administered with two sets of sandpapers in random order and instructed to arrange them from rough to smooth grade with tip of index finger. The blind subjects used the index finger of the hand used by them to read Braille while the sighted subjects used index finger of their dominant hand. Subjects were instructed to arrange sandpapers as quickly and correctly as possible. The number of sandpapers arranged correctly within 30 seconds was expressed as percentage accuracy of haptic texture discriminative task. Subjects were allowed to continue haptic manual exploration at the end of 30 seconds and time required to arrange sandpapers in set I and set II from rough to smooth gradation was measured and taken as speed of haptic texture discriminative task.

The data collected from all the subjects was recorded on an MS Excel sheet and subjected to statistical analysis. An unpaired t-test was applied to analyze the data by using SPSS software (Version 15).

Result

Time taken by blind and blindfolded sighted subjects to arrange sandpapers from rough to smooth grade is shown in Table I. Blind subjects were able to arrange sandpapers on an average 30 to 31 seconds faster than blindfolded sighted subjects. This difference was consistent for both the sets of

TABLE I: Comparison of Time (in seconds) required for Texture Identification in blind and control groups.

Variable	*Groups*				*Unpaired t-test applied*	
	Blind		*Control*			
	Mean (sec)	*S.D.*	*Mean (sec)*	*S.D.*	*p-value*	*Difference*
Time required for Texture Identification – Set I	12.50	2.60	42.08	12.72	0.0000000000	Significant
Time required for Texture Identification – Set II	16.44	8.20	47.71	10.19	0.0000000000	Significant

TABLE II: Comparison of percentage accuracy of texture identification between Blind and Control Groups.

Variable	Groups				Unpaired t-test applied	
	Blind		Control			
	Mean (%)	S.D.	Mean (%)	S.D.	p-value	Difference
Texture Identification – Set I	98±8	0.08	77±16	0.16	0.0000000175	Significant
Texture Identification – Set II	93±14	0.14	72±17	0.17	0.0000043882	Significant

sandpapers tasted and was statistically significant.

Blind subjects also showed significantly higher accuracy for arrangement of sandpapers compared to blindfolded sighted subjects. This difference, again, was consistent for both the sets of sandpapers (Table II).

Discussion

For texture discrimination, congenitally blind participants significantly outperformed blindfolded sighted participants both in terms of speed and accuracy suggesting that congenitally blind get the benefit of haptic experience. Precise reason for this specific advantage of haptic experience is not clear probably the practice and habit of using touch as a main navigation for active exploration of surrounding environment might have helped.

Lederman et al. proposed two models for haptic processing of information. Direct apprehension model suggests that haptic system has unique representation of objects, independent of any visual representation. While according to image mediation model, haptic system translates tactile information into visual image, which is then perceived by visual system (4, 5). Thus perception of surrounding environment is highly integrated and requires sensory representation from different modalities, mainly visual and haptic sense for blinds. However contribution of one sense over another has long been debated and found to be dependent upon the nature of the work (6). Touch perceives roughness of texture more appropriately while vision is helpful for differentiating boundaries of texture (7).

In a comparison for texture perception among sighted, late blind and early blind, similar performance was observed, suggesting that there was no extra advantage of visual experience and visual coding of tactile stimuli is not necessary as touch itself is advantageous for detection of smoothness of surfaces (3).

When blind individuals were compared with sighted subjects in 3 different tactile discriminative tasks, it was found that blind subjects were more superior in tactile discriminative task. For grating oriented threshold and vibrotactile frequency discrimination threshold, similar performance was found between them. All blinds were fluent Braille readers and possibly the similarity between raised dot surface and Braille characters had resulted into superior performance in them (8).

A tactile image recognition study done on early blind children using different techniques and materials showed that early blind children were better at recognizing textured images than other illustrations. The study also showed that adequate exposure and practice is a key factor for improvement (9).

Our finding that congenitally blind subjects are significantly better than blindfolded sighted subjects both in speed and accuracy in haptic texture discriminative task suggests that haptic sense is very well developed in congenitally blinds and they need not depend upon visual experience for texture perception. Tactile sensitivity and awareness are necessary for performing higher haptic task. With the help of previous tactile experiences and memory, congenitally blinds develop their own spatial skills by using body centered cues and active tactile exploration (10, 11). So proper training and adequate exposure to the surrounding environment in critical age is at most necessary for development of these

special skills (12). Congenitally blind participants of present study were living in residential blind training school and were exposed to different sorts of academic, social and cultural environment and were trained in Braille reading from young age. This might have helped them to develop a strong haptic special sense and succeed in texture discriminative task.

We hope that our finding regarding superiority of congenitally blinds in texture discriminative task and importance of early exposure to tactile training will be useful for developing various tactile devices and strategies.

We would like to do extensive research on late blinds also to know their encoding patterns during haptic exploration, which in turn may help us to train them to develop strategies to use their haptic sense.

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Original Article

Correlation of Age with Lung Parameters in Asthmatic Patients with Positive Parental History

S. Subashini[1], Rashmi Ramanathan[2]*, Mohan Jayapal[2], Manishankar Subramaniyam[2], Jyothi Sivalingiah[2] and Rajeswari[3]

Department of Physiology[1], Community Medicine[3]
Karpagam Faculty of Medical Sciences and Research,
Othakalmandapam, Coimbatore

Department of Physiology[2]
Coimbatore Medical College, Coimbatore

Abstract

Patients with positive family history of asthma among their first degree relatives have increased risk by 3–6 times. Pulmonary functions were assessed using computerised spirometer RMS Helios 702 in asthmatics aged 20 to 40 years with positive parental history. They were classified based on GINA (Global Initiative for Asthma) 2017 guidelines into controlled, partly controlled and uncontrolled group and subdivided into 20-30 yrs and 31-40 yrs. In this study patients belonging to 20-30 years in uncontrolled group show positive correlation between age and FEV_1/FEC ratio whereas negative correlation noted in controlled and partly controlled groups. Positive and significant ($P<0.05$) correlation was found between age and Peak expiratory Flow Rate (PEFR) in controlled and uncontrolled groups among 20-30 years. The uncontrolled group with mild symptoms progress to severe forms within a short period of time due to genetic susceptibility. Therefore earlier the onset of illness, more profound is their impact on pulmonary function.

Introduction

Bronchial asthma has grown to be one of the major chronic health problem worldwide affecting 300 million. The global prevalence ranges from 1%-18%. It affects all age groups with rising treatment costs and burden to the community. Risk factors are genetic predisposition, environmental factors like allergens, pollution, infections, active and passive smoking, temperature variation, pet hair, perfume, over exertion and stress or anxiety.

Release of acetylcholine from parasympathetic nerves activates post junctional muscarinic receptors present on airway smooth muscle, sub mucosal glands and causes bronchoconstriction and mucus secretion (1). John Floyer defined asthma as “laborious respiration with lifting of the shoulders and wheezing with intermittent episodes” and that the treatment needs rescue and controller therapy (2). Symptoms are

***Corresponding author :**

Dr. Rashmi Ramanathan. New No.: 163 NSR Road, Saibaba Colony, Coimbatore – 11, Tamilnadu, India; Mobile: 9486127040; Email: rashmikumar82@gmail.com

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shortness of breath, coughing and wheezing. Asthma has no set pattern. It can flare up from time to time and then not appear for longer periods. Bateman ED et al. asserts that "it is reasonable to expect that in most patients with asthma, control of the disease can be achieved and maintained" and recommends a change in approach to asthma management (3).

Computerized spirometry is a physiological test that measures how an individual inhales or exhales volume of air as a function of time. The primary signal measured in spirometry may be volume or flow (4). The most common measurements are FVC and FEV_1. FEV_1 is the forced expiratory volume in first second i.e, volume of forced vital capacity expired in 1st second of exhalation (83%). FVC is the volume of air that is expelled into the spirometer following a maximum inhalation effort. An obstructive pattern affects the rate at which air can be expelled from the lungs with normal FVC, reduction in FEV_1 and low FEV_1/FVC ratio. Reversibility of FEV_1 by more than 400 ml or 20% suggests a diagnosis of asthma. Asthma management decisions based on symptom control appear to have greater practical utility in a primary care setting (5). GINA (Global Initiative for Asthma) guidelines provides a comprehensive and integrated approach for categorizing and treating asthma patients. Halbert RJ et al. suggested that measuring asthma control is the first step to effective patient management (6).

Bill Cookson et al. found that any one of brothers and sisters affected by allergy-type illnesses had inherited the relevant gene, situated on maternal chromosome 11 (7). Burke W et al. in his study emphasised family history as a predictor of asthma risk (8). Recent researches have suggested that the risk of allergies in young children is much greater when the mother is allergic than the father. This is due to the influence of maternal antibodies on the immunity of offspring.

Fernando martinez in his study showed that the development of asthma is determined by complex interactions between genetic and environmental factors. Breast milk contains antibodies and dietary substances (9) which alters infant's immunity and reduces the risk of developing allergies in adult life. There are studies which say, influence of heredity on the risk of developing asthma declined over age (10). Earlier the onset of illness, more marked the pulmonary function parameters deterioration. Hence this study was undertaken to assess the impact of age on onset and severity of asthma leading to deterioration of lung parameters in patients with positive parental history.

Materials and Methods

This cross sectional study was conducted in the physiology clinical laboratory. Asthmatics with positive parental history between the age group 20-40 years of both sex were taken as subjects after informed consent. Smoking history, drug history, disease duration, respiratory symptoms, remissions and exacerbation, hospital admissions, education level were elicited. Pulmonary parameters were obtained using computerised spirometer RMS Helios 702.

Inclusion criteria

1. Confirmed cases of asthma as per GINA guidelines
2. Both males and females
3. Age group 20-40 years attending outpatient department.

Exclusion Criteria includes patients with pneumonia, diabetes mellitus, pregnancy, hypertension, tuberculosis, taking drugs like anti-epileptics, sympathomimetics.

Prior to Pulmonary function tests, the patient should avoid :

– Smoking for 24 hours.

– Vigorous exercise for at least 30 minutes.

– Wearing any tight clothing.

– Eating a large meal for at least 2 hours.

– Taking short-acting bronchodilators for 2 hours.

– Taking long-acting beta-2-agonist inhalers for 12 hours and theophylline-based drugs for 24 hours as per Association of Respiratory Technicians and Physiologists-ARTP and British Thoracic Society (11).

Procedure

The patient was allowed to sit and relax for 5-10 min. The patient was instructed to take the maximal breath, close the nostrils with nose clips and exhale through mouthpiece for at least 6 seconds followed by deep inspiration. Three breathing maneuvers were recorded during the procedure and the highest of three trials is used for evaluation of breathing. After investigation, 25 patients were enrolled in each of the three groups (controlled, partly controlled and uncontrolled) based on FEV_1 and clinical symptoms.

Statistical analysis

Data was recorded and analysed using SPSS Version 22.0. The statistical significance was set at P£0.05. The correlation of age with FEV_1/FVC and PEFR were calculated using Pearson product moment correlation analysis. When r³0.75 it is interpreted as strong relationship, when 0.50£r£0.74 interpreted as moderate relationship and when r£0.49 interpreted as weak relationship.

Results

TABLE I: Comparing Age, BMI & FEV1/FVC in the three asthmatic groups.

Asthmatics	*Controlled (n=25)*		*Partly controlled (n=25)*		*Uncontrolled (n=25)*	
	20-30 yrs	*31-40 yrs*	*20-30 yrs*	*31-40 yrs*	*20-30 yrs*	*31-40 yrs*
Mean agein years	24.3±3.020	35.2±3.004	24.92±3.28	34.77±2.919	25±2.631	35.818±3.572
Mean BMI Kg/m^2	22.4±2.8	25.84±3.2	25.64±3.6	26.92±2.8	24.8±3.8	27.2±4.2
FEV1 (L)	2.63±0.188	2.89±0.325	1.469±0.406	1.718±0.363	1.206±0.312	1.391±0.402
FVC (L)	3.178±0.43	3.618±0.452	2.225±0.631	2.759±0.660	2.98±0.659	3.018±0.821
FEV1/FVC (%)	80.07±1.502	80.14±2.82	66.577±6.01	63.607±2.894	40.819±5.61	46.018±2.868
PEFR/Sec	4.67±0.098	5.01±0.15	3.72±0.19	3.97±0.20	2.585±0.28	2.91±0.23

TABLE II: Correlation of Age with FEV1/FVC ratio in different asthmatic groups.

	Asthmatics	*Age group*	*95% CI of 'r'*	*'r' value*	*'p' value*
1.	Controlled group	20-30 yrs	–57.75 to –53.78	0.406	0.244
		31-40 yrs	–46.903 to –42.98	0.260	0.349
2.	Partly controlled group	20-30 yrs	–46.33 to –36.989	–0.182	0.571
		31-40 yrs	–32.146 to –25.53	–0.773	0.002*
3.	Uncontrolled group	20-30 yrs	–18.742 to –12.90	0.434	0.121
		31-40 yrs	–13.158 to –7.24	0.078	0.819

CI = Confidence interval, r = Correlation coefficient, NS = Not significant. *-Significant

TABLE III: Correlation of Age with PEFR in different asthmatic groups.

	Asthmatics	*Age group*	*95% CI of 'r'*	*'r' value*	*'p' value*
1.	Controlled group	20-30 yrs	17.531 to 21.74	0.842	0.002**
		31-40 yrs	28.504 to 31.87	–0.221	0.430
2.	Partly controlled group	20-30 yrs	19.11 to 23.28	0.086	0.792
		31-40 yrs	28.99 to 32.61	–0.343	0.251
3.	Uncontrolled group	20-30 yrs	20.98 to 23.84	0.600	0.02*
		31-40 yrs	30.56 to 35.256	0.368	0.265

CI = Confidence interval, r = Correlation coefficient, NS = Not significant. *-Significant

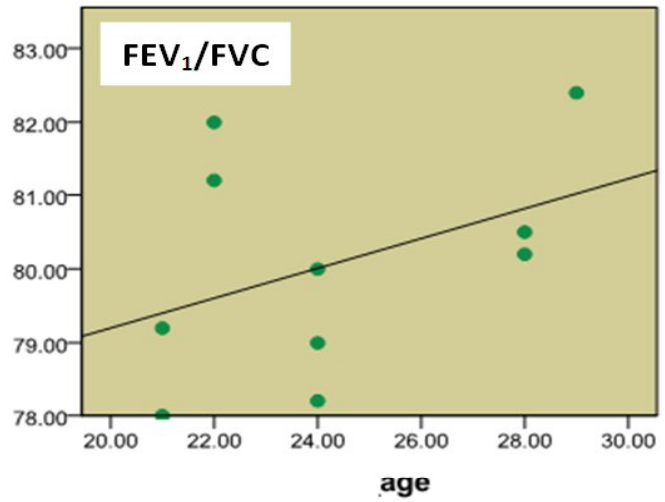

Fig. 1 : Correlation of age and FEV_1/FVC ratio in Controlled group in 20–30 years.

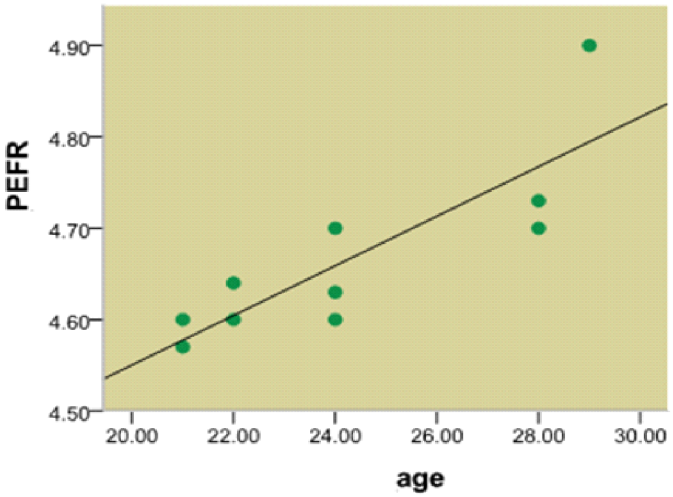

Fig. 2 : Correlation of age and PEFR ratio in Controlled group in 20–30 years.

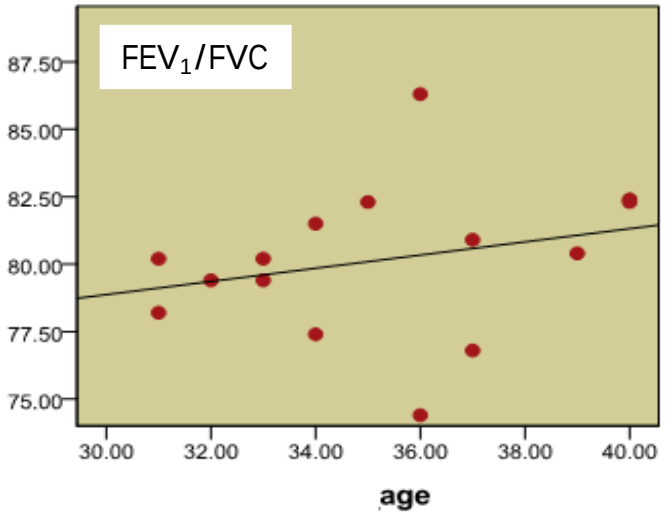

Fig. 3 : Correlation of age and FEV_1/FVC ratio in Controlled group in 31–40 years.

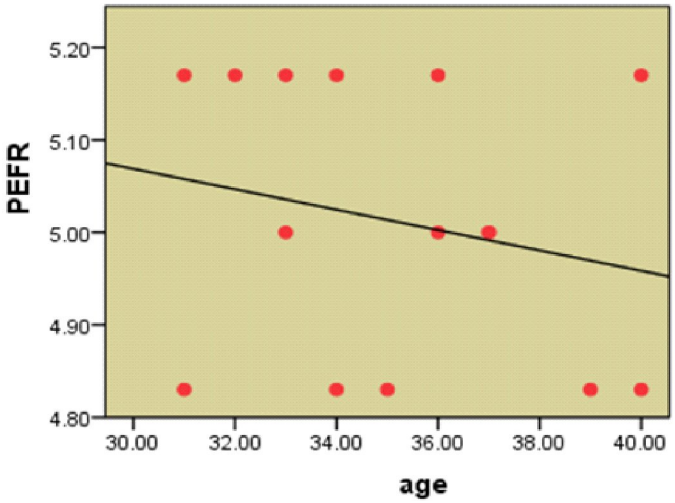

Fig. 4 : Correlation of age and PEFR ratio in Controlled group in 31–40 years.

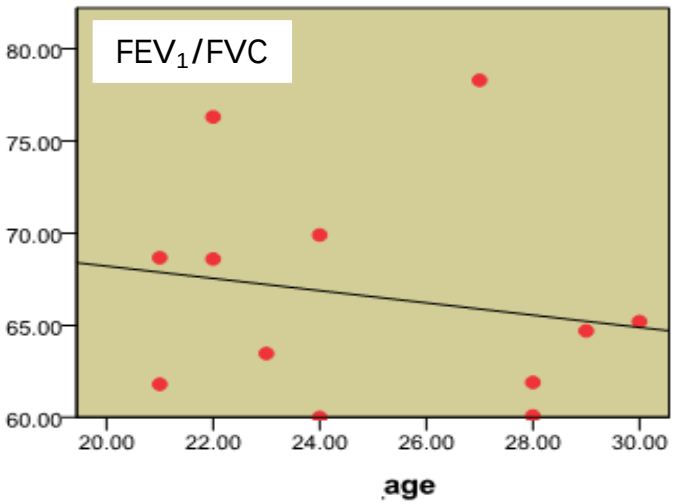

Fig. 5 : Correlation of age and FEV_1/FVC ratio in Partly Controlled group in 20–30 years.

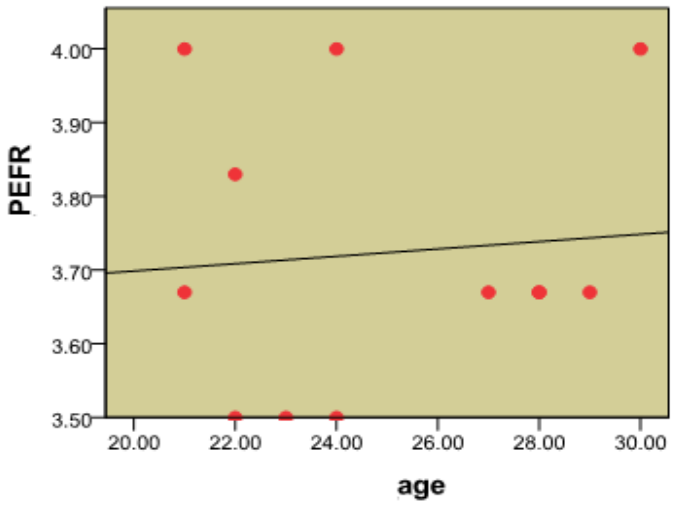

Fig. 6 : Correlation of age and PEFR ratio in Partly Controlled group in 20–30 years.

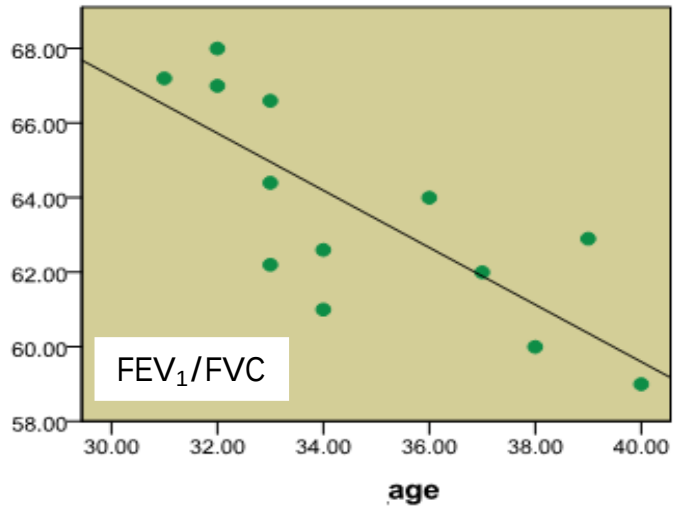

Fig. 7 : Correlation of age and FEV_1/FVC ratio in Partly Controlled group in 31–40 years.

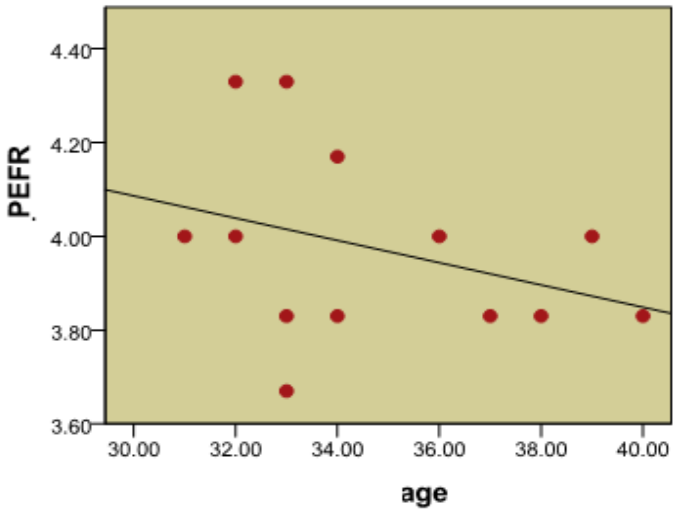

Fig. 8 : Correlation of age and PEFR ratio in Partly Controlled group in 31–40 years.

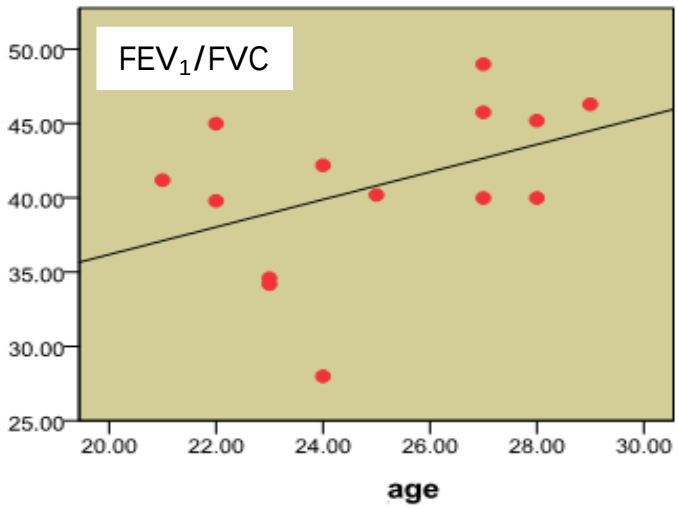

Fig. 9 : Correlation of age and FEV_1/FVC ratio in Uncontrolled group in 20–30 years.

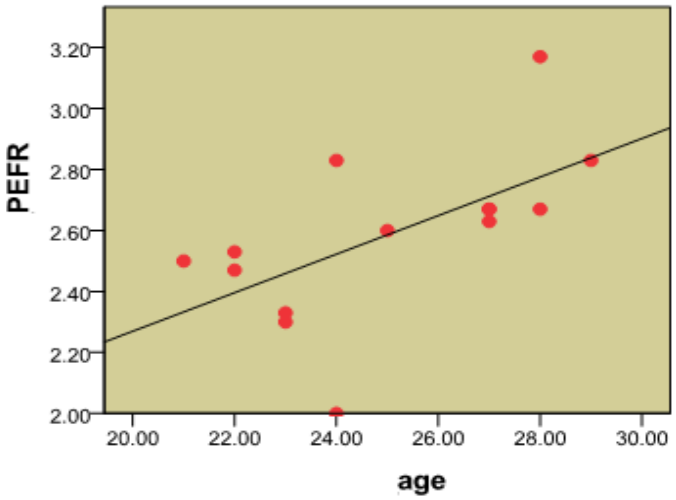

Fig. 10 : Correlation of age and PEFR ratio in Uncontrolled group in 20–30 years.

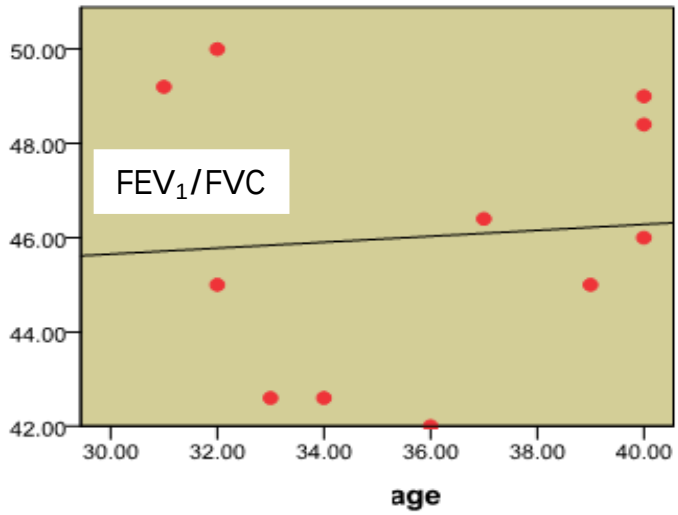

Fig. 11 : Correlation of age and FEV_1/FVC ratio in Uncontrolled group in 31–40 years.

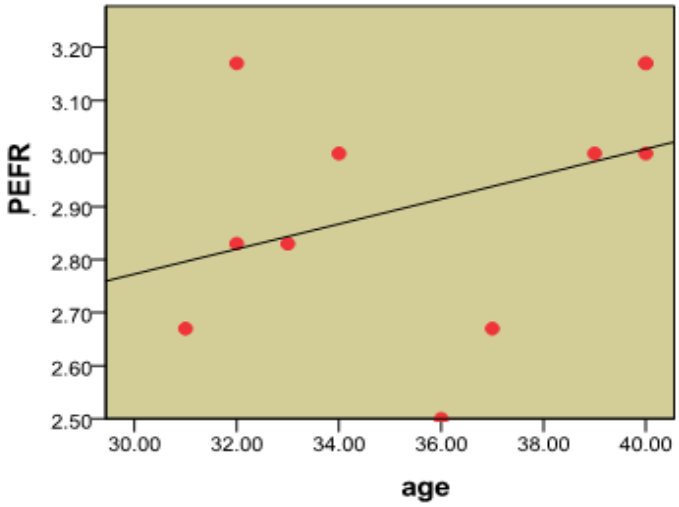

Fig. 12 : Correlation of age and PEFR ratio in Uncontrolled group in 31–40 years.

Discussion

Modern clinical practice has revolutionized to identify and remove the exciting causes of asthma to alleviate the bronchospasm during the paroxysm and to treat complications and sequelae by following strategic GINA guidelines to optimize diagnosis and management (12).

In controlled group population, FEV_1 is near normal but they presented with one or two episodes of day time asthmatic attacks per week. FEV_1 was lowered in partly controlled along with few limitations of activity and nocturnal symptoms. In the uncontrolled group, FEV_1 is significantly lowered along with exacerbations any time in a week, limitation of activity and nocturnal symptoms. FEV_1 and symptoms were reversible with bronchodilator therapy.

Decrease in the rate of maximal expiratory air flow due to the increased resistance and a reduction in forced vital capacity (FVC) correlate with the level of hyperinflation of the lungs as these patients breathe at such high lung volumes near the top of the pressure-volume curve, where lung compliance greatly decreases.

In our study asthmatics with positive family history was included to evaluate the impact of age of disease onset on lung parameters. A statistically significant association of bronchial asthma with family history of asthma was observed (13). Most patients with positive family history presenting with mild symptoms progress to severe forms early in their life within a short period of time than others due to genetic susceptibility.

In case of Asthmatics, FEV_1 Value and FEV_1/FVC ratio decreases in the descending frequency among controlled, partly controlled and uncontrolled groups respectively. In this study, Controlled group asthmatics in the age group of 20-30 years have mean FEV_1, mean FEV_1/ FVC and mean PEFR less than those in the age group of 31-40 years. Also, partly controlled group asthmatics in the age group of 20-30 years have mean FEV_1, mean FEV_1/ FVC and mean PEFR less than that in the age group of 31-40 years. The patients in uncontrolled group in the age group of 20-30 years have mean FEV_1, mean FEV_1/ FVC and mean PEFR less than that in the age group of 31-40 years.

One may expect the pulmonary function to deteriorate with age. It was noted in our study that asthmatic patients with positive family history and early onset of symptoms had decreased lung function parameters. This is in accordance with London et al. who found that early onset persistent asthma with reduced pulmonary function was strongly associated with parental history (14).

Among the controlled asthmatics in the age group of 20-30 years and 31-40 years, positive correlation ($r=0.406$, $r=0.260$ respectively) which is less significant ($p>0.05$) was noted between age and FEV_1/FVC ratio. In partly controlled asthmatics of 20-30 years negative correlation ($r=-0.182$) which is less significant ($P>0.05$) was found between age and FEV_1/FVC ratio whereas in the 31-40 years group, negative correlation ($r=-0.773$) which is significant ($p<0.05$) was observed. In Uncontrolled asthmatics of 20-30 yrs and 31-40 age groups, positive correlation ($r=0.34$, $r=0.078$ respectively) which is less significant ($P>0.05$) was found between age and FEV_1/FVC ratio. The results were similar with the study evidence of Stephaine J & W James et al. that parental and siblings history of asthma and allergy were generally more strongly associated with early onset persistent asthma compared with early transient or late onset asthma (15).

PEFR is the sensitive index to respiratory muscles strength and reflects mainly the calibre of the bronchi and larger bronchioles. Usually PEFR declines with age but in our study mean PEFR is more in 31-40 years and less in 20-30 years. The inverse relationship between familial asthma risk and people's age at onset may reflect a stronger genetic component (16).

Positive and Significant ($P<0.05$) correlation was found between age and PEFR in controlled and uncontrolled asthmatic groups in the 20-30yrs age group and positive insignificant correlation exists

between them in partly controlled group. Higgins and Keller et al. found that a statistically significant correlation exist between ventilatory function in children and their parents (17). Negative and insignificant (P>0.05) correlation was found between age and PEFR in controlled and partly controlled asthmatic groups in the 31-40 yrs age group.

Leeder in his study emphasise that, the earlier the onset of the illness, the more marked was its effect on ventilator function (18). Our study also confirms the positive association between family history of asthma and the earlier onset of symptoms.

Conclusion

Identifying positive parental history of asthma provide a basis for targeted prevention efforts and aimed at reducing exposure to environmental risk factors. Unless prevented, asthma attacks can interrupt everyday activities causing a significant impact on the quality of life. Asthma flare-ups are stressful and require hospitalizations. Patients with positive family history presenting with mild symptoms progress to severe forms early in their life within a short period of time due to their genetic predisposition. The early onset of symptoms was associated with decreased lung function parameters in asthmatic patients with positive parental history.

Prevention is better than cure. Since genetic susceptibility is a non modifiable factor, we conclude by emphasising that the avoidance of triggering and exacerbating factors like allergens, pollutants and passive smoking in genetically susceptible persons reduce the exacerbation of asthma and minimize the use of unnecessary drugs.

Acknowledgements

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Conflict of interest :

None

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Original Article

Effects of Tobacco Smoking on Innate Immunity: A Study Based on Neutrophil Phagocytic Index

Tanu Thakur[1], Arpana Bhide[2]*, Abhijit Chaudhury[3], Asha Thota[4], Latheef Kasala[5] and Narendra Hulikal[6]

Medical Student[1], Kasturba Medical College,
Manipal, Karnataka.

Departments of Physiology[2], Microbiology[3], Pathology[4], Cardiology[5] and Surgical Oncology[6]
Sri Venkateswara Institute of Medical Sciences,
Tirupati, A.P., India.

Abstract

The present study was undertaken to find out the effects of tobacco smoking on innate immune mechanism of the body. A total of 60 adult consenting men in the age group of 30 to 50 years were recruited of which 30 were chronic smokers and the rest were non smoking controls. 5ml of venous blood was drawn from each of the subjects and the following parameters were assessed: phagocytic index of neutrophils (which is an index of neutrophil function and is defined as number of neutrophils positive for ingested microbes per 100 neutrophils), total leucocyte count (TLC), differential count of neutrophils. The values from smokers were compared with those from non-smokers. There was a statistically significant decrease in the phagocytic index among smokers when compared to non-smokers (9.44±6.62 vs 28.16±7.31; p<0.0011). An increase in TLC and neutrophil percentage was found in smokers which were not statistically significant (p=0.37 and p=0.12 respectively). Hence it can be concluded that tobacco smoking adversely affects the capacity of neutrophils to ingest microbes and so has suppressive effect on the innate immune mechanism.

Introduction

The tobacco use, either in the form of smoking or smokeless tobacco is the leading preventable cause of death worldwide with more than 5 million deaths per year. As per an estimate, by 2030, the tobacco attributable death is expected to reach 8 million per year (1). A recent study from India estimated that tobacco smoking accounts for about 930000 deaths annually and one in ten adult deaths is related to smoking (2).

It is well known that the smokers are susceptible to a plethora of diseases and conditions like stroke, vascular diseases including myocardial infarction, chronic obstructive pulmonary disease (COPD), multiple cancers, hypertension and osteoporosis. Further, a less recognized fact is that, the smokers

***Corresponding author :**

Dr. Arpana Bhide, Assistant Professor, Department of Physiology, Sri Venkateswara Institute of Medical Sciences, Tirupati, A.P., India.

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are more susceptible to various infections (3). There is a growing evidence to suggest that the tobacco smoking might impair the immune system and increase susceptibility to infections (4–6). One of the methods to assess the innate immunity in vitro is calculating the phagocytic index of neutrophils. The phagocytic index is defined as the number of neutrophils with ingested microbes per hundred neutrophils (7). This is calculated by treating one's blood with the candida suspension in a suitable environment and counting the number of neutrophils with ingested candida. With this background, a study was proposed to find out the effect of smoking on the innate immunity which predisposes the smokers to infections.

Material and Methods

This was a study planned to assess the effect of smoking on innate immunity. Following institutional research committee and ethical committee approvals, a total of 60 willing adult men of the age group 30 to 50 years with 30 men being chronic smokers (defined as those with a history of minimum 20 pack years of smoking, study group) and other 30 being never smokers (controls) were recruited. Sample size calculation was performed using OpenEpi version 3 online sample size calculator with prevalence of smokers 30%, power 80% and 95% confidence levels. The minimum sample size required was 58 (29 cases and 29 controls) (8).

Inclusion & exclusion criteria

Study Group (Smokers group) : This group consisted of 30 willing men in the age group 30 to 50 years with a history of minimum 20 pack years of cigarette or beedi smoking (9). Pack year is equal to ([number of cigarettes or beedi smoked per day X number of years] ÷20) (10). To confirm the smoking status, serum cotinine levels were measured using the Qualisa ELISA kits and a value of more than 12.5 ng/ml was taken as the cut off (11). Those men who were on antibiotics, anti-hypertensive drugs or suffered from any ailment at the time of study including diabetes mellitus, COPD and hypertension were excluded. A detailed history regarding current smoking status, number of cigarettes smoked per day and years of smoking was obtained by using a pre-tested questionnaire.

Control group (non-smokers group) : This group included willing men of 30 to 50 years of age with no history of past or present tobacco smoking. The non-smoking status was confirmed by serum cotinine levels (<12.5 ng/ml) (10). Similar to the study group, those who were taking antibiotics, anti-hypertensive drugs or suffered from any ailment including diabetes mellitus, COPD and hypertension at the time of study were excluded.

Collection of the blood sample

Under strict aseptic conditions, 5 ml of peripheral venous blood was drawn from the antecubital vein of the subjects, collected in heparinized sterile bottles and transferred to sterile test tubes immediately for evaluation. The serum cotinine, TLC, differential count (DC) of neutrophils and phagocytic index of the neutrophils were measured on each of the samples drawn. The TLC, using automated analyser (Mindray BC 5300 5 parts differential analyser) and DC, using Leishman's stain technique were estimated (12).

Phagocytic index (7) : The neutrophils in the peripheral blood ingest microbes, when optimal conditions are provided in a medium. The number of neutrophils positive for ingested microbes per 100 neutrophils gives the 'Phagocytic Index'. After half an hour of incubation at 37°C in a suitable medium, normal neutrophil may contain anywhere from 0 to 4 microbes/cell.

Reagents required for estimating phagocytic index

Heat killed Candida suspension, Pooled sera of AB blood group of different healthy individuals, Phosphate buffer saline (PBS), Hanks medium and Lieshman's stain.

Preparation of heat killed microbial suspension

Candida was grown in Sabouraud's 2% dextrose medium for 48 hours at 37°C to obtain organisms in the yeast form. These colonies were then mixed with the PBS using a sterile loop. This mixture

was boiled for 15 minutes and then centrifuged at 3000 rpm for 10 minutes. The deposits were washed with 5 ml PBS and stored at 4°C. This heat killed microbial suspension was counted in improved Neubauer's chamber and optimum amount of the suspension required for the procedure was standardized.

Procedure for estimating phagocytic index

The heparinised blood sample was centrifuged at 2500 rpm for 10 minutes and plasma was discarded. The buffy coat was aspirated carefully and transferred to another test tube. 200 µl of pooled sera, 100 µl of candida suspension and 200 µl of Hank's medium were added to the buffy coat preparation from the test subject. The tube was kept in a water bath for incubation at 37°C for 30 minutes. The test tube was centrifuged at 1500 rpm for 5-10 minutes. The clear supernatant solution was discarded and the buffy coat was aspirated, taken on glass slides and smears were prepared.

Staining

The smears were stained with the Leishman's stain and examined under oil immersion for the presence of microbes inside the neutrophils (Fig. 1). The number of neutrophils positive for ingested microbes per 100 neutrophils was recorded as the 'Phagocytic Index' (13).

Statistical analysis

The data was recorded on a predesigned proforma and managed using MS-Excel 2007 (Microsoft Corporation, Redmond, WA). The descriptive statistics such as mean and standard deviation were calculated. The unpaired student's *t*-test was performed to compare the means between cases and controls. A p-valued ≤0.05 was considered significant. All statistical analysis were performed with the help of SPSS (Statistical Package for Social Sciences) version 20.0 (IBM Corp., Armonk, NY).

Results

The mean age of the study group was 39.2 (±8.2) years, mean height was 164.7 (±7.23) cm and the mean weight was 55.76 (±9.5) kilograms. Among the smokers, majority smoked cigarettes (86.7%), and 50% were light, 26.7% were moderate and 23.3% were heavy smokers. All smokers had serum cotinine

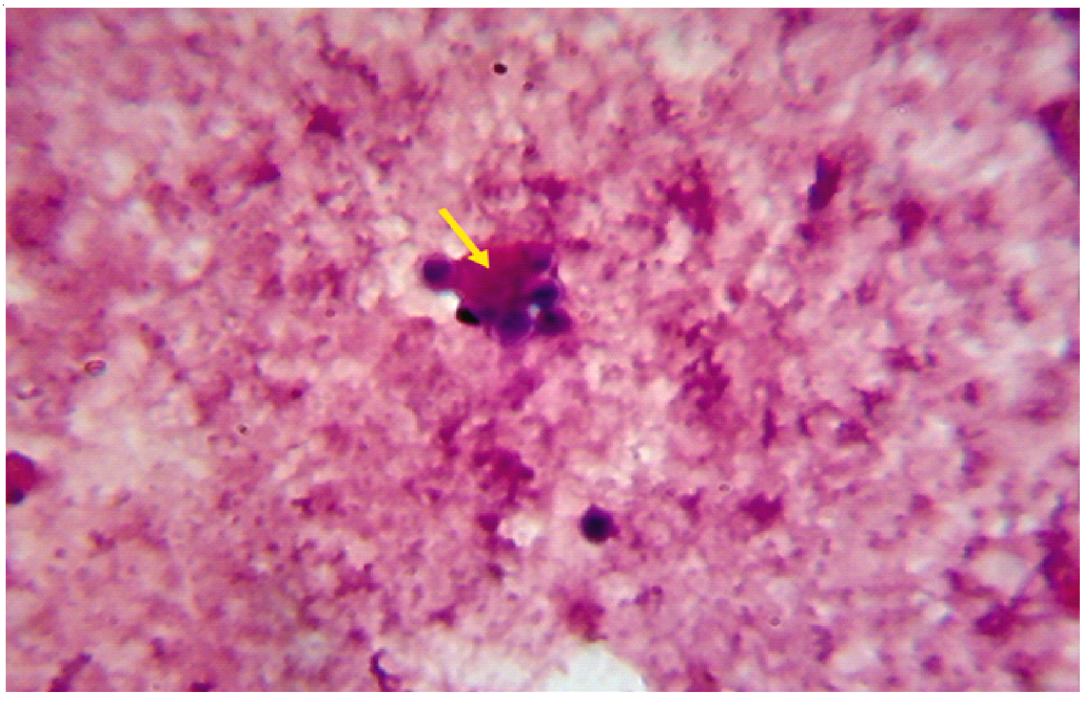

Fig. 1: Polymorphonuclear cell with ingested Candida in chronic smoker (arrow mark, x 100).

levels > 12.5 ng/ml and non-smokers had serum cotinine levels less than 12.5 ng/ml. There was a statistically significant decrease in the phagocytic index in the study group (smokers) when compared to the control group (non-smokers) [9.44±6.62 vs 28.16±7.31; p<0.0001] (Fig. 2). An increase in TLC [8261.29±2728.57 vs 7677.41±1676.24] and DC of neutrophils [58.26±8.33 vs 55.26±8.48] were also observed in the smokers when compared to non-smokers, but these were not statistically significant [p=0.37 and p=0.12 respectively].

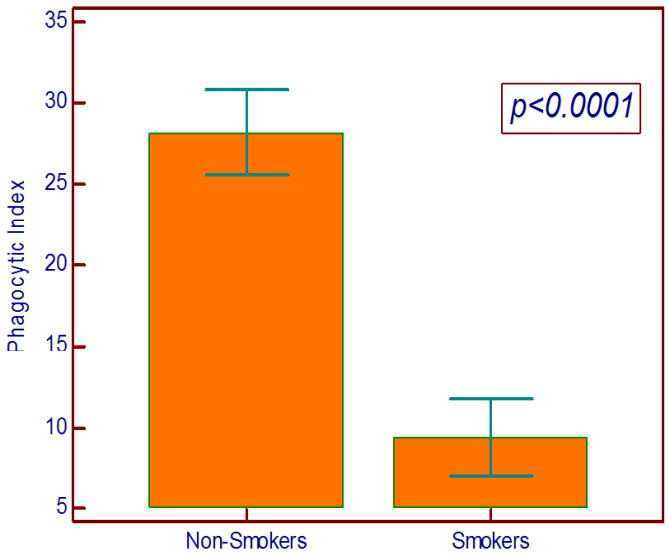

Fig. 2 : Mean±SD (n=30/group) Phagocytic index of PMN from non-smokers vs smokers.

Discussion

Tobacco smoking affects both the innate and adaptive immunities and this in turn possibly predisposes the smokers to a number of infectious diseases (14, 15). The tobacco smoke consists of several toxic and carcinogenic substances such as tar, nicotine, ammonia, carbon monoxide, carbon dioxide, acrolein, formaldehyde, hydroxyquinone, acetone and cadmium (16). These substances have harmful effects on human health such as increased susceptibility to respiratory infections, cardiovascular diseases including myocardial ischaemia, stroke, COPD and lung cancer (17). Tobacco smoke affects the functioning of white blood cells and hence has got an immunosuppressive effect (18, 19). Some earlier studies have suggested that PMNs in smokers exhibited depressed migration and chemotaxis when compared to non-smokers (20–22). Nicotine is one of the key ingredients of tobacco smoke which has a dose and duration dependent toxic effect on immune system (18). However the global influence of tobacco smoke on immunity is still not clear (23).

The present study was conducted to assess the impact of smoking on innate immune mechanism in a sample of apparently healthy chronic smokers. Neutrophils are an important component of innate immunity. Whenever neutrophil functioning or number is decreased, an increased susceptibility to infections is observed (24). The study showed that the phagocytic function of neutrophils was adversely affected by the tobacco smoking. This is in accordance with studies by Guntsch A et al and M Srinivas et al (4, 5). The process of phagocytosis involves various steps like margination, diapedesis, chemotaxis, opsonization, engulfment, degranulation and finally killing phase (25). Further research could throw light on which of these steps are affected by the tobacco smoke.

In the present study, increase in TLC and DC of neutrophils were found which were not statistically significant. The possible reasons could be inflammation of bronchioles, chronic tissue damage and nicotine induced catecholamine release (26, 27).

Infectious diseases are one of the major causes of morbidity and mortality from smoking along with cancer, heart disease and chronic lung disease. The findings emphasize the need to include smoking cessation as a part of preventive and therapeutic plan for smokers suffering from serious infections. These findings could be extrapolated to environmental exposure to tobacco smoke, so called second hand smoking or passive smoking among household contacts of an active smoker, who are at an increased risk of developing infections (28).

The findings also stress the need to control second hand tobacco smoke exposure (passive smoking) for prevention of infectious diseases especially among individuals who are constantly exposed to active smokers.

Conclusions

The present study confirms that in chronic smokers, the phagocytic ability of the PMNs is actually reduced. A larger study may be advisable to confirm the findings.

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Indian J Physiol Pharmacol 2018; 62(2) : 187–194

Original Article

Fluorescent Light Induced Oxidative Damage in Wistar Strain Albino Rat : Possible Protective Effect of LED Light Induced Photobiomodulation

A. Ahamed Basha, D.C. Mathangi* and R. Shyamala

Department of Physiology,
Chettinad Hospital and Research Institute,
Chettinad Academy of Research and Education,
Rajiv Gandhi Salai, Kelambakkam,
Chennai – 603 103, India

Abstract

Fluorescent light exposure affects normal physiology in both animal models and human. Primary objective of this study was to elucidate the effects of 1800 lux fluorescent light exposure on oxidative stress markers. Additionally, effects of 670 nm LED light exposure on fluorescent light induced changes were also elucidated. Wistar albino rats were divided into 10 groups based on fluorescent / LED light exposure for 1, 15 or 30 days group. Oxidative stress markers like lipid peroxidation, total reduced glutathione and super oxide dismutase in brain, heart, kidney, liver, skeletal muscle and blood were analysed. One-way ANOVA and Tukey's multiple comparison tests were used for statistical analysis. Exposure to fluorescent light resulted in oxidative damage and LED light offered protection against this oxidative damage. Protective effect of LED light against fluorescent light induced damage might be due to photooxidation and enhanced antioxidant activity.

Introduction

Fluorescent lighting has become an unavoidable source of light in both day and night time, making human to adapt to 24 hour active society. However, increase in the use of fluorescent lighting during night time produce undesirable side effects known as "light pollution". Light pollution cascade numerous physiological changes in a living organism at the cellular level. Even minor deviation in the intensity and duration of fluorescent light at a given time of day/night can alter or disrupt physiology. Various studies on animal models have suggested that use of fluorescent light especially during night time results in multisystem deleterious and harmful effects. Cardiovascular, neuro muscular, hepato renal and even metabolic and endocrinological disturbances have been reported (1-15). Hence, deleterious effect of light exposure at night has to be taken into consideration.

Oxidative stress is implicated in the development of

***Corresponding author :**

D.C. Mathangi, Department of Physiology, Chettinad Hospital and Research Institute, Rajiv Gandhi Salai, Kelambakkam, Chennai – 603103, India; Email:mathangidc@hotmail.com

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various pathological states in humans as well as experimental animals. Alteration in circadian rhythm and melatonin levels is implicated in the oxidative damage due to artificial fluorescent light exposure. The cellular damage is reflected by the change in concentrations of Lipid peroxidation (LPO), glutathione (GSH) and superoxide dismutase (SOD). The present study aims at finding the possible link between tissue damage and oxidative stress which results due to light pollution (16). However, there are conflicting reports on the levels of antioxidants and their byproducts in the available literature. Constant light exposure at night in rat for a period of 12 hours upto 21 days increases liver, kidney as well as circulatory thiobarbituric acid reactive substance (TBARS) and decreases antioxidative enzymes GSH and SOD (17). Light exposure of 500 lux (lx) between 9.00 am to 1.00 pm for a period of 7 days to rats increases brain TBARS and decreases GSH and SOD (18). In contrast, mice exposed to 30 min light for 2, 7 and 21 days showed gradual increase in liver SOD activity with increase in the acclimation period (4). Another study shows that continuous light exposure increases GSH and SOD level in rat brain (19). Studies have also explored the therapeutic potentials of fluorescent light exposure (20, 21). Based on the available literatures, it is clear that light exposure causes imbalance in oxidative process, though some are conflicting with tissue specific variations in antioxidant defense mechanism in different tissues. However, tissue specific interactions as well as time dependent modification in light induced oxidative stress have not been documented extensively. Hence, we investigated the effect of fluorescent light exposure on three key oxidative stress markers LPO, GSH and SOD.

Melatonin supplements are being commonly used as treatment to overcome light induced damages (22). However, it is expensive and time consuming and so there is a need for an alternative. One such alternative is LED (Light Emitting Diode) light exposure, because it is known to have antioxidant activity. Hence, utility of LED exposure in fluorescent light induced biochemical changes were also evaluated in this study.

Methods

Toxic effects of light at night in animal model mimic similar effects on humans (23, 24). Rat is a suitable model to study the effects of light induced stress (25). Hence, Wistar strain albino rats have been chosen for the study. On obtaining the ethical clearance from Institutional animal ethics committee, male Wistar rats weighing between 150-170 gms were divided into 10 groups (n=6 each). The animals were divided based on their exposure as described below.

Group 1 [CL] was control animals maintained at normal ambient animal house temperature, illumination and light cycle (12L: 12 D). Group 2, 3, 4 were one day exposure groups (Fluorescent light exposure of 1800 lx [FL_1], LED light exposure + fluorescent light exposure of 1800 lx [LL_1] and only LED light exposure [OL_1]). Similarly the animals were grouped based on the exposure regimen and days of exposure into fifteen day groups - Group 5[FL_{15}], 6[LL_{15}], 7 [OL_{15}] and thirty day groups - Group 8[FL_{30}], 9[LL_{30}] and 10[OL30]. The experimental set up and methods utilized for fluorescent light exposure of 1800 lx and LED light of 670 (±10) nm in the near infra red range with energy density of 9J/ cm^2 are as described in detail in our earlier publication (26). Fluorescent light exposure was between 8 p.m – 8 a.m daily for 1, 15 or 30 days. LED light of 670 nm for duration of 6 min was exposed independently or prior to fluorescent light exposure as per the group criteria.

By using pentothal sodium as a mode of anaesthesia, animals belonging to each group were euthanized and blood, brain (cerebral cortex), heart (cardiac muscle), liver, kidney and thigh muscle (skeletal muscle) of the animals were harvested for LPO (27), GSH (28) and SOD (29) estimation. Rats belonging to 1 day exposure group were sacrificed immediately after the exposure, however rats belongs to 15 and 30 day groups were sacrificed 24 hours after their final exposure to avoid acute exposure effects on the parameters studied. After the harvest of the organs and tissues, the carcasses were disposed as per the CPCSEA guidelines.

All statistical computations were performed using SPSS statistical package (Version 17.0). Values of each group are given as graphical representation. One-way ANOVA and Tukey's multiple comparison tests were used to determine statistical significance. $P<0.05$ was considered statistically significant.

Results

Control vs fluorescent light

Tissue and plasma lipid peroxidation showed a significant rise after exposure to fluorescent light for a period of 15 (FL_{15}) and 30 (FL_{30}) days (Table I). This was accompanied with a fall in total reduced glutathione. Reduction in the levels of reduced glutathione however was observed after exposure to one day of fluorescent light (FL_1) itself, except in the skeletal muscle where this change was observed only after 15 and 30 days of exposure (Table II). The antioxidant enzyme, superoxide dismutase level was significantly lowered in all the three points (FL_1, FL_{15} & FL_{30}) studied in the liver. This fall however was observed only after 15 days of exposure in the brain, muscle and hemolysate. No significant change were observed in the heart and kidney (Table III).

Control vs LED pre exposure

LED pre exposure groups (LL_1, LL_{15} and LL_{30}) showed significant change in the parameters studied when compared with the control group. Lipid peroxidation was higher in skeletal muscle at all the three time points studied. Similar higher values were observed in the brain and liver after exposure for 15 and 30 days and in the kidney of the 30 day group alone. Liver is the only organ which showed a significant lower value for lipid peroxidation after 30 days of LED pre exposure (Table I). Total reduced glutathione showed significant decrease in the LL_1 group in all the tissues except skeletal muscle. A rise was observed after 15 and 30 day LED pre exposure (LL_{15}, LL_{30}) in the tissues studied. Liver was one of the tissues studied where lower levels of total reduced glutathione was observed both in 1 and 15 day exposure and the rise observed only after 30 days exposure (Table II). A similar change was observed with Superoxide dismutase. Though the levels of SOD after one day exposure (LL_1) showed a decrease in the brain, heart and kidney they were statistically not significant (Table III).

Control vs only LED exposure

A significant change in the parameters studied was observed in all the tissues after exposure to only LED light for a period of 30 days (Table: I, II and III). The rise in GSH was significant in the plasma, brain, heart and kidney, with a concomitant fall in LPO observed only in the brain and plasma in the OL_{30}

TABLE I: Lipid Peroxidation (µmols of MDA/ml of plasma or homogenate).

	Days	*CL*	*FL*	*LL*	*OL*
Plasma	1 day	7.482±0.574	6.826±0.154	6.796±0.338	7.774±0.551
	15 days	7.482±0.574	16.599±0.226*$	11.975±0.358*#$	7.745±0.225
	30 days	7.482±0.574	13.419±0.832*$@	6.637±0.458#@	5.747±0.199*$@
Brain	1 day	6.870±0.174	7.132±0.267	6.972±0.754	7.060±0.483
	15 days	6.870±0.174	15.257±0.440*$	8.226±0.749*#$	6.111±0.299$
	30 days	6.870±0.174	12.398±0.175*$@	11.391±0.335*#$@	6.155±0.181*$
Heart	1 day	6.520±0.271	6.214±0.139	6.330±0.417	6.666±0.129
	15 days	6.520±0.271	14.498±0.443*$	7.541±0.279*#$	6.593±0.404
	30 days	6.520±0.271	14.571±0.525*$	9.422±0.514*#$@	6.399±0.174
Kidney	1 day	7.293±0.415	8.926±0.248*	7.060±0.208#	6.884±0.126
	15 days	7.293±0.415	8.795±0.161*	6.826±0.404#	6.651±0.126
	30 days	7.293±0.415	14.119±0.434*$@	11.348±0.259*#$@	6.680±0.259
Liver	1 day	9.408±0.210	9.393±0.320	8.926±0.943	8.999±0.153
	15 days	9.408±0.210	14.484±0.388*$	8.955±0.215#	8.941±0.235
	30 days	9.408±0.210	15.009±0.732*$	6.432±0.153*#$@	9.072±0.615
Skeletal Muscle	1 day	3.894±0.204	5.951±0.196*	6.301±0.309*	3.326±0.360
	15 days	3.894±0.204	8.970±0.494*$	5.411±0.331*#$	3.296±0.757
	30 days	3.894±0.204	9.145±0.876*$	7.176±0.469*#$@	3.325±0.355

TABLE II : Reduced Glutathione (µg/ml of hemolysate or homogenate).

	Days	CL	FL	LL	OL
Hemolysate	1 day	19.092±0.413	14.502±1.631*	16.227±1.855*	18.669±0.800
	15 days	19.092±0.413	9.359±1.197*$	22.689±2.281*#$	19.238±0.347
	30 days	19.092±0.413	15.406±0.591*$	25.629±0.418*#$	23.293±0.922*$@
Brain	1 day	11.865±0.262	9.229±1.235*	9.131±1.060*	11.507±0.618
	15 days	11.865±0.262	7.048±0.354*$	16.683±1.072*#$	11.605±0.246
	30 days	11.865±0.262	10.726±0.450*@	23.177±0.119*#$@	13.346±0.138*$@
Heart	1 day	36.833±1.334	17.448±2.055*	17.301±2.317*	36.182±1.199
	15 days	36.833±1.334	27.588±1.044*$	50.049±1.610*#$	36.751±1.454
	30 days	36.833±1.334	26.812±1.665*$	59.629±0.565*#$@	40.333±2.096*$@
Kidney	1 day	37.646±1.528	23.112±3.027*	22.314±4.224*	36.230±1.452
	15 days	37.646±1.528	28.955±2.665*$	47.868±2.014*#$	36.426±1.171
	30 days	37.646±1.528	11.545±0.206*$@	40.199±0.323#$@	41.814±2.768*$@
Liver	1 day	50.684±1.517	21.322±3.173*	21.094±3.221*	48.739±0.871
	15 days	50.684±1.517	29.248±0.669*$	33.919±2.645*#$	49.007±2.429
	30 days	50.684±1.517	25.379±0.415*$@	54.001±1.365*#$@	50.102±0.685
Skeletal Muscle	1 day	29.948±1.033	29.370±0.423	27.976±1.869	29.097±0.514
	15 days	29.948±1.033	26.774±0.354*$	32.861±0.329#$	29.346±2.533
	30 days	29.948±1.033	21.425±0.412*$@	33.160±0.282*#$	29.187±0.259

TABLE III : Superoxide Dismutase (50% inhibition of pyrogallol auto oxidation/min/ml of hemolysate or homogenate).

	Days	CL	FL	LL	OL
Hemolysate	1 day	27.037±1.418	14.074±1.913*	15.926±3.285*	28.148±1.210
	15 days	27.037±1.418	21.481±0.855*$	42.963±1.711*#$	31.111±1.711*
	30 days	27.037±1.418	17.574±1.211*$@	52.482±3.289*#$@	32.259±4.271*
Brain	1 day	26.296±2.222	20.370±2.530	20.741±6.735	26.667±1.210
	15 days	26.296±2.222	24.444±0.855$	33.704±2.222#$	29.259±2.804
	30 days	26.296±2.222	17.100±1.510*@	41.100±4.530*#$	39.900±5.745*$@
Heart	1 day	22.347±1.235	17.037±1.913	18.148±5.042	26.667±2.095
	15 days	22.347±1.235	22.222±1.711$	28.148±3.421$	37.778±0.855*$
	30 days	22.347±1.235	18.670±2.630	53.907±2.332*#$@	46.019±3.021*$@
Kidney	1 day	25.593±1.298	26.167±0.333	27.241±2.650	28.815±1.532
	15 days	25.593±1.298	21.481±0.855$	40.000±5.132#	28.889±0.855
	30 days	25.593±1.298	21.519±2.927$	35.044±4.318#	39.963±1.587*$@
Liver	1 day	36.898±3.067	22.593±3.285*	21.852±6.667*	32.593±3.825
	15 days	36.898±3.067	18.519±0.855*	22.963±2.566*	34.074±3.421
	30 days	36.898±3.067	23.822±2.133*@	46.578±5.369*#$@	43.022±3.740$@
Skeletal muscle	1 day	26.259±2.182	20.370±5.324	21.852±5.722	26.296±4.895
	15 days	26.259±2.182	18.519±0.855*	41.111±1.418*#$	27.407±1.913
	30 days	26.259±2.182	20.278±1.078*	44.157±1.950*#$	42.226±2.979*$@

Values given are Mean±SD. Each group consisted of 6 animals.
Groups: CL - control, FL - fluorescent light exposed group, LL - LED pre exposure + fluorescent light exposed group, OL - only LED exposed group
ANOVA was performed followed by Tukey's multiple comparison if F test ratio was significant.
Level of significance was set at $p<0.05$
Comparison between groups denoted by superscripts:
*denotes significant difference with CL
#denotes significant difference between FL and LL
Effect of Time denoted by superscripts:
$denotes comparison with their respective 1 day group
@denotes comparison between their respective 15 and 30 days group

group. Levels of SOD showed marked rise in the plasma and heart after exposure to only LED light for 15 and 30 days. This rise was observed in other tissues only after 30 days of exposure.

Fluorescent light vs fluorescent light + LED pre exposure

Pre exposure of animals to LED light before exposing them to fluorescent light showed a significant lower level of lipid peroxidation in all the organs studied in the LL_{15} and LL_{30} groups of animals (Table I). In these tissues similar change was observed in the levels of SOD and GSH following pre exposure to LED light (Table II & III). In the plasma, LPO showed no change in the LL_1 and LL_{30} group, however a significant rise was observed in the LL_{15} group alone. Superoxide dismutase and total reduced glutathione levels showed significant rise similar to the changes observed in the tissues studied in the LL_{15} and LL_{30} group.

Discussion

Radiation exposure from manmade sources as simple as X ray and television affects human physiology, metabolism and behavior. The degree of damage by the various radiation exposures depends upon the source, time as well as duration of exposure. In the same way, artificial light exposure has the ability to disrupt the physiological mechanism of living organisms. Fluorescent light exposure during the night causes more damage than exposure during the day (30). In our study, lipid peroxidation, peroxidation of polyunsaturated fatty acids, GSH - natural antioxidant and SOD - first line defense enzyme against reactive oxygen species (ROS) in the cell were assayed to quantify the degree of oxidative damage. The levels of these markers were altered in the blood as well as in other tissues following exposure to fluorescent light, which was duration and tissue specific.

Light exposure at night can result in oxidative stress in a duration and intensity dependent manner. One of the important causes for the oxidative stress is decreased levels of the antioxidant hormone, melatonin which is altered with light exposure at night as seen in our earlier study (31). From the present study, it is evident that exposure of rats to fluorescent light at night results in increased lipid peroxidation in the tissues examined right from day one of exposure. This result is in agreement with earlier studies using light of other lower lx. Even exposure of rats to constant light in a standard animal house lighting (325 lx) results in increased lipid peroxidation in the brain, liver and kidney when exposed to 2 weeks (32) and in the blood following a period of 21 days (17). Hence, it is clear from the results obtained in our study that fluorescent light exposure induces oxidative damage and is dependent on the duration of exposure and is also tissue specific (26, 31).

Mechanisms for fluorescent light induced oxidative damage have been documented in earlier studies. Increase in lipid peroxidation is probably due to the generation of photo-oxidants and reactive radicals (32), stimulation of flavins, an endogenous photosensitizer, to initiate generation of free radical (33). Photosensitized reaction can occur by means of type I and II reactions, where it leads to the production of ROS (34). Photo oxidative reaction resulting in increased lipid peroxidation of brain tissue following fluorescent light exposure has been studied and explained with glutamate N methyl – D aspartate (NMDA) receptor and calcium ion concentration. These finally result in the production of toxic hydroxyl radicals (17).

Change in glutathione system was time dependent and the first to occur with a decrease in its concentration seen in almost all the tissues. This could be due to over utilization of GSH to scavenge the fluorescent light mediated lipid oxidation. Other possible reason for decrease in GSH might be due to the low melatonin concentration (35) or direct inhibition of glutathione peroxidase activity by the fluorescent light (36). Decrease in SOD in the FL group of animals in the various tissues and time points indicates over utilization of this catalytic enzyme to quench the free radicals generated by fluorescent light exposure. Earlier reports following exposure to fluorescent light of varied lx have also observed similar changes as observed in our study with respect to GSH and SOD levels in the blood

and other tissues (17, 32). Few of the tissues studied did not show any significant change. This can be attributed to variation in the dominant antioxidants in these tissues or quantum of light required to produce oxidative damage could be tissue specific.

Exposure to 30 days of LED increased the total reduced glutathione and SOD level significantly. Protective effect of LED on oxidative stress and oxidative damage has been evaluated extensively. Exposure to LED of 670 nm with an intensity 9 J/cm^2, as used in the current study, for 18 days as well as 14 weeks has shown partial protection in diabetic rats as observed in the liver antioxidant status and damage (37). Similarly another animal study with the same wavelength of LED exposure however with intensity of 6 J/cm^2 also exhibited similar protective response by inhibiting free radical induced early lesions in diabetic retinopathy (38). In the current study, this protective effect of LED was evident in the LED pre exposure followed by exposure to fluorescent light as well. LL_{15} group animals showed significant change in the oxidative stress parameters, when compared to FL_{15} group and their values were near control values. Most of these changes returned to baseline or control value in the LL_{30} group. This indicates that that LED pre exposure offers a significant protection in all the tissues studied from free radical induced oxidative damage effect of fluorescent light. As the changes observed in the LL_1 group were identical with FL_1 group it indicates that LED light protective effects depend on duration of exposure (26).

The LED protective effect might be due to stimulating effect of the redox signaling in cells, enhanced antioxidant activity and/or direct effect on reducing free radical damage (37, 39, 40). Evidences from the previous literature show that low power laser irradiation in the red to near infrared region enhances cellular metabolic functional activity and reducing lipid peroxidation in cell (41). LED light exposure in the near infrared region might also exhibit the protective response via a similar mechanism. In our earlier study, we found that LED light exposure of 670 nm for period of thirty days showed significant protection against 1800 lx fluorescent light induced retinal damage. This was evident with increase in outer nuclear cell count and thickness (26, 31). Light exposure in the near infra red (NIR) wavelength act as a therapeutic/optical window in biological tissues, as this wavelength of light has a better and deeper light tissue interactions (42).

Chromophores are responsible for photobiostimulation. Endogenous porphyrins, mitochondrial membrane cytochromes and flavoproteins act as photo acceptors in visible light and NIR light region (43). Among this, the key role is played by cytochrome c oxidase, as the absorption spectra of cytochrome c oxidase and action spectra of low intensity red and NIR light are the same for any biological response (42). However, one should take into account that cytochrome c oxidase act as a primary photo acceptor only in partially reduced form. In addition, red and near infrared light exhibit a biphasic response, i.e., induces as well as decreases ROS production depending upon the intensity and duration of exposure (44). Hence, a short duration of LED pre exposure, as used in our study (26, 31), might result in activation of cytochrome c oxidase, alters redox status, reduces the oxidative stress and radical damage (37). It could also enhance transcription factor activity or production and increase energy metabolism (41). In this study we have seen that LED light photobiomodulation is contributed by the biochemical and cellular changes at macroscopic level, like changes in oxidation and reduction reactions of glutathione system and antioxidative enzymes like SOD.

Conclusion

Fluorescent light exposure results in oxidative stress. LED light therapy improves the antioxidant defense system and protects the tissues from oxidative damage thereby indicating that 670 nm LED light photobiomodulation may be broadly applicable to reverse fluorescent light induced oxidative stress. The current study was done for specified period of exposure to fluorescent light/LED light exposure which is a limitation of our study. Additional studies are necessary to elucidate the exact mechanism(s) of this protective effect.

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Original Article

Effect of co-administration of Green Tea (*Camellia sinensis)* on Clove- (*Syzygium Aromaticum)* Induced Hepatotoxicity and Oxidative Stress in Wistar Rats

Joseph A. Adeyemi[1]*, Olatunbosun K. Arowolo[2], Solomon T. Olawuyi[3], Daniel Alegbeleye[1], Aderopo Ogunleye[1], Olufemi Samuel Bamidele[4] and Chris O. Adedire[1]

[1]Department of Biology,
School of Sciences, Federal University of Technology,
P.M.B. 704, Akure, Ondo State, Nigeria

[2]Department of Biological Sciences,
Environmental Management and Toxicology Unit,
Faculty of Science, Elizade University,
P.M.B. 002, Ilara-Mokin, Ondo State, Nigeria

[3]Department of Anatomy,
School of Health and Health Technology,
Federal University of Technology,
P.M.B. 704, Akure, Ondo State, Nigeria

[4]Department of Biochemistry,
School of Sciences, Federal University of Technology,
P.M.B. 704, Akure, Nigeria

Abstract

The study was designed to investigate the potential of oil extracts of clove (*Syzygium aromaticum)* to induce oxidative stress and hepatotoxicity in Wistar rats. The ameliorative effect due to co-administration with green tea, *Camellia sinensis* was also determined. Adult Wistar rats were exposed via oral gavage to one of the following: mineral oil (negative control), 5% green tea (GT), 12.5 mg/kg/day chlorpyrifos (CHL, positive control), 360 mg/kg/day clove oil (CO), green tea + chlorpyrifos (GT + CHL) and green tea + clove oil (GT + CO). Experimental treatment lasted three weeks, after which the animals were sacrificed and the following indices of oxidative stress and hepatotoxicity were determined in the plasma: levels of reduced glutathione (GSH), activities of catalase, glutathione peroxidase (GPx), aspartate amino transferase (AST), alanine amino transferase (ALT), and alkaline phosphatase (ALP). There was a significant decrease in plasma levels of GSH in the chlorpyrifos and *S. aromaticum* treated groups compared to the control rats. The activities of AST and ALT were higher in the chlorpyrifos and *S. aromaticum* treated groups compared to the control, however these data were only significant in the chlorpyrifos treated group. The activities of GPx, catalase

***Corresponding author :**
Joseph A. Adeyemi, Departments of Biology, School of Sciences, Federal University of Technology, P.M.B. 704, Akure, Ondo State, Nigeria
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and ALP did not differ significantly among the groups. The co-administration with *C. sinensis* resulted in less depletion of GSH as well as reduced levels of plasma AST and ALT. Overall, the results of this study show that the co-administration with *C. sinensis* has the potential to ameliorate the clove- induced oxidative stress and hepatotoxicity in rats.

Introduction

Clove (*Syzygium aromaticum*) is one of the most valuable plants widely used as food preservative and for many medicinal purposes including antimicrobial, anti-inflammatory, antioxidant and anticancer activities (1). The major constituent of clove includes phenolic compounds (eugenol acetate, gallic acid, beta-caryophyllene, vanillin and eugenol) which possess cosmetic, pharmaceutical, food and agricultural applications (2). Although, there are reports on the antioxidant property of eugenol (3, 4), however at high concentrations, eugenol could be a prooxidant, thereby leading to cytotoxicity, reactive oxygen species (ROS) production, and alteration of intracellular glutathione levels (5). Recently, studies have shown that extracts and derivatives of *S. aromaticum* were toxic to certain insect pests and microcrustaceans (6-8) while data on the toxic effect on mammals are not common. The pesticidal property of *S. aromaticum* has made it an effective and efficient alternative to conventional synthetic pesticides which are less environmentally friendly. However, since there is the possibility of residual accumulation on crops and food items, studies focusing on toxicity of *S. aromaticum* on mammalian models are imperative.

Green tea (*Camellia sinensis*) is probably the most widely drunk beverage all over the world, with estimated consumption of over 3 billion cups per day (9, 10). Several beneficial health claims have been attributed to the consumption of green tea amongst many others including the improvement of asthenia, diarrhea, bronchitis, asthma, hyperlipidemia, cellulitis, and abscesses as well as weight reduction (11, 12). Some other studies have also shown that green tea consumption is associated with a reduced risk of cardiovascular diseases, degenerative diseases, and cancer (13, 14). The potential health benefits associated with green tea consumption have been partially attributed to the antioxidative properties of polyphenols which include (-)- epigallocatechin-3-gallate (EGCG), (-)-epicatechin-3-gallate (ECG), (+)-gallocatechin (GC), (-)-epicatechin (EC), gallocatechingallate (GCG) and catechin (15, 16). Notwithstanding the aforementioned health benefits linked to consumption of green tea, there are reports of green tea resulting in liver damage when consumed at higher quantity (16, 17).

From the foregoing, studies focusing on evaluation of toxic effect of *S. aromaticum* should be encouraged especially using mammalian models. This present study is therefore designed to investigate the potential toxic effects of essential oil obtained from *S. aromaticum* using various indices like levels of reduced GSH, catalase and GPx enzymes activities (oxidative stress), and the plasma levels of enzymes such as AST, ALT, and ALP (hepatotoxicity), and also to investigate the protective role of the green tea, *C. sinensis* on *S. aromaticum* induced toxicity in Wistar rats.

Materials and Methods

Extraction of essential oils from S. aromaticum

Essential oil was extracted from clove flower buds following the procedures of Ileke and Ogungbite (18). Briefly, dried flower buds of *S. aromaticum* were obtained from a local market within Akure metropolis, and grinded using a blender. Acetone extracts of *S. aromaticum* were obtained using cold extraction method. This was done by soaking 100 g of the powder in an extraction bottle containing 300 ml of acetone. The mixture was stirred occasionally with a glass rod and extraction was terminated after 72 hours. The extract was filtered through Whatman filter paper (pore size; 0.7 microns). The extraction solvent

was evaporated using a rotary evaporator set at 56°C. The resulting extract was air dried in order to remove traces of solvent.

Experimental animals

Adult male Wistar rats weighing approximately 200 g were obtained from a commercial farm within Akure metropolis, and were placed individually in polypropylene cages, with laboratory grade pine shavings as bedding. Rats were allowed to acclimatize to experimental room conditions for two weeks prior to commencement of experiments. Rats were fed with rat chow and tap water *ad libitum*, throughout the period of experiment.

Experimental Design

The animals were randomly allocated into six groups (5 = 5 per group), and were exposed through oral gavage to one of the following treatments; oil (vehicle for extracted clove oil, thus serving as negative control), 5% green tea (GT), 12.5 mg/kg/day chlorpyrifos (CHL, positive control), 360 mg/kg/day extracted clove oil (CO), green tea + chlorpyrifos (GT + CHL) and green tea + extracted clove oil (GT + CO). Experimental treatment was done every day and lasted for three weeks. At the end of the third week of treatment, animals were sacrificed using cervical dislocation, and the blood was collected into anticoagulant bottles. The following indices of oxidative stress and hepatotoxicity were determined in the plasma: levels of reduced glutathione (GSH), activities of catalase, glutathione peroxidase (GPx), aspartate amino transferase (AST), alanine amino transferase (ALT), and alkaline phosphatase (ALP). The green tea, *Camellia sinensis* used in the study was purchased as a processed product from a commercial store in Akure, Nigeria. Experimental animals were treated and sacrificed following the approved guidelines for the use of animals by the Federal University of Technology, Akure, Nigeria.

Determination of GSH, GPx and Catalase activity in the plasma

The level of reduced GSH in the blood was determined spectrophotometrically following the methods of Ellman (19) using 5-52 -dithio-bis(2-nitrobenzoicacid(DTNB) as the substrate. GSH levels were expressed as µM GSH/ml. GPx activity was determined by the method of Paglia and Valentine (20). The principle of determination is based on the decrease in absorbance of NADPH at 340 nm, and the activity was expressed as mmol NADPH/min/ml while catalase activity was determined following the procedures of Aebi (21), the principle being the rate of H_2O_2 and its activity was expressed as rate constant of H_2O_2 decomposition (k) per ml.

Determination of plasma activity levels of AST, ALT and ALP

The plasma activity levels of AST, ALT and ALP was determined using RANDOX® diagnostics kits (Randox Laboratories Ltd, Crumlin, UK) following the manufacturer's instruction. The activities of the enzymes were expressed as Units/ml.

Statistical analyses

The plasma GSH levels, catalase, GPx, AST, ALT and ALP activities data were subjected to one-way analysis of variance test, so as to determine the difference among the different treatment groups. Tukey's multiple comparison tests was later performed in circumstances of significant difference. Statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA). For reporting purposes, data were expressed as mean±SE, and statistical significance was assumed at $p \leq 0.05$.

Results

Plasma levels of GSH, GPx and catalase activities

The results of levels of GSH, GPx and catalase activities are presented in Figs. 1, 2, and 3 respectively. There was a significant difference in the levels of GSH among the groups (p=0.0031). The rats that were treated with the extracted clove oil and chlorpyrifos had significant lower levels of GSH in comparison to the oil and green tea treated groups. There was no significant difference between the mixture groups (GT + CO & GT + CHL) and the oil-treated control group and the green tea treated group. The plasma GPx and catalase activities did not differ significantly among the groups (p=0.2671 and 0.5112

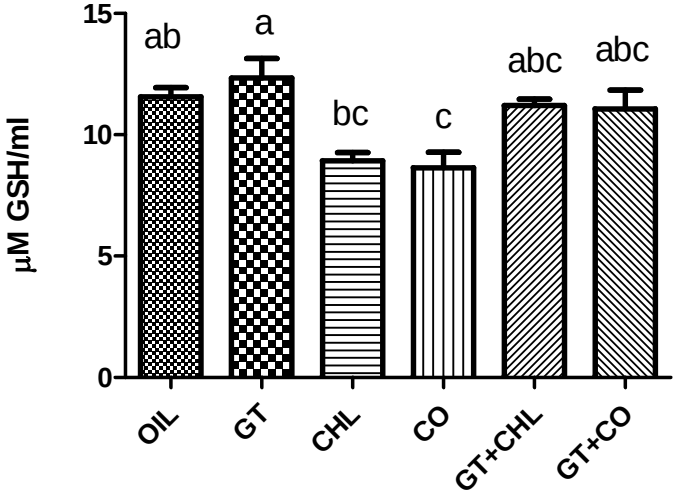

Fig. 1 : GSH levels (µM GSH/ ml) in the plasma of Wistar rats. Each point is the mean±standard error (n=5). Bars with different letters are significantly different in pairwise comparison.

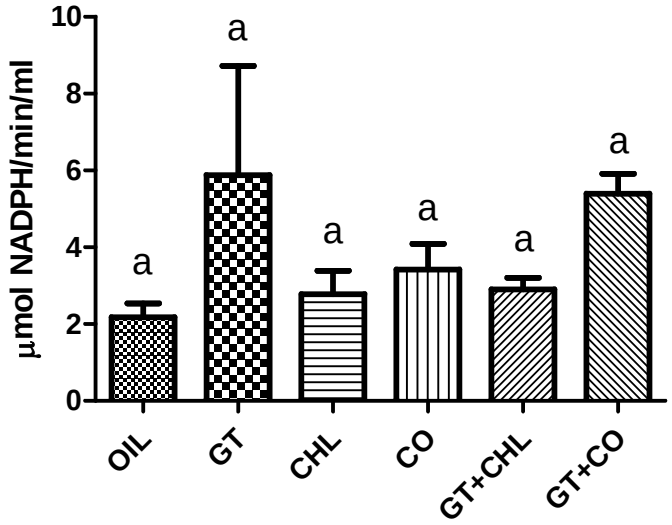

Fig. 2 : GPx activity (mmol NADPH/min/ml) in the plasma of Wistar rats. Each point is the mean±standard error (n=5). Bars with different letters are significantly different in pairwise comparison.

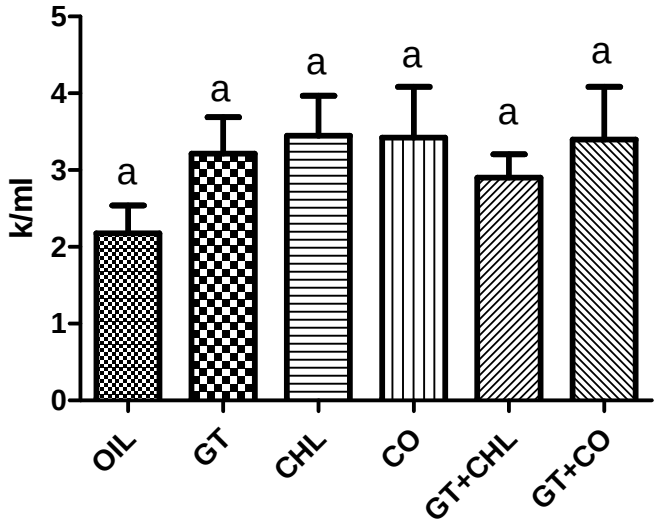

Fig. 3 : Catalase activity (rate of H_2O_2 decomposition (k)/ml) in the plasma of Wistar rats. Each point is the mean±standard error (n=5). Bars with different letters are significantly different in pairwise comparison.

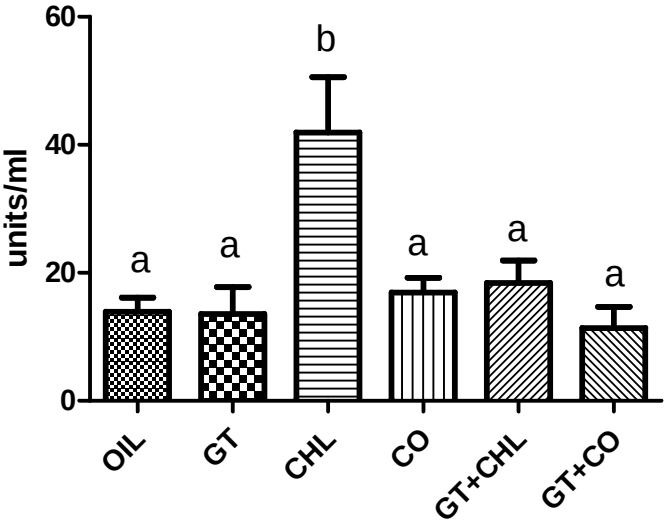

Fig. 4 : AST activity (units/ml) in the plasma of Wistar rats. Each point is the mean±standard error (n=5). Bars with different letters are significantly different in pairwise comparison.

for GPx and catalase respectively). There was preponderance for decreased GPx activity in the groups that were treated with chlorpyrifos, clove oil and the mixture of green tea and chlorpyrifos but this trend was not statistically significant.

Plasma AST, ALT and ALP activities

The plasma activity levels of AST, ALT and ALP are shown in Figs. 4, 5 and 6 respectively. There were significant differences in the activities of AST and ALT among the groups (p=0.0012 and 0.0358; AST and ALT respectively). Wistar rats administered with chlorpyrifos had significantly higher activities of AST and ALT compared to other groups in which the activities of AST and ALT were statistically the same. Also, pairwise comparison between the groups treated with chlorpyrifos and the mixture of chlorpyrifos and green tea indicated a significant reduction in the activities of AST and ALT. The activity of ALP did not differ significantly among the

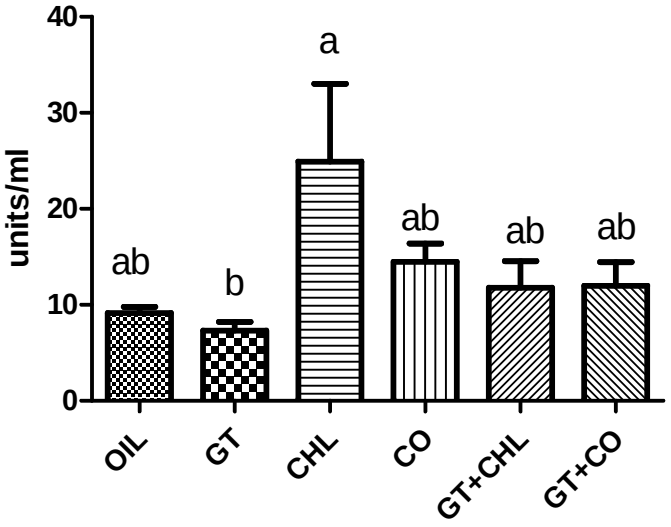

Fig. 5 : ALT activity (units/ml) in the plasma of Wistar rats. Each point is the mean±standard error (n=5). Bars with different letters are significantly different in pairwise comparison.

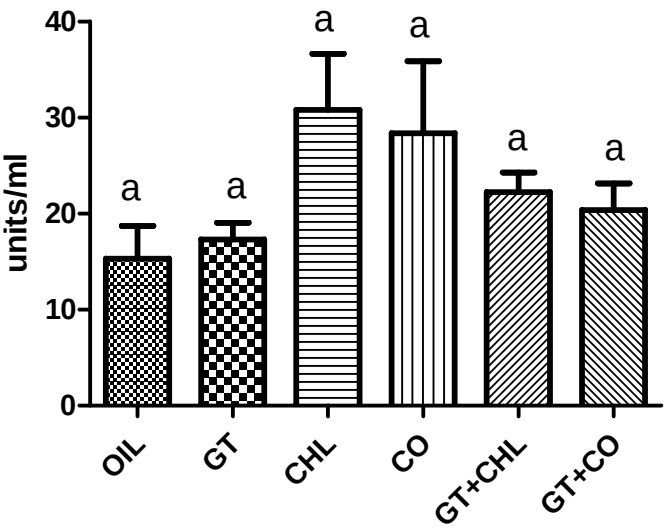

Fig. 6 : ALP activity (units/ml) in the plasma of Wistar rats. Each point is the mean±standard error (n=5). Bars with different letters are significantly different in pairwise comparison.

groups (p=0.1496). The groups that were treated with chlorpyrifos and extracted clove oil appeared to have higher ALP activities compared to the other groups although this difference was not statistically significant.

Discussion

The cellular levels of reduced glutathione have been widely used as a biomarker of oxidative stress in animals. As a defense mechanism against oxidative stress, glutathione in its reduced state donates its thiol group to the reactive oxygen species, thus neutralizing them, and consequently leading to depletion of the cellular level of reduced glutathione (22). The significant reduction in the levels of reduced glutathione in the rats that were treated with clove oil and chlorpyrifos was an indication of GSH depletion in response to oxidative stress. Previous studies have shown that the exposure of animals to certain organophosphate pesticides resulted in depleted level of reduced glutathione (23, 24).

The activity levels of antioxidant enzymes such as catalase and glutathione peroxidase has often being employed as biomarkers of oxidative stress in animals. Catalase acts to detoxify hydrogen peroxide by converting it to water and molecular oxygen while glutathione peroxidase in addition to its role in detoxifying hydrogen peroxide also converts lipids hydroperoxides to their corresponding alcohols. In the present study there was no significant difference in the activity levels of both catalase and glutathione peroxidase among the treatment groups. The lack of difference in activity of the two enzymes measured in this study, notwithstanding the significant difference in the levels of reduced glutathione could be wrongly interpreted to mean lack of oxidative stress due to treatment with clove oil or the known toxic chlorpyrifos. This suggests the need to employ multiple biomarkers when studying the toxic effects of certain substances on animals. The results of this study therefore demonstrated that the treatment of Wistar rats with clove oil has the tendency to cause oxidative stress. The result is in tandem with the findings of Cortes-Rojas et al (1). which reported that eugenol, the major constituent of clove, acts as an antioxidant at low concentrations (5-10 µmol/ L) but could serve as a prooxidant at a high concentration (500 µmol/L) resulting in increased production of reactive oxygen species.

The liver has been shown to be the centre of assault to numerous toxic substances in the body showing various abnormalities like degeneration of hepatocytes, infiltration with inflammatory cells, vacoulation of cells, hypertrophy etc. (24, 25). Also,

the biochemical quantification of activities of liver enzymes such as AST and ALT has been used as biomarkers of liver damage (26, 27). A high level of these enzymes in the blood is an indication of injury to the liver (28). The present data showed that the treatment of Wistar rats with chlorpyrifos caused significant damage to the liver cells with little support for *S. aromaticum* induced hepatotoxicity. Previous studies have actually shown that chlorpyrifos is hepatotoxic (29, 30). Although, the data from the present study do not find significant hepatotoxic effect due to clove oil administration, however few studies have shown that clove oil was hepatotoxic to rodents and man (31, 32).

The green tea, *Camellia sinensis* has been reported to be of significant health importance to man and other animals (13, 14). In this study, rats that were co-administered with clove oil and green tea, there was approximately 28% less GSH depletion while the AST and ALT levels were reduced by approximately 33 and 17% respectively. Similarly, in rats that were co-administered with chlorpyrifos and green tea, the depletion of GSH was less by 25% while the inhibition of glutathione peroxidase was reduced by almost 97%. The plasma levels of AST and ALT were reduced approximately 56 and 57% respectively. The findings were in agreement with the data from other studies that have reported the potential for *C. sinensis* to have anti oxidative and anti hepatotoxic effects in animals (33, 34). The antioxidant and hepatoprotective property of green tea may be due to the presence of catechin and (-)- epigallocatechin-3-gallate (EGCG) (15, 35).

In conclusion, we show in this study that the administration of *S. aromaticum* and chlorpyrifos to Wistar rats resulted in oxidative stress evidenced by decreased level of plasma GSH and decreased activity of glutathione peroxidase. The relative high level of liver enzymes in the plasma is also an indication that extracted oil of *S. aromaticum* and chlorpyrifos could cause liver damage in rats. Overall, the results of this study provide minimal support for protective ability of *C. sinensis* against *S. aromaticum* induced oxidative stress and hepatotoxicity in rat.

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Original Article

Plastic Toxin Bisphenol-A Depresses the Contractile Activity of Rat Ileum and Colon *in vitro*

Kumari Nirja, Parul Sharma, Anil Kumar Tiwari and M. B. Mandal*

Department of Physiology,
Institute of Medical Sciences,
Banaras Hindu University, Varanasi

Abstract

Bisphenol A (BPA), a plastic toxin, is required in the production of various plastic items including water bottles, baby feeding bottles and other food and beverage containers. Since, the primary source of human exposure to BPA is the leachate from food and beverage containers, gastro intestinal tissues are particularly susceptible to BPA-induced changes. Therefore, the present study was undertaken to explore the possible effects of BPA on contractility in adult rat ileum and colon. In an organ bath preparation, isometric contractions were recorded from segments of dissected out colon and ileum, with the help of force transducer and digitized data acquisition system. The results indicated that BPA (1-100 µM) significantly ($p<0.05$) depresses contractile tension and frequency of ileum and colon in a dose dependent manner. Further, the exploration of possible mechanisms for BPA-induced decline in contractile responses revealed that the decrease in contractility was independent of estrogen receptors, nitric oxide and cholinergic system.

Introduction

The chemical, 4, 4'-Isopro-pylidenediphenol, commonly known as Bisphenol A (BPA), is produced in a high volume and used primarily in the production of polycarbonate plastics and epoxy resins (1). Polycarbonate plastics are mainly used to make various types of plastic products including water bottles, baby feeding bottles and various food and beverage containers. Epoxy resins are used for lining metal cansto avoid erosion and maintain quality of canned food and beverages. However, it is known that BPA leaches from these plastic products into the foods contents and thus, humans are exposed to the BPAduring the consumption of foods, water and drinks served in plastic containers (2). Some dental sealants and composites may also play as important sources of BPA for human exposure (3). Because of extensive use of BPA in plastic industry, there iswidespread and well documented human exposure of BPA. BPA has been detected in various body fluids and tissues in a large number of people across the world. For example, the national health and nutrition examination survey on U.S. population above 6 yrs. of age, reported detectable urinary BPA levelsin more than 90% of population (4). Human exposure to BPA in several Asian countries has also been

***Corresponding author :**

Prof. M. B. Mandal, Department of Physiology, Institute of Medical Sciences, Banaras Hindu University, Varanasi; Email: maloy_mandal@yahoo.com

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documented (5). BPA has been known as endocrine disruptor and mimics estrogen in its mode of action (6). Owing to estrogenic activities, it produces various reproductive and behavioral toxicities (7). Also alteration in function of coronary smooth muscle (8) and depression of the atrial contractility in rat (9) has been reportedly induced by BPA.

Given that the main route of exposure to BPA is oral, intestinal tissues remains to be vulnerable to BPA-induced changes. Although, some studies have shown that BPA alters gut barrier and immune responses (10, 11), its effect on intestinal motility has not been adequately addressed.A recent study has reported that BPA inhibits duodenal movement via nitric oxide mediated pathway (12). However, its effect on ileum and colonic motility is not clearly understood.Therefore, the present study was undertaken to explore the possible effects of BPA on contractility of rat ileum and colon using*in vitro* experiments in organ bath preparations.

Methods

The present experiments were carried out on Charles Foster strain of rats after the approval of institutional ethical committee for animal experiments. The adult rats of either sex weighing 150-200 gm (4-6 months old) were procured from institutional animal house. Rats were housed in the departmental animal house in an environment of controlled temperature (25±0.5ºC), and light (12: 12 hr light dark) with *ad libitum* supply of rat feed and potable water.

Dissection of animal

Rats, fasted overnight, were sacrificed by cervical dislocation. The abdomen was opened quickly by midline incision. The ileum and proximal colon were dissected out and immediately placed in a petri dish containing 100% oxygenated fresh Krebs-Ringer solution and the intestinal contents, if any, were flushed out by this solution with the help of a syringe.

Mounting and recording of contractile response

The procedure for mounting and recording of contractile responses has been described earlier (13, 14). Briefly, after cleaning the tissues, the segments of 1-1.5 cm of intestine were placed in Krebs-Ringer solution filled organ bath (15 ml) maintained at 37°C±1°C and continuously bubbled with 100% O_2. One end of tissue segment was fastened to a glass tube support, and the other end was fixed to a force transducer (MLT 0210, AD instruments, Australia). Strips were mounted vertically for primarily recording of contractions of longitudinal muscle. The tissue segment was placed under optimum resting tension (0.5 gm.) and then left to equilibrate for 30 minutes, with replacement of Krebs-Ringer solution every 15 minutes. After stabilization, the initial recordings of spontaneous contractions were made for 30 minutes. Before, as well as after recording the contractile responses, calibration for the tension (0-10 g) was performed. After recording of contractions, the segment of tissue was removed from the organ bath and placed on blotting paper for lightly soaking the extra water from the tissue. The two ends of the strips were cut to remove the injured parts. The wet tissue was then weighed in a fine balance to express the contractile response per unit weight of tissue (g/g wet tissue).

Isometric contractions were amplified by bridge amplifier and digitized via an analog/ digital interface (Power Lab 4/ST system) to acquire onto a personal computer. The recordings were displayed and analyzed with the help of software Chart-5 for windows (AD Instruments, Sydney, Australia).

Drugs and solutions

The physiological solution (Krebs-Ringer solution) was prepared with following compositions (in mmol): NaCl, 119; KCl, 4.7; $CaCl_2.2H_2O$, 2.5; KH_2PO_4, 1.2; $MgSO_4.7H_2O$, 1.2; $NaHCO_3$, 5; and glucose, 11, with pH adjusted to 7.4. BPA was obtained from HIMEDIA laboratories Pvt. Ltd, Mumbai. It was dissolved in 50% ethanol to have stock solution (10 mM). For dose response curve, 1 µM 10 µM, 30 µM and 100 µM concentrations were used. In experiments where tissue was pretreated with various antagonists, 100 µMconcentration of BPA was used.

Antagonists- L-NAME (N – nitro – L – arginine methyl ester, a Nitric Oxide synthase inhibitor), Tamoxifen

(an estrogen receptor blocker), Atropine (a muscarinic receptor blocker), Hexamethonium (a ganglion blocker) were procured from Sigma Chemicals Inc. (St Louis MO, USA). The stock solution (10 mM) of these chemicals was prepared in distilled water. The stock solution was refrigerated and required dilutions were made in Krebs – Ringer solution just before the experimentations. The concentration used in experiments was 10 µM for Tamoxifen and 100 µM for all other antagonists.

Experimental protocol

There were three sets of experiments. Initially tissue was allowed to stabilize for 30 min and control recording was taken. In first set after stabilization, tissue was exposed to cumulative concentration of BPA (1, 10, 30, 100 µm), to assess the cumulative dose response of BPA. For each concentration, tissue was exposed to BPA for 10-15 minutes, followed by exposure to next higher concentration, without wash. In second set the tissue was exposed to equi-volumes of ethanol present in respective BPA concentrations. In third set, the gut tissue was exposed to one of four antagonists in different subsets for 15 min & subsequently it was exposed to BPA at concentration of 100 µm for 15 min. At the end of each experiment the tissue segment was removed, blotted and weighed.

Parameters studied and statistical analysis

Parameters studied were contractile tension and frequency**.** The tissue strips were subjected to initial tension of 0.50 gm. The maximum height of contractions were converted to tension (gram) with help of chart-5 software and then the tension so developed was expressed as tension per unit mass (g/g wet tissues) using the tissue weight determined at the end of the experiments. Frequency of contractions was calculated frequency per minute.

The values were then pooled to calculate Mean±SEM. The statistical significance of differences between mean values was determined by using paired *t*-test and unpaired *t*-test as applicable. One or two-way ANOVA were applied for multiple comparison depending on the requirement. A *p*-value of < 0.05 was considered statistically significant.

Results

Characteristics of contractions in normal recordings in ileum and colon of rat

The contractions observed in the ileum were phasic type (short lasting) and in colon were tonic type (long lasting). The Mean±SEM values of spontaneous contractions and frequency of contractions in control samples of ileum and colon were observed as in Table I.

TABLE I : Mean±SEM values of spontaneous contractions expressed as g/g wet tissue and frequency of contractions/min in ileum (n=6) and colon (n=6) of rats in untreated samples.

Parameters	*Untreated samples*	
	Ileum	*Colon*
Contractile tension (g/g wet tissue)	10.13±1.78	11.31±2.48
Frequency (contractions/min)	18.17±0.98	1.33±0.23

Effect of different concentrations (1, 10, 30 and 100 µM as cumulative doses) of BPA on spontaneous contraction in ileum tissue.

There was concentration dependent decrease in the maximum height of contractions after application of each concentration of BPA (Fig. 1a). There was significant ($p<0.05$, one way ANOVA), decrement in the response/tone at higher dose of BPA (30 and 100 µM); (Fig. 1b). There was a significant decrease in frequency when the concentration was increased to 30 and 100 µM of BPA (Fig. 1c). The frequency became zero, indicating no spontaneous contraction at 100 µM bath concentration (Fig. 1c).

Effect of different concentrations (1, 10, 30 and 100 µM as cumulative doses) of BPA on spontaneous contraction in colon tissue.

The effect of BPA on colon tissue was similar to ileum (Fig. 2a). On comparison of responses induced by different concentrations (1-100 mM) of BPA in colon and ileum, it was observed that there was continuous decrease in contractile tension with increasing concentration of BPA in colon (Fig. 2b), while in ileum response remains almost same up to

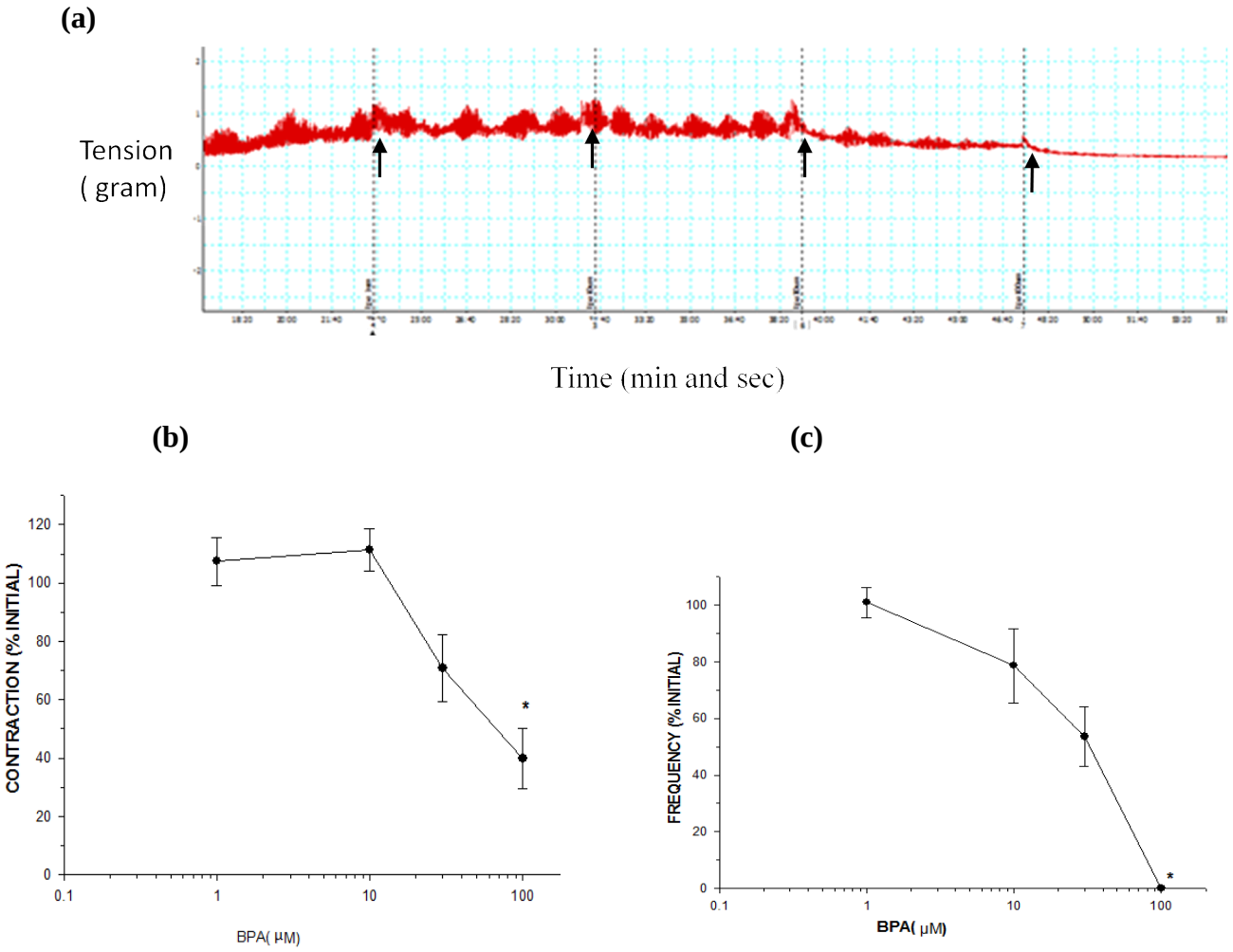

Fig. 1 : Above (a) showing original representative recording of dose response of ileum on application of different concentrations (1, 10, 30 and 100 mM) of BPA. Arrows indicate the point of application of BPA, vertical & horizontal calibrations represent the tension (gram) and time (min. and sec.) respectively. Below graphs showing the effect of BPA on contractile tension (b) and frequency (c) of contraction per min (Mean±SEM, n=6). The asterisk indicate significantly (p-value <0.05, one way ANOVA) different from previous concentrations.

10 mM concentration and then it decreased with 30 and 100 mM concentration. However, the frequency of contractions declined progressively and reached to zero at highest concentration (100 mM) of BPA in both (Fig. 1c, 2c).

Effect of Ethanol (Vehicle) on spontaneous contractions in ileum and colon

There was no statistically significant(p-value >0.05, one way ANOVA) change in contractions/tone as well as frequency of contractionswith different concentration of ethanol used to dissolve respective concentrations of BPA (Table II).

BPA (100 µM) response after pre-treatment of various antagonists

Pre-treatment of all antagonists viz. estrogen receptor antagonist tamoxifen, nitric oxide synthatase inhibitor L-NAME, ganglion blocker hexamethonium and atropine failed to block BPA (100 µM) induced depression in contractile responses in ileum (Fig. 2b) as well as colon (Fig. 3b), because there was no significant (p>0.05) difference between the response of BPA (100 µM)with or without pretreatment of antagonists (Fig. 3a, 3b). The frequency of contraction were not recordable in all the pre-treated groups, as the recording was flattened, therefore the

TABLE 2 : Mean±SEM values of contractile tension (g/g wet tissue) and frequency of contractions (per min) expressed as percentage of initial (i.e. control as 100%) and after application of different concentrations of ethanol (v/v%) from six experiments in rat ileum and colon. Please note that there was no significant($p>0.05$) change in contractility and frequency of contraction induced by various concentrations of ethanol.

	Ethanol (v/v %)									
	Control		*0.005*		*0.05*		*0.15*		*0.50*	
	Ileum	*Colon*	*Ileum*	*Colon*	*Ileum*	*Colon*	*Ileum*	*Colon*	*Ileum*	*Colon*
Contractions	100.00± 0.00	100.00± 0.00	118.79± 7.71	115.34± 7.37	119.63± 10.15	116.24± 10.05	118.36± 6.58	109.75± 6.49	114.66± 8.13	114.05± 7.40
Frequency/min	100±0	100±0	88±2	96±8	94±5	98±10	86±6	96±14	87±3	89±16

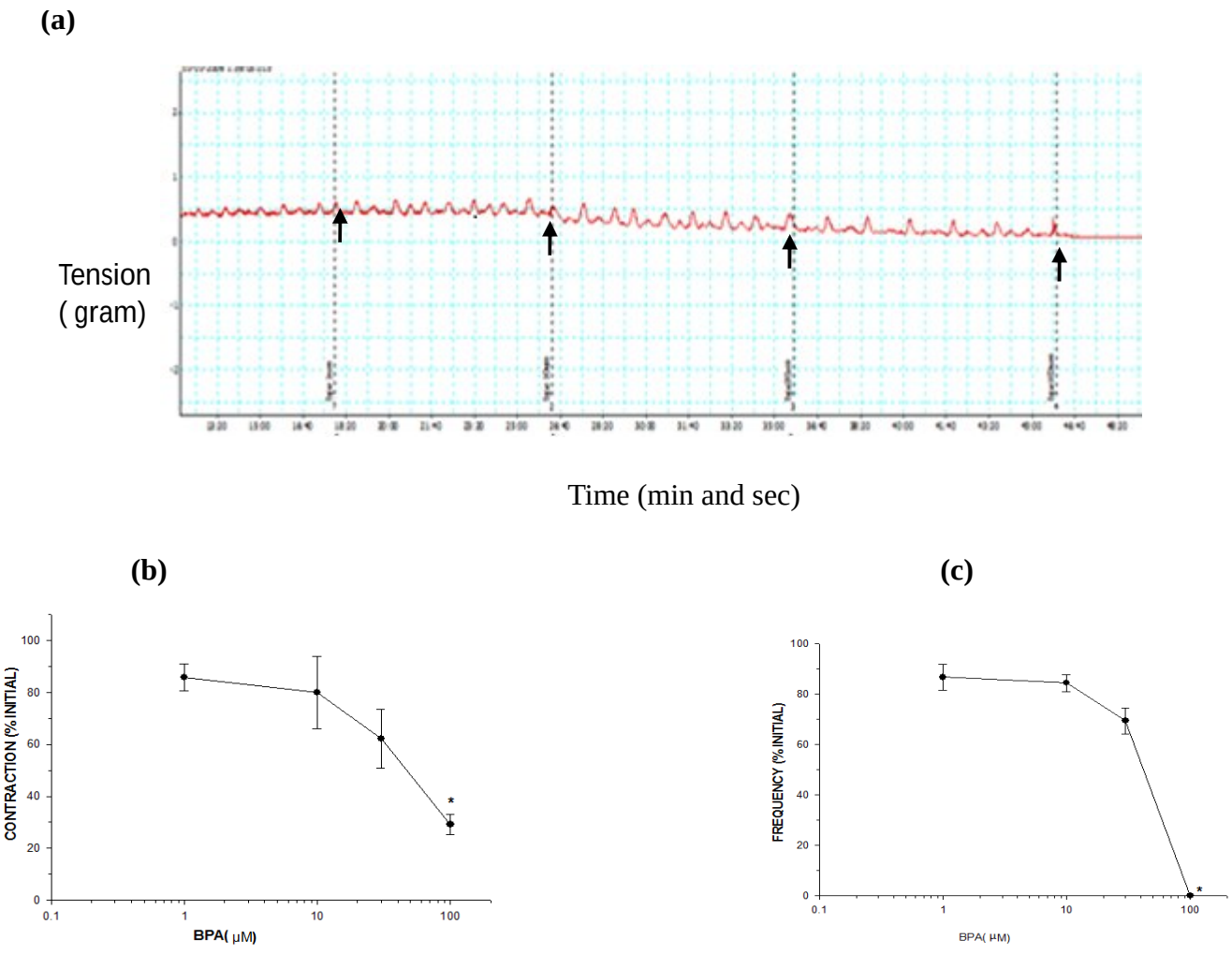

Fig. 2 : Representative recording (a), contractile tension (b), frequency (c) from colon issue (n=6), after application of different concentrations (1, 10, 30 and 100 mM) of BPA. Rest as described in Fig. 1.

frequency was considered zero after the addition of BPA 100 µM.

Discussion

The present study clearly demonstrated that BPA decreased contractile tension as well as frequency of spontaneously occurring contractions in segments of both small and large intestine (ileum and colon) of rats. It was apparent that the inhibition was by per se action of BPA and not the vehicle (i.e. ethanol), because the vehicle control experiments clearly

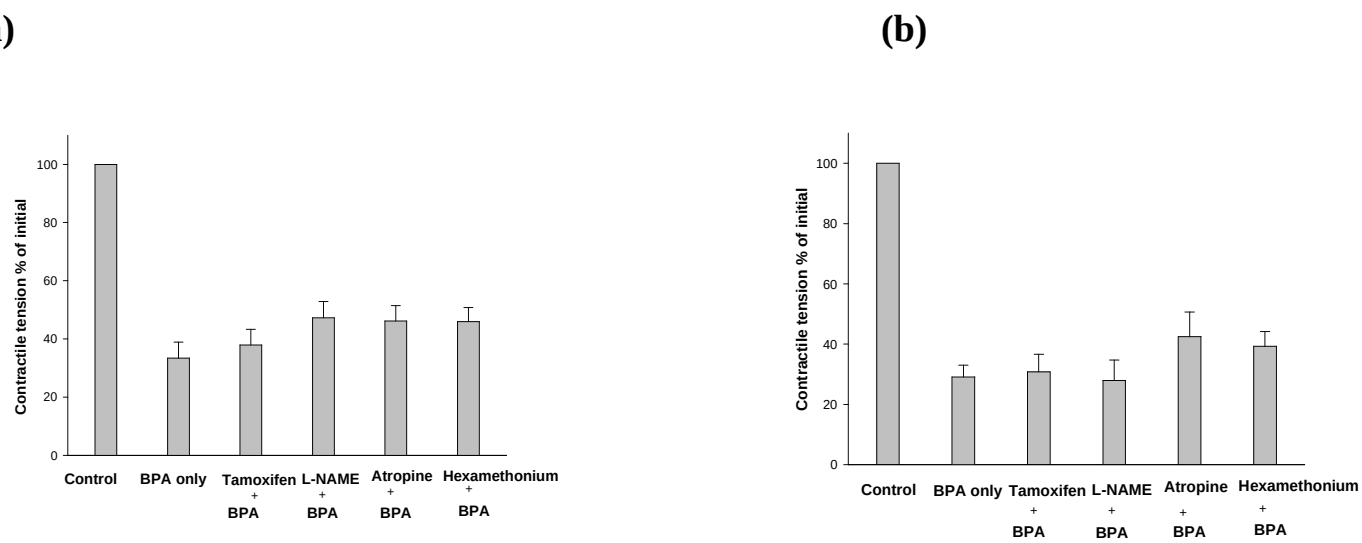

Fig. 3 : Bar diagrams showing (Mean±SEM, n=6) the contractile tension in ileum (a) and colon (b) after addition of BPA only (100 µM) and addition of BPA after treatment with L-NAME(100 µM), Tamoxifen (10 µM), Atropine (100 µM) and Hexamethonium (100 µM), expressed as percentage of initial.

showed that the amount of ethanol used for dissolving various concentration of BPA, in the present study, did not alter the smooth muscle contractile activity significantly.

BPA level has been detected in human urinary samples in several studies (4, 5). BPA concentrations used in our experiments are higher than these human urinary BPA levels. The various doses (1-100 µM) were used to assess the dose response of BPA and its toxicity, as used in studies carried out in other tissues in *in-vitro* experiments (9, 12).

It was observed that the depression of contractile functions was characterized by reduction of both contractile tension and frequency. The observation, therefore, signified that BPA might affect contractile machineries for reduced tension generated in intestinal smooth muscle and also interstitial cells of Cajal for observed change in the frequency of contractions.In the present study, an attempt has been made to assess the mechanisms of BPA induced inhibition of intestinal smooth muscle contractions. BPA is known to have estrogen like action (15) and estrogen has been found to impair contractile activity of gut muscle (16). Therefore, the decreased contractile functions of gut observed in the present experiments may be attributed to the estrogen like activity of BPA. Estrogen is known to act via two types of estrogen receptors (ER), namely ERá and ERâ. The ERâ is known to be expressed in intestine (17). However, in our study the estrogen receptor antagonist tamoxifen failed to block the BPA induced inhibitory response, thus indicating the ER-independent mechanisms for the action of BPA. It was postulated that the BPA might mediate its action via neural elements in enteric nervous system. However, the experiments using ganglion blocker hexamethonium discarded this proposition too. Further, BPA has been found to depress the atrial activity through nitric oxide (NO) mechanisms (9). In order to evaluate the involvement of NO mechanisms, some experiments with nitric oxide synthase inhibitor, L-NAME were also carried out in this study. It was observed that prior application of L-NAME failed to protect BPA-induced contractile impairment, thus, suggesting non-involvement of NO mechanisms in mediating the BPA induced attenuation of contractile responses.However a recent study reported that BPA inhibits the movement of the duodenum through NO mediated mechanisms (12). Further, any cholinergic involvement also was excluded by experiments having pre-treatment with atropine.

Thus, it appeared that the inhibitory response of BPA was brought about by its ER-independent action on intestinal smooth muscle. Similar ER-independent action of estrogen through activation of potassium

channels or inhibition of calcium channels has been proposed earlier (18). However, it is not possible to confirm if similar mechanisms are responsible for inhibitory action of BPA in the present investigation.

It may be concluded that, BPA diminish the contractile functions of small and large intestine in rats. The inhibitory mechanisms seem to be operating without involvement of estrogen receptors, nitric oxide and intrinsic neural plexuses. The exact mechanisms for the reduced contractile activity could not be ascertained from the present experiments. Further, the BPA-induced impaired contractile function of intestine is likely to have clinical implications like constipation and other gastrointestinal motility disorders and warrants further critical evaluation.

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Original Article

Effect of Meditation on Heart Rate, Blood Pressure and Exercise Performance in Coronary Artery Disease Patients

Shashank Shekhar Sinha[1]*, Ajay Kumar Jain[1], Sanjay Tyagi[2] and Aarti Sood Mahajan[1]

[1]Department of Physiology,
Maulana Azad Medical College,
New Delhi, India

[2]Department of Cardiology,
Maulana Azad Medical College and Associated GB Pant Hospital,
New Delhi, India

Abstract

Introduction: Coronary artery disease (CAD) is the leading cause of morbidity and mortality worldwide. This study is to evaluate the role of Meditation in improving physiological parameters like heart rate (HR) and systolic (SBP) and diastolic blood pressure (DBP) and exercise performance in known CAD patients.

Method: Sixty CAD patients are divided into two groups of which one group did Meditation and other did not. HR, SBP, DBP and Metabolic Equivalents (METs) were measured before and at end of 6 months of study in both the groups.

Result: At the end of study significant decrease in HR, SBP and DBP was seen in patients who practiced Meditation as compared to other group but there was no significant improvement in exercise performance in Meditation group.

Discussion: Meditation may modulate the physiological response to stress via neurohumoral activation, which may be a novel therapeutic target for the treatment of CAD but has no statically significant effect on exercise performance.

Introduction

Coronary artery disease (CAD) is epidemic in world and the leading cause of death worldwide (1). Previously thought to affect primarily high-income countries, CAD now leads to more death and disability in low- and middle-income countries (2), with rates that are increasing disproportionately compared to high-income countries. CAD affects people at younger ages in our country thereby having a greater economic impact (3). Advances in therapy like angioplasty, stenting and by-pass surgery address effectively the problem of individual patients. However, they are very expensive and beyond the

***Corresponding author :**
Shashank Shekhar Sinha, Department of Physiology, Maulana Azad Medical College, New Delhi, India.
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reach of the majority of patients in our country. Besides, these procedures are focused upon treating the manifestations of disease and not on the underlying cause of disease.

With increasing understanding of various risk factors as causative agents of CAD, lot of interest is generated in prevention of modifiable risk factors like tobacco smoking (4), high blood cholesterol (5), hypertension (6), physical inactivity (7), obesity (8) and diabetes mellitus (6). It has been also recognized that stress, anxiety and depression are also important in aetiology and progression of CAD (9) Cumulative exposure to chronic stressors may be a risk factor for CAD (10). Individuals who are exposed to high levels of demands and low levels of control at work, to distressing marriages, and to low social support are more likely to have incident CAD than their less stressed counterparts (10–12). Dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis is one of the pathways through which chronic stress may affect CAD risk. In healthy individuals, cortisol has a distinct diurnal pattern with the peak cortisol occurring in the early morning, declining throughout the day, and reaching a nadir around 2 or 3 AM (13). Cortisol also exhibits sizable, short-term increases during the first hour after awakening and in response to a lunch meal or a threat-provoking stressor. Dysregulation can take the form of altered overall levels of cortisol or a smaller decline in cortisol throughout the day and evening, i.e., flatter slope.

The physiological underpinning of this link may involve excessive sympathetic nervous system activation (14). Ornish et al (15) were the first to document the beneficial effects of lifestyle changes in reversing the coronary heart disease. Manchanda et al (16) in their study similarly showed encouraging results with their yoga lifestyle intervention. However, both these studies included only a small number of patients.

“Meditation” is a set of attentional practices leading to an altered state or trait of consciousness characterized by expanded awareness, greater presence, and a more integrated sense of self. Practice of concentrating focus on an imaginary point on forehead (between eyebrows), sound or object increase awareness of the present moment, reduce stress, promote relaxation, and enhance personal and spiritual growth. Meditation practice self-regulates the body and mind, thereby affecting mental events by engaging into a specific attentional set. These practices are a subset of other practices used to induce relaxation or altered states such as hypnosis, progressive relaxation and trance-induction techniques (17). Meditation is a simple mental technique which has well documented benefits for health and wellbeing (18, 19). It can be learned easily by anyone regardless of age, educational background, or culture. The technique is effortless and requires no belief or any change in lifestyle or diet.

During Meditation mental activity settles down in a natural way, while alertness is maintained and even enhanced. Meditation produces a specific physiological response pattern that involves various biological systems. Mechanism most frequently suggested that meditation produces effects including metabolic, autonomic, endocrine, neurological, cardiovascular and psychological responses on a multidimensional interactive basis.

Mental states can markedly alter physiologic function. For example, stressful situations result in a hypermetabolic state, with increased oxygen consumption, heart rate and blood pressure. In contrast, the majority of scientific studies show meditation to be a wakeful state accompanied by a decreased metabolism resulting in decreased breathing pattern, decreased heart rate, and decreased blood pressure (16). There is also marked decreased in the level of oxygen utilization and carbon dioxide elimination by muscles verified by innumerable studies (20).

Methods

The study was conducted in Department of Physiology and Cardiology, Maulana Azad Medical College and associated G. B. Pant Hospital from June 2011 to January 2012. The study group comprise of sixty angiographically proven (criteria: 50% or more obstruction in any coronary artery) coronary artery disease patients. These patients were

randomly selected and were equally divided into two groups, Meditation and Control group, each group consisting of thirty patients. Out of sixty patients, fifty six (93.3%) were males and four (6.7%) were females.

Group I : Meditation group contains CAD patients with medication and on prescribed meditation (concentrative meditation) and dietary modifications

Group II : Control group contains CAD patients with medication and dietary modifications.

Inclusion criteria were 1) Age group 30-70 years of either sex, 2) Angiographically proven coronary artery disease, 3) non-smokers. Exclusion criteria were 1) Patients with a history of acute myocardial infarction in recent past (two months), 2) Patients with unstable angina pectoris, 3) Patients with clinical cardiac failure, those with ejection fraction of below 30% by echocardiography, 4) Patients who had undergone coronary angioplasty or by-pass surgery, 5)Patients with heart ailments other than CAD such as congenital heart disease, cardiac myopathies, etc., 6) Patients with endocrine disorders like thyrotoxicosis, 7) Patients with neurological or psychiatric disorders, 8) Patients who had participated in athletics/sports activity or routinely following yogic exercises.

A complete general physical and detailed systemic examination was done on each patient to rule out any other major systemic illness. Heart rate was recorded with the help of a stethoscope placed on anterior left chest wall (precordium) to count heart beats for complete one minute and blood pressure were recorded with the help of a mercury sphygmomanometer using palpatory and auscultatory method.

The TMT was done in Department of Cardiology, G. B. Pant Hospital, New Delhi. The test was done using a computerized machine (manufacturer: Mortara Xscribe) with built-in Bruce protocols. The tests were done by a medical officer in collaboration with a cardiologist. Male patients were instructed to come chest clean shaven and female patients were instructed to come with proper inner wears. All of them were asked to accompany a relative to take care in case of any unforeseen problem. Graded metabolic workloads were administered for fixed intervals of 3 minutes. Continuous ECG recordings were made throughout the test. Blood pressure was recorded at the end of each stage through automatic blood pressure recording machine. Patients following the conventional Bruce treadmill protocol started the test at the speed of 1.7 mph, with an elevation of 10% grade (for 3 min) and continued in 3-minute intervals (i.e., at 2.5 mph, 12% grade; at 3.4 mph, 14% grade; at 4.2 mph, 16% grade; 5.0 mph, 18% grade; and 5.5 mph, 20% grade, respectively). The test was discontinued in the event of limiting symptoms like pain in leg, breathlessness, fatigue, angina, abnormalities of rhythm or blood pressure, or marked and progressive ST-segment deviation (more than 1mm from the baseline) or when the target heart rate (THR) was achieved. THR was calculated by subtracting the patients age from 220, 85% of this value was THR. Metabolic equivalents (METs), a measure of energy expenditure, were automatically calculated by the testing device during the exercise testing (1 MET equals approximately 3.5 mL of oxygen consumed per minute per kilogram of body weight). After the test, patients were allowed to recover for about 3 to 5 minutes till his/her heart rate reached the baseline value. Continuous ECG recording was taken till the patient recovers.

Protocol for Meditation in Meditation group: Patients were called in group of 10 twice a week (Monday and Thursday) at 9 AM in the Department of Cardiology, G. B. Pant Hospital. They were instructed to come empty stomach, wearing clean, simple and loose clothing. They were made to sit comfortably on the floor and allowed to relax for about five minutes. This was to allay any apprehension associated with the class. To ensure free and fresh ventilation all the windows of the room were opened. The room's ambient temperature was maintained on all days between 16ºC-20ºC. The room was clean, noise-free and dim lighted. Meditation technique was demonstrated each day for first few days until they had learned the technique perfectly; subsequently they followed the procedure themselves. Special emphasis was laid on breathing technique practiced

by each patient individually and the same was checked on each subsequent visit.

Meditation technique

Concentration on body : Sitting relaxed on the floor, patients were asked to focus attention on their body. Asked to put their attention at the area of forehead, and just sweep the body, feeling every part of body sensations, tensions. If they felt any tensions in their body, they were asked to just be aware of those tensions; don't try to resist or control those tensions and continue sweeping the body.

Concentration on breathing : Patients were taught to allow their body to breathe naturally, and focus their attention wherever they feel the sensation of the breath in the body. While inhaling, be aware, be conscious of inhaling; when exhaling, be aware, and be conscious of exhaling. Be with this movement of the breath; just come back to it as an anchor.

Distress to de-stress : Patients were taught that whenever they experience tension, stress or anxiety, try to focus their attention on other things: perhaps the sounds they hear, the sensations in their body, the touch of their clothing, movements in their body, their heart beating, or the rise and fall of the abdomen during breathing. They were made to learn to be aware of other things that are happening while they are experiencing stress.

Forgiveness

Patients were taught to gently soften their thought towards themselves, accept themselves as they are, without any notion of what they should become. Making friends with whom they are - and really feel that friendship, that kindness. Then only they can extend that friendship, gentleness, softness even to those who have hurt, disappointed or frustrated them. Letting go of the hurts and wounds they have been carrying by learning to forgive, by learning to accept the common humanness.

To ensure whether patients were doing meditation properly or not heart rate and blood pressure were recorded before (after 5 minutes of rest) and after doing meditation. Patients in meditation group were asked to maintain a Record Diary in which they entered days on which they did meditation and for how long. To ensure their compliance to program at home, they were subjected to stress management intake questionnaire. Any patient found not following instructions properly or doing meditation for less than 5 times in a week was not included in the study.

Follow-up: 1) All the patients were directed to fill up the requisite information with respect to the medication prescribed routinely as per performa given, 2) In the meditation group of patient they were instructed to routinely follow up the meditation process and to make the entries in the record diary, 3) Each patient in control group was instructed to report for follow up regularly at an interval of 15 days, 4) Each patient was instructed to immediately contact the investigator in case of any problem, 5) At the end of 6 months physiological parameters were studied in both the group of patient.

Results

Name of the software used for statistical analysis is IBM SPSS Statistics Data Editor. The data was normally distributed. Name of the test used is student's t-test. Physical characteristics of two groups of patients are shown in Table I. It can be seen that the physical characteristics in the two groups of the patients showed no statistical difference in the age, height, weight and body surface area. Hence the two groups are statistically comparable

TABLE I: Mean±SD of baseline value of anthropometry in the two groups of patients.

		Mean	*Std. Deviation*	*p value (Gr. I compared with Gr. II)*
Age (years)	Group I	53.9	9.8	0.328 (NS)
	Group II	56.2	7.2	
Height (cms)	Group I	165.5	4.9	0.690 (NS)
	Group II	166.1	5.9	
Weight (kgs)	Group I	69.5	6.2	0.173 (NS)
	Group II	67.2	6.3	
BSA (m^2)	Group I	1.8	0.1	0.345 (NS)
	Group II	1.7	0.1	

to assess the effect of meditation on CAD patients.

A thorough clinical examination was done in each of the two groups of patients. The data obtained from them is presented in Table II. Mean±SD value of HR, SBP and DBP were obtained and the data suggested that the patients in the two groups can be presumed to be normally distributed.

Intra-group comparison of physiological parameters before and after study in the two groups of patients

Mean±SD value of physiological parameters viz. heart rate and blood pressure were measured in the two groups of patients before and after study and the values are given in Table II and III. Mean±SD of heart rate, systolic and diastolic blood pressure in group I patients were found to be higher before the study as compared to that of after the study and the difference was statistically highly significant.

On the other hand Mean±SD of heart rate, systolic and diastolic blood pressure in group II patients showed minor differences before and after the study and were statistically insignificant (Table IV).

Metabolic equivalents (METs) were automatically calculated by the Treadmill during the exercise testing in both the groups of patients before and after study. Mean±SD value of METs in group I patients before and after study was 10.1±1.8 and 10.0±2.0 respectively. Mean±SD value of METs in group II patients before and after study was 9.7±1.7 and 9.6±2.0 respectively (Table V). Although the value of METs decreased in both the group of the patients at the end of the study but its statistical significance was not established (p>0.05).

TABLE II : Mean±SD of baseline physiological parameters in the two groups of the patients. (n=30 for each group).

		Mean	*Std. Deviation*	*p value (Gr. I compared with Gr. II)*
Heart rate at rest (bpm)	Group I	72.2	4.6	0.600 (NS)
	Group II	71.6	4.2	
SBP (mmHg)	Group I	139.9	12.4	0.729 (NS)
	Group II	140.9	11.3	
DBP (mmHg)	Group I	81.9	9.4	0.390 (NS)
	Group II	83.9	7.8	

p value: >0.05 not significant (NS); <0.05 significant (S); <0.01 highly significant (HS); SBP=systolic blood pressure; DBP=diastolic blood pressure; bpm=beats per minute; mmHg=millimetre of mercury.

TABLE III : Mean±SD values of physiological parameters in group I patients before and after study (n=30).

Parameters	*Before study*	*After study*	*p value*
Heart rate (bpm)	72.2±4.6	68.0±4.4	0.000 (HS)
SBP (mmHg)	139.8±12.4	130.5±10.2	0.000 (HS)
DBP (mmHg)	81.9±9.4	76.4±9.2	0.000 (HS)

p value: >0.05 not significant (NS); <0.05 significant (S); <0.01 highly significant (HS); SBP=systolic blood pressure; DBP=diastolic blood pressure; bpm=beats per minute; mmHg=millimetre of mercury.

TABLE IV : Mean±SD values of physiological parameters in group II patients before and after study (n=30).

Parameters	*Before study*	*After study*	*p value*
Heart rate (bpm)	71.6±4.2	72.9±3.9	0.018 (S)
SBP (mmHg)	140.9±11.3	138.9±9.9	0.077
DBP (mmHg)	83.8±7.8	84.5±6.7	0.460

p value: >0.05 not significant (NS); <0.05 significant (S); <0.01 highly significant (HS); SBP=systolic blood pressure; DBP=diastolic blood pressure; bpm=beats per minute; mmHg=millimetre of mercury.

TABLE V : Mean±SD value of METs in both the group of patients before and after study (n=30 for each group).

Parameter	*Group*	*Before study*	*After study*	*p value*
METs	I	10.1±1.8	10.0±2.0	0.099 (NS)
METs	II	9.7±1.7	9.6±2.0	0.127 (NS)

p value: >0.05 not significant (NS); <0.05 significant (S); <0.01 highly significant (HS); METs=Metabolic equivalents.

Discussion

Coronary artery disease (CAD) remains one of the major causes of morbidity and mortality in India. A number of risk factors have been identified to be strongly associated with CAD, stress and behaviour patterns are one of them. Hence the present study was chosen to study the effect of stress relieving technique i.e., meditation on physiological parameters

on coronary artery disease patients.

Meditation has a number of positive effects on the physiology of the human body. It has been shown to reduce the heart rate, blood pressure (21, 22, 23) and thus, the practice of meditation significantly helps in the management and the prevention of CAD by reducing the risk factors which are associated with the same.

In our study, we found that resting heart rate in the patients practicing meditation for a period of 6 months was decreased significantly as compared to those in the non-meditating subjects. The statistical analysis showed that the differences were highly significant ($p<0.01$). In a study which was conducted in 1984 on 25 rajyogis, both males and females, by the medical wing of Rajyoga Education and Research Foundation, an overall decrease in the mean values of the heart rate, systolic and diastolic blood pressure was observed (21). Meditation is associated with a blunted sympathetic activity as is shown by a reduction in the heart rate after regular meditation for a period of 6 months. Similar trends in the heart rate were noted in other studies (22, 23). The Mean±SD value for systolic blood pressure in the meditators was 130.5±10.2 and in the non-meditators, it was 138.9±9.9. Similarly, the Mean±SD value for diastolic blood pressure in the meditator subjects was 76.4±9.2 and in the non-meditator subjects, it was 84.5±6.8. Statistically, these results were found to be significant ($p<0.001$).

In other studies, there was a significant reduction in the systolic and diastolic blood pressure, serum cholesterol and the incidence of ischaemic heart disease in the meditators (24, 25). It was reported that not only in hypertensive individuals, but in normotensive individuals also, the regular practice of meditation could reduce the ambulatory blood pressure levels and hence, it could give significant protection from cardiovascular diseases (26).

Improvements in the cardiovascular parameters in the present study were similar to those which were found in other studies on meditation (27-30). The decrease in the diastolic and systolic blood pressure and the heart rate may be because of the activation of the parasympathetic state (31). Meditation, by modifying the state of anxiety, reduces the stress induced sympathetic over-activity, resulting in lowering of the diastolic blood pressure and the heart rate. It makes the person relaxed and thus decreases the arterial tone and the peripheral resistance (21, 32). This could be another reason for the fall in the diastolic blood pressure. Environmental conditions and a variety of behavioral factors such as stress, anxiety and the affective and attitudinal disposition of the individual influences the cardiovascular responses. Meditation affects the hypothalamus and brings about a decrease in the diastolic and systolic blood pressures through its influence on the vasomotor centre, which reduces the sympathetic tone and the peripheral resistance (32).

Prior studies of meditation and blood pressure have been criticized because of the quality of the trials (33, 34), potential side effects of meditation (34), and potential bias of investigators (33, 34). Different studies stated that the benefits of meditation and relaxation could only be maintained by the regular practice and integration of these techniques in the day to day life (35). Also, the amount of the practice of meditation does not correlate with the amount of blood pressure reduction after the training. Regular meditation is required to maintain positive effects on the blood pressure and the heart rate (36) for at least 60 minutes/day.

Conclusion

In the present study, it was concluded that there is significant decrease in the heart rate and systolic and diastolic blood pressure in CAD patients practicing meditation for a period of 6 months. Our findings are also in compliance with the study conducted by Lang et al. (37) and Wenneberg et al. (38) on the effect of Meditation on heart rate and blood pressure by regulating sympathetic activity. The results of the present study demonstrate that practicing meditation reduces heart rate and blood pressuren in CAD patients and that change were more marked in yoga group. It can be concluded from the present study that meditation helps in improving health status of an individual including heart rate and blood pressure. Furthermore, the practice should be adopted and continued for a long duration.

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Original Article

Gemcitabine Sensitivity Evaluation in Pancreatic Ductal Adenocarcinoma Following Inhibition of ABCG2 and Wnt/β-Catenin Pathway

Somayeh Layeghi, Mehdi Fazeli*, Jafar Jalaei and Maryam Dehghani

Department of Pharmacology and Toxicology,
School of Veterinary Medicine,
Shiraz University, Shiraz, Iran

Abstract

Purpose: Pancreatic cancer is one of the most lethal malignancies worldwide. Gemcitabine based therapy is currently the first-line treatment and usually fails to treat the patients with advanced pancreatic ductal adenocarcinoma (PDA) because of rapid acquisition of resistance to chemotherapy. Thus, identification of agents that re-sensitize gemcitabine-resistance pancreatic cancer cells to gemcitabine and exploring more about molecular mechanisms of gemcitabine resistance are essential to develop new therapeutic approaches for pancreatic cancer.

Methods: In the present study, we report that FTC (an ABCG2 inhibitor) and IWR-1 endo (a Wnt/β-catenin signaling inhibitor) combination re-sensitize resistant AsPC-1 cells to gemcitabine as shown by MTT and western blot analysis. *Main findings:* IWR-1 and FTC co-incubation with different concentrations of gemcitabine (0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.28, 2.56 µmol) for 72 hours showed significant cell death effects, in which gemcitabine IC50 after IWR-1 endo or FTC added on AsPC-1GR (gemcitabine-resistance) cells were 1.35 and 1.58 (µmol), respectively, while cell's viability remarkably reduced following co-treatment of IWR-1endo plus FTC with gemcitabine (gemcitabine IC50=0.1 µmol) ($p \leq 0.05$).

Principal conclusion: Results of the current study suggest that gemcitabine co-treatment with combination of FTC and IWR-1 endo, could represent a novel therapeutic strategy for pancreatic cancer patients with gemcitabine resistant characterization.

Introduction

Pancreatic Ductal Adenocarcinoma (PDAC), is one of the most deadliest cancers, mainly occurs in elderly people (1). The annual statistic in the USA recently announced PDA as the fourth leading cause of cancer-related deaths in the world (2). In many of non-metastatic pancreatic cancer patients, nonsurgical therapy is not currently curative, and relapse after surgery commonly occurs (3). This is the result of intrinsic resistance to cytotoxic agents, knowing as the main characterization of pancreatic cancer (4). MDR tumors (Multi Drug Resistance) are the major barriers to achieve optimal treatment and suggested to be fundamental contributors to death

***Corresponding author :**
Mehdi Fazeli, Department of Pharmacology and Toxicology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran; Email: mfazeli@shirazu.ac.ir

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by pancreatic cancer. The high priority of cancers with MDR characterization is understanding the molecular basis and developing strategies or clinical reagents to treat resistant tumors or prevent the occurrence of resistance (5). Because the resistance issue in PDAC has not been properly addressed, identifying responsible biomarkers for therapeutic resistance is crucial in PDAC patients (6). One of the main transporter protein involved in multidrug resistance is human ATP-binding cassette subfamily G member 2 (ABCG2) protein, a semi-membrane protein, which originally isolated from breast cancer cells. ABCG2 together with multidrug resistance protein (MRP) and P-glycoprotein belong to the ABC membrane transport proteins superfamily (7). In tumor cells, overexpression of ABCG2 is tightly associated with multidrug resistance. Indeed, high expression of ABCG2 has been indicated in many cancers like pancreatic cancer (8). ABCG2 overexpression in these tumor tissues is often accompanied by fast progression of malignancy, unsuccessful chemotherapy and poor prognosis (9). Another critical regulator of chemo-resistance in many tumors is Canonical Wnt signaling, mediated through â-catenin. Inhibition of Wnt/â-catenin signaling has been reported to increase chemotherapeutic drugs sensitivity in cancers (10). Also, the crucial role of dysregulation of Wnt/β-catenin signaling has been found in many recent pancreatic cancer chemo resistance studies (4). Since the sole approach treatment did not achieve absolute satisfaction, combination therapy concerned as a new and effective solution in cancer treatment in many investigations (11). The present study was designed to characterize the effect of combined Wnt/β-catenin and ABCG2 inhibitors on PDAC cells to re-sensitize resistant-gemcitabine cells to gemcitabine.

Materials and Methods

Cell culture and cell line

Gemcitabine resistant AsPC-1 cells were selected from the PDA parental cell line grown in graduated concentrations (0.01 µmol to 1.0 µmol) of 4-hydroxy-Gemcitabine (Sigma Aldrich, Castle Hill, NSW, Australia) over six months (12). Cells were grown in RPMI 1640, supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin in a humidified incubator at 37°C and 5% CO2. For maintaining, cells were passaged two times a week before reaching complete confluence; and were seeded into 96 well plates with 4000 cells/well; optimal cell confluence was based on doubling time.

Proliferation assay

Cell viability was measured using MTT Cell Proliferation Kit I (Roche, Basel, Switzerland) following the manufacturer's instructions. Briefly, AsPC-1 and AsPC-1GR cells were seeded into a 96-well plate with a concentration of 4000 cells in each well. After treatment with different drug concentrations for the final 72 hours, all cells were incubated with MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in a humidified atmosphere of 5% CO2 at 37°C for 4 hours. The absorbance was subsequently measured at 572 nm on a micro plate reader (SpectraMax M2), readouts averaged and adjusted accordingly.

Western blot analysis

The cells protein profiles were determined using western blot analysis. Cells were collected and washed three times with PBS (Phosphate Buffered Saline). Then, in the fresh RIPA protein lysis buffer (a lysis buffer used to lyse cells and tissue, for radio immune precipitation assay) containing 1% PMSF (phenyl methanesulfonyl fluoride), the cells were lysed on ice. BCA assay (bicinchoninic acid assay**)** was employed for the total protein concentration determination. After separation by SDS–PAGE electrophoresis, the samples were transferred to PVDF membrane (polyvinylidenedifluoride). Ponceau S staining was used to visualize protein blots. ABCG2 and Wnt/ â catenin primary antibodies were added for an overnight incubation at 4°C. The membrane was then incubated with the secondary antibody (1:10,000) at room temperature for 1 h and washed three times with TBST buffer. ECL chemi-luminescence reagent and Bio-Rad exposure apparatus were used for exposure (13). Membrane bound secondary antibodies were visualized by enhanced chemiluminescence (ECL) detection kits (Santa Cruz Biotechnology). Mouse monoclonal antibody b-actin (TA-09) (Zhongshan Goldenbridge

Biotechnology, diluted 1:2000) was used as a total protein internal control.

Statistical analysis

Viability values were converted to log 10 scale prior to statistical analysis. Graphsand their statistical comparisons were done using GraphPad Prism software with unpaired and two-tailed Student's t-test ($p \leq 0.05$).

Results

Development of gemcitabine-resistant cell line to create stable resistant pancreatic cancer cell

AsPC-1 cells were exposed to increase the concentrations of gemcitabine. Specifically, cells in log phase were first exposed to 10 n mol gemcitabine, which resulted in greater than 95% cell death. Once surviving cells reached 80% confluence, they were passaged twice in the same concentration of gemcitabine, the process was then repeated with intermittent increase in the doses of gemcitabine until the selected cell population demonstrated 50 folds greater IC 50 to gemcitabine. Resultant AsPC-1 cells were resistant to 1000 n mol gemcitabine. The resulting cell line was designated AsPC-1 GR. The gemcitabine-resistant phenotype has been stable over 20 passages during experiments.

Gemcitabine Effect on cell viability

MTT assay was performed on AsPC-1 and AsPC-1GR cells. Cells were exposed to different concentrations of gemcitabine (0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.28, 2.56 µmol) for 72 h. The gemcitabine IC50 for AsPC-1 and AsPC-1GR cells were 0.43 and 2.37µmol, respectively (Fig. 1A). Resistant cells morphology was different from their parent as shown

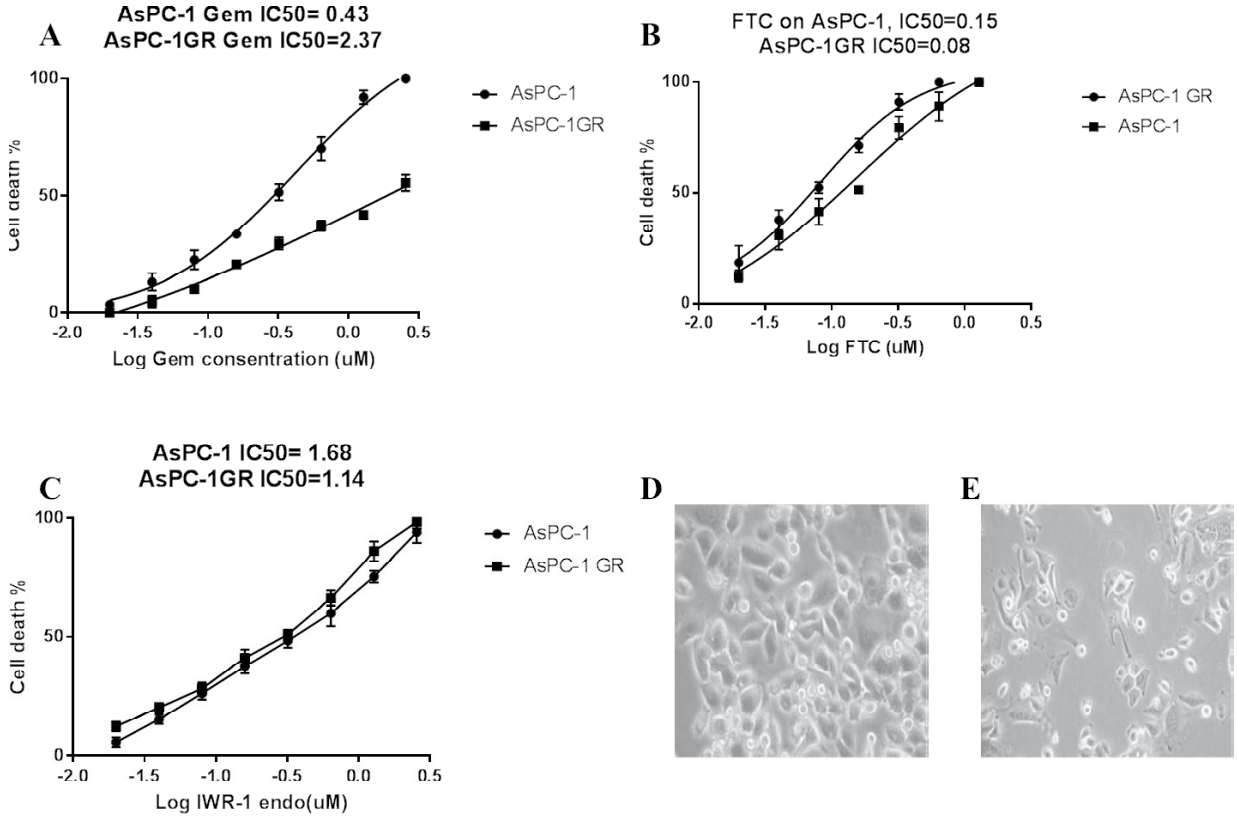

Fig. 1 : MTT proliferation assay on normal and resistant AsPC-1 cells. A: Viability of parental AsPC-1 and AsPC-1 GR (gemcitabine resistant) cells treated with increasing doses of Gemcitabine (0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.28, 2.56 µmol), cell media was used as control.The IC50 of FTC (C) and IWR-1 endo (B) was measured on AsPC-1 and AsPC-1GR cells. D: AsPC-1 parenteral and E: AsPC-1 GR cells morphology. The values shown are the Mean±Standard deviation (S.D.) of three different experiments measured simultaneously. The measured IC 50 is based on (µmol) and the P value was calculated using two-tailed Student's t-test ($p \leq 0.05$) (A, B and C).

in Fig. 1D and E; the resistant cells were more rounds, larger, flatter and exhibited more mesenchymal phenotype than the parental cell line AsPC-1. Fumitremorgin C (FTC, an ABCG2 inhibitor) and IWR-1 endo (a Wnt/β-cateninin inhibitor) (Sigma Aldrich, Castle Hill, NSW, Australia) were treated with resistant and non-resistant cells to evaluate the cell-death effect. There were not significant differences between their IC50 in normal or resistant cancer cells. As shown in Fig. 1, the IC50 for FTC and IWR-1 endo in AsPC-1 cells determined as 1.68 and 0.15 µmol respectively, while their IC50 on AsPC-1GR for FTC was 1.14 µmol (Fig. 1C) and for IWR-1 endo was 0.08 µmol (Fig. 1B). These results showed that the inhibitors alone could not increase cell death in resistant cells as much as when they co-treated with gemcitabine.

Western blotting of β-catenin and ABCG2 protein expressions

Western blotting was performed to determine relative β-catenin and ABCG2 protein expressions in both cell lines. As expected, β-catenin and β-actin were detected as single bands at 92 and 42KDa, respectively. ABCG2 is a 72 KD as it shown in Fig. 3. The β-catenin and ABCG2 expressions were higher in AsPC-1 GR than those in normal AsPC-1cells. As it shown in Fig. 2, gemcitabine separately co-treatment with FTC or IWR-1 endo, decreased ABCG2 and β-catenin expression, however their all combination treatment on resistant cells decreased both protein expression significantly (Fig. 2B).

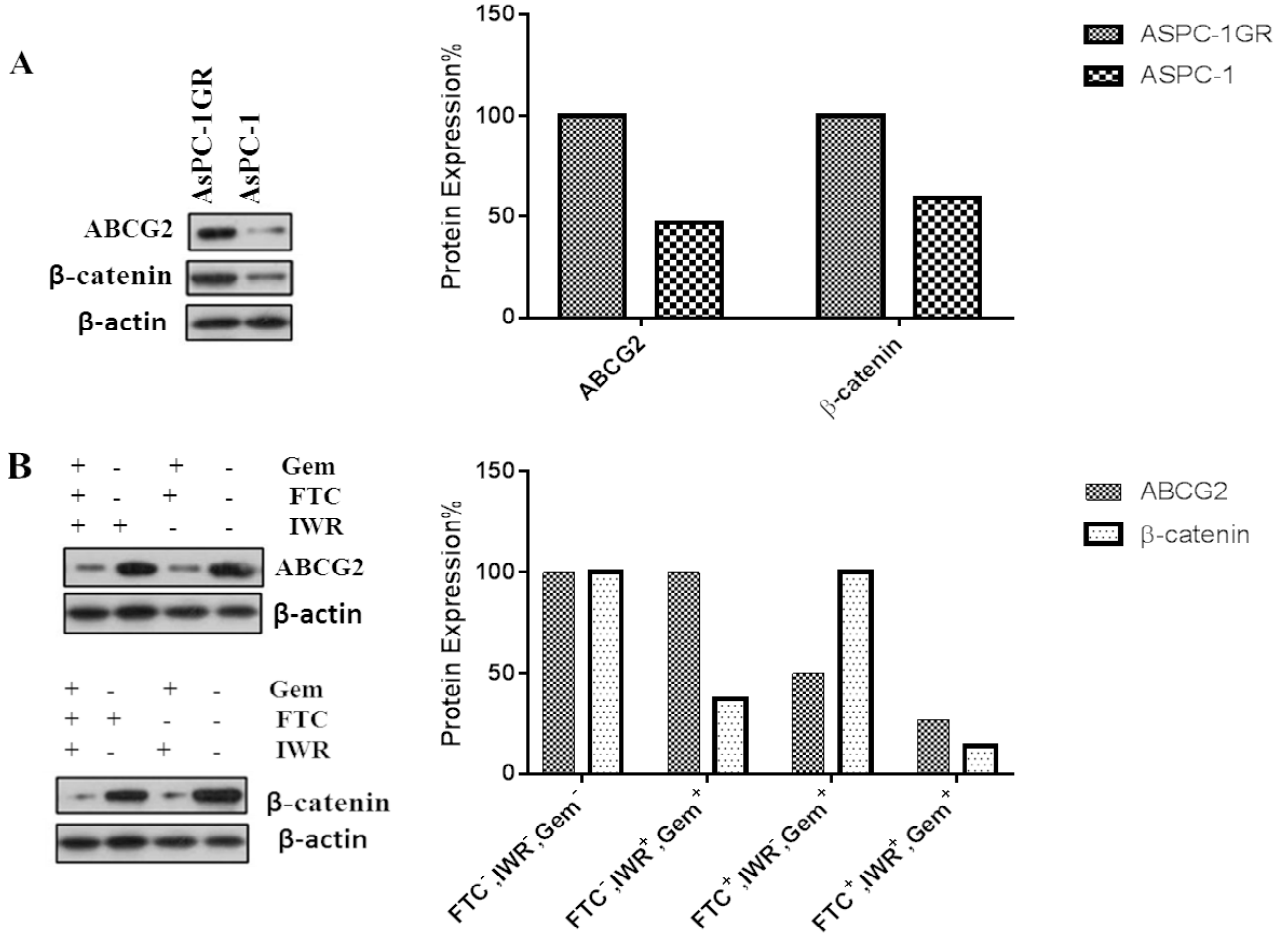

Fig. 2 : α-catenin and ABCG2 protein expression by western blot analysis in AsPC-1 and AsPC-1GR cells. A: β-catenin and ABCG2 protein expressions were significantly higher in resistant cell line than those in AsPC-1 cells. B: β-catenin and ABCG2 protein expressions were decreased after IWR-1 and FTC added in response to their inhibition effect in gemcitabine-resistant cells. The combination of inhibitors and gemcitabine effects were notable on ABCG2 and β-catenin expression in resistant cells. All graph's results normalized with β-actin as control.

ABCG2 and Wnt/β-catenin inhibitors alone and their combination significantly re-sensitize gemcitabine-resistant pancreatic cancer cells to gemcitabine.

Treatment with 1 µmol of gemcitabine induced death in ~ 70% of AsPC-1 cells; it also reduced cell viability in AsPC-1 GR cells (Fig. 1) however, it was remarkably lower than normal cancer cells. AsPC-1 GR cells were treated with different concentrations of gemcitabine (0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.28, 2.56 µmol) and co-incubated with ABCG2 inhibitor, Wnt/β-catenin inhibitor and these inhibitors combination. The results confirmed that ABCG2 and Wnt/â-catenin inhibitors alone stimulated gemcitabine-induced cell death in AsPC-1 GR cells. Gemcitabine IC50 after 72 h was 1.58 µmol and 1.35 µmol in order following addition of FTC and IWR-1 endo. These inhibitors combination co-incubation with gemcitabine resulted in further increase cell death due to gemcitabine re-sensitization in resistant cells, which was confirmed by gemcitabine IC50=0.14 µmol (Fig. 3). As it was shown in Fig. 2 B and C, these inhibitors had not significant effect on cell death without gemcitabine, while their combination with gemcitabine improved gemcitabine cell-death effect. These results indicated that ABCG2 and Wnt/β-catenin inhibitors alone and their combination notably inhibit the activated signaling pathway and specific proteins with crucial role in the gemcitabine-resistant cells.

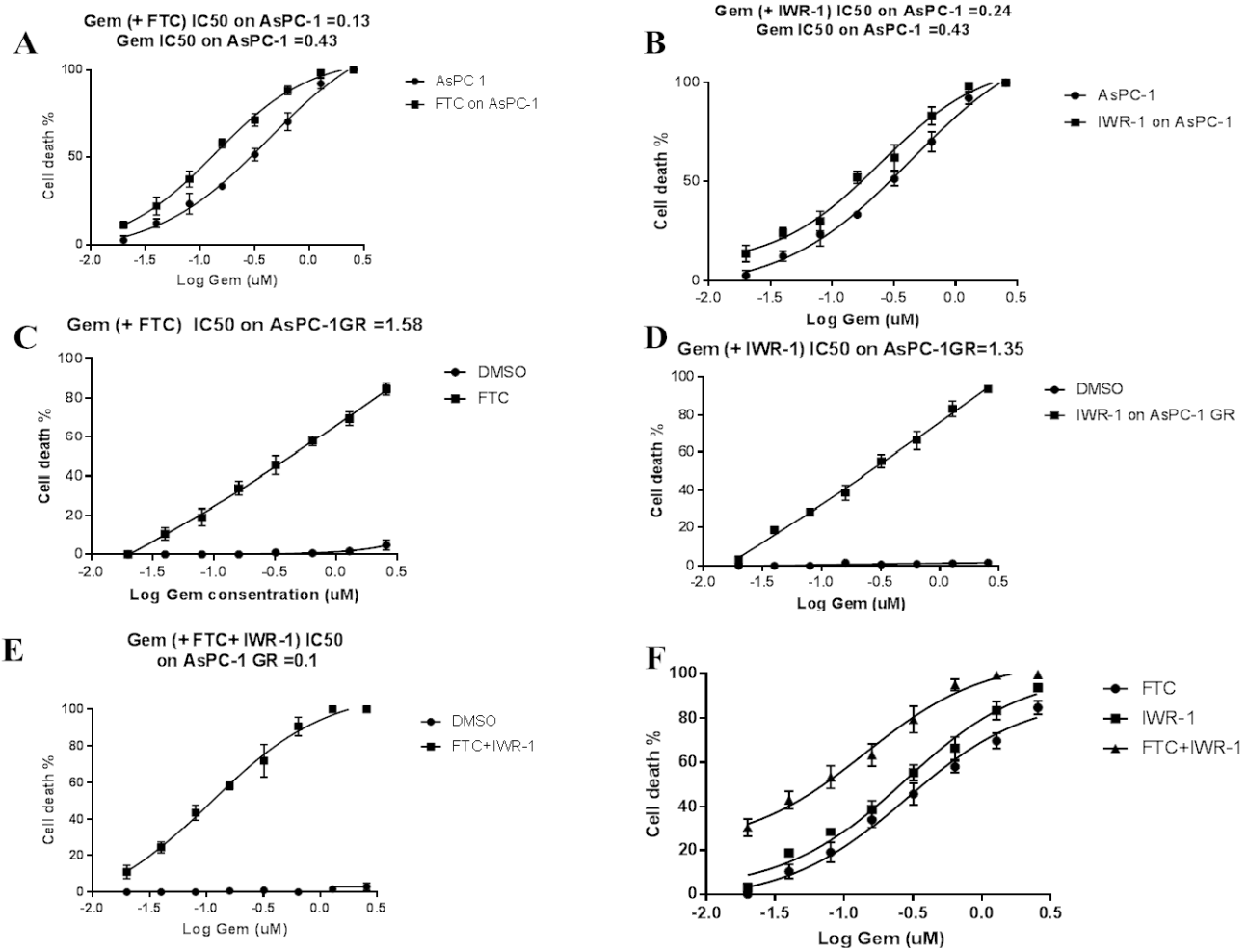

Fig. 3 : The inhibitory effects of Wnt inhibitor (IWR-1 endo) and ABCG2 inhibitor (FTC) on proliferation of AsPC-1 GR cells. A: Gemcitabine IC50 on AsPC-1 cells with and without FTC, B: Gemcitabine 1C50 on AsPC-1 cells with and without IWR-1 endo; C: Gemcitabine 1C50 on AsPC-1GR cells co-treated with FTC, D: Gemcitabine 1C50 on AsPC-1GR cells co-treated with IWR-1 endo . E: Gemcitabine IC50 on AsPC-1GR cells co-treated with IWR-1 endo and FTC combination, and F: Gemcitabine IC50 on AsPC-1GR cells co-treated with FTC alone, IWR-1 endo alone and their combination in comparison. The measured IC 50 is based on (µmol) and the P value was calculated using two-tailed Student's t-test ($p \leq 0.05$).

Discussion

Although gemcitabine based treatment is the main approach for pancreatic ductal adenocarcinoma, survival remains poor because of resistance acquisition to gemcitabine in pancreatic cancer cells. Combination therapy is one of the best ways to over drug resistance. In this survey for the first time we identified significant effect of combining ABCG2 and Wnt/β catenin pathway inhibitors on AsPC-1 gemcitabine resistant cell line *in vitro*. This is the first report to show that FTC and IWR-1 endo combination re-sensitizes gemcitabine-resistant cultured pancreatic cancer cells to gemcitabine-induced cell death. Using gemcitabine-resistant cells is an important aspect of this study, which was established by gradually increasing exposure to gemcitabine, for drug screening and the following analysis. Cultured resistant cell line have been mainly used for such screening before (14). Resistant cell analyzing has provided new insights into finding new treatments for pancreatic cancer (15). Similarly, our screening method found that FTC and IWR-1 endo combination has high potential as a useful biochemical tool to achieve better cancer treatment results. Several studies showed effective pancreatic cancer treatment by combination therapy; for example positive combination effects of sorafenib which is a multikinase inhibitor and an isothiocyanate found in broccoli, named sulforaphane, reduced pancreatic cancer cells drug resistance (16). Moreover, significant increased sensitivity of pancreatic cancer cells to different chemotherapeutic drugs including gemcitabine with sulforaphane found by Kallifatidis (17). Zhou et al, showed synergistic effects of sulforaphane and quercetin that target pancreatic cancer stem cells, which their role in pancreatic cancer drug resistance have been proved in many studies (18). In the present study, treatment of AsPC-1GR cells with IWR-1 endo resulted in pronounced inhibition of Wnt/ â catenin pathway due to higher activity of this pathway in resistant cells (4). Since pancreatic cancer cell re-sensitization to gemcitabine induced by IWR-1 endo and gemcitabine co-incubation in AsPC-1GR cells was not remarkable, it was assumed that inhibition of different ways which are involve in pancreatic cells drug resistance may give more significant and decisive results. In this regard, FTC an inhibitor of ABCG2 was added to complex of IWR-1 endo and gemcitabine. Different gemcitabine concentrations (0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.28, 2.56 µmol) co-incubated with IWR-1 and FTC. The gemcitabine IC50 in AsPC-1GR cells were 1.35 and 1.58 µmol, respectively, while gemcitabine IC50 following addition of IWR-1 endo + FTC combination on these cells was 0.14 µmol. This means that combinatory effects of these inhibitors in re-sensitizing pancreatic cancer cells to gemcitabine were several folds more than FTC and IWR-1 endo alone co-treatment with gemcitabine. The main aspect of present study is demonstration of effective re-sensitization of pancreatic tumors cells to gemcitabine with FTC plus IWR-1 endo co-administration with gemcitabine compared to gemcitabine alone or gemcitabine with just one of these inhibitors. Therefore, suggesting that combined treatment may serve as a best choice to target gemcitabine resistance pancreatic cancer cells. ABCG2 overexpression in AsPC-1 cells promotes migration and invasion; however the mechanism by which this transporter becomes overexpressed is not clearly understood (19). Moreover, one of the main characterizations of pancreatic cancer stem cells which have a vital role in tumor resistance is ABCG2 overexpression, which determines stem cell chemosensitivity after its inhibition (20). On the other hand, Wnt/β catenin pathway inhibition could reverse multi-drug resistant in many solid tumors like pancreatic cancer (21). ABC transporters including ABCG2 downregulation by inhibiting Wnt- signaling pathway has been reported in many studies (22-24). In a recent one, a combinatorial treatment (celecoxib and imatinib) re-sensitized K562 imatinib resistant cells through downregulation of several ABC transporters such as MDR1, MRP1-5, ABCA2 and ABCG2 by inhibiting Wnt/β catenin pathway signaling (25). In conclusion, our data demonstrated that FTC, an inhibitor of ABCG2, in combination with IWR-1 endo, a Wnt/β catenin pathway inhibitor, could represent a novel therapeutic strategy for pancreatic cancer patient with gemcitabine resistant characterization.

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Original Article

Impaired Glucose Tolerance Secondary to Partial Intestinal Ischemia is Reversed by Sitagliptin

Mohamed-Ahdy Saad[1], Emad-El-Deen M. El-Henawy[2]*, Adel H. Omar[2], Magda A. Mansour[3], Hatem M. Samy[4], Mahmoud H. El–Odemi[2], Mohamed Abouelkheir[1] and Nagat M. Saeid[5]

Department of Clinical Pharmacology[1],
Faculty of Medicine, Mansoura University

Departments of Pharmacology[2], Histology[3] and Biochemistry[4],
Faculty of Medicine Menoufiya University

Department of Pharmacology[5],
Faculty of Medicine, Tripoli University, Libya

Abstract

While diabetes is a predisposing factor for intestinal ischemia, mesenteric ischemia is associated with impaired glucose tolerance. We evaluated the effect of partial intestinal ischemia on glucose tolerance, GLP-1 and insulin level in forty non-diabetic SD rats. Rats were randomly assigned into 4 groups; SHAM; SHAM + sitagliptin (30 mg/kg/day); intestinal ischemia; and intestinal ischemia + sitagliptin (30 mg/kg/day). The superior mesenteric artery was partially occluded. At the 11th day, glucose tolerance, plasma GLP-1 and insulin levels were measured along with histological changes in the pancreas and insulin-secreting beta cells. Intestinal ischemia resulted in significant impairment of glucose tolerance with reduction of plasma levels of GLP-1 and insulin. Treatment with sitagliptin partially ameliorated these changes. Beta cells in the pancreas were not affected by ischemia. These results suggest that impaired glucose tolerance with intestinal ischemia might be secondary to functional impairment of beta cells secondary to decreased basal GLP-1 secretion.

Introduction

Small intestine is a major source of glucagon-like peptide-1 (GLP-1) (1). GLP-1 has several physiological actions which are considered beneficial in diabetic patients. It is a potent secretagogue for insulin (2). GLP-1 potentiates insulin action and increases insulin sensitivity (3). Moreover, it has been shown that GLP-1 has hemodynamic and cardioprotective capacities (4). The half -life of GLP-1 is only 1-2 minutes due to its rapid metabolism (5) by an enzyme called dipeptidyl peptidase-4 (DPP-4) (6).

The interaction between GLP-1 and diabetes is complex. GLP-1 secretion in response to a

***Corresponding author :**
Emad-El-Deen M. El-Henawy, Department of Pharmacology, Faculty of Medicine Menoufiya University

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standardized meal was found to be significantly decreased in patients with type II diabetes in comparison to glucose tolerant subjects (7). Whether impaired GLP-1 secretion is a primary phenomenon in the pathogenesis of diabetes or a consequence of diabetes itself is yet to be revealed. First-degree relatives of patients with type II diabetes have normal GLP-1 secretion in response to oral glucose challenge; suggesting that the GLP-1 secretion abnormality seen in diabetes may be acquired (8).

As GLP-1 is secreted from the intestine, it would be interesting to know if any changes in the intestinal hemodynamics could affect the secretion or metabolism of this hormone. The aim of the present study was to evaluate the effect of partial intestinal ischemia on glucose tolerance, insulin and GLP-1 levels in non-diabetic rats and whether a DPP-4 inhibitor, namely sitagliptin, could modify these effects.

Methods

The experiments were conducted in accordance with regulations specified by the local ethical committee, Faculty of Medicine Menoufiya University, Egypt.

Animals

Forty adult pathogen-free Sprague Dawley rats (weighing 200-250 g) under similar housing conditions.

Reagents

ELISA kits were used for the assay of Glucagon like peptide-1 (Phoenix Pharmaceuticals, INC., USA) and insulin (Phoenix Pharmaceuticals, INC., USA). Monoclonal antibodies against insulin (Dako, Glostrup, Denmark) were used for immunohistochemical study of beta-cell mass in the tail of pancreases. The anesthetic, ketamine, was provided by Amoun (Egypt) as 10 ml vials (Ketamar) with a concentration of 50 mg ketamine/ml. Sitagliptin was obtained from Merck Sharp & Dohme Corp.

Experimental protocol

The 40 rats were randomly divided into the following groups (n = 10 in each group):

Group (I): SHAM: Animals in this group were subjected to all steps in the surgical induction of intestinal ischemia except narrowing of the superior mesenteric artery (SMA).

Group (II): SHAM and sitagliptin: As group (I) but starting from day 5 after the operation, rats were treated with sitagliptin (30 mg/kg/day) orally for 7 days.

Group (III): Intestinal ischemia: As group (I) but rats were subjected to the partial narrowing of the superior mesenteric artery (SMA).

Group (IV): Intestinal ischemia and sitagliptin: As group (III) but starting from day 5 after the operation, rats were treated with sitagliptin (30 mg/kg/day) orally for 7 days.

Induction of intestinal ischemia

Rats were anesthetized by ketamine (50-100 mg/kg, i.p.) (9). A midline laparotomy was performed and the SMA was partially constricted below the origin of the middle colic artery to avoid affecting the blood supply of the inferior pancreatic-duodenal artery that supplies the pancreas. The SMA was partially occluded by placing a single ligature of 3-0 silk around both the SMA and blunt-tipped needle (using a 22-gauge needle), placed alongside the SMA. The needle was then removed from ligature, creating a callipered constriction of SMA. The procedure resulted in about 30-40% reduction of SMA blood flow as verified by a Doppler flow meter. After that, sterile saline (1 ml) was injected into the peritoneal cavity for rehydration, and the abdomen was closed in layers.

The animals had 4 days for postoperative recovery. At the 11th day, rats were anaesthetized and subjected to intravenous glucose tolerance test (IVGTT) over 50 minutes. By the end of the test,

blood samples from the femoral vein were used to measure plasma levels of GLP-1 and insulin. Animal were then sacrificed and SMA partial narrowing with the absence of gangrenous intestinal loops was then verified. Pancreatic tissue was dissected for histological examination.

Intravenous glucose tolerance test.

Rats were fasting for 16-18 hours (overnight) prior to the glucose tolerance test. The animals had free access to drinking water during the fasting period. Prior to performing the glucose tolerance test, baseline glucose level was recorded for each rat (blood samples were taken from the tail). D-glucose (10 g/dl) was injected in the femoral vein over 1 minute in a dose of 1 mg glucose/gram body weight (2.5 ml in 250-g rat). At 2, 5, 20, and 50 minutes after injection, blood samples were collected from the tail vein (10). Measurement of blood glucose was done by using the blood glucose checker [GLUCOTREND®2 (Roche Group, UK)] and (Accu-Chek® Active) strips (Roche Group, UK).

Histological and immuno-histochemical study of beta-cells

Tissue samples (tail of the pancreas) were dissected, and fixed in 10% formalin for 5 days, then processed as usual to make paraffin blocks. Sections of 5 microns were stained with haematoxylin and eosin (H and E) for routine histological study. Identification of insulin-secreting beta-cells of pancreas was performed by using immuno-stain with anti-insulin monoclonal antibodies as described by the manufacturer.

Statistical analysis

Data is expressed as Mean±SEM. Data was analyzed using SPSS version 16. Variables were tested for normality distribution using Kolmogorov-Smirnov test. Comparison between groups was done by analysis of variance (ANOVA) followed by Tukey test. A *p* value of less than 0.05 was considered statistically significant.

Results

1. IVGTT:

After injection of glucose, the blood glucose level at the 2nd minute showed a significant increase and remained significantly higher at 5th and 20th minutes in comparison to the basal level. With exception of the ischemia only group, the 50th minute measure was not significantly different from the basal level indicating that 50 minutes were enough for the homeostatic mechanisms to readjust the blood glucose level (Table I). Regarding the comparison between groups, partial intestinal ischemia led to significant elevation of basal level of blood glucose as well as all values in the different time points of IVGTT. Treatment with sitagliptin partially ameliorated the changes induced by partial intestinal ischemia (Table I).

2. Insulin and GLP-1 levels:

In comparison to SHAM group, the ischemic group had a significant decrease in both insulin and GLP-1 levels. Again, treatment with sitagliptin partially ameliorated the changes induced by partial intestinal ischemia. Of note, treatment with sitagliptin resulted in elevation of insulin and GLP-1 levels in group II in comparison to SHAM group (Table II).

TABLE I: Effect of superior mesenteric artery ischemia and sitagliptin on intravenous glucose tolerance test [mean±SEM] (n=10).

Group	*Basal blood glucose mg/dl*	*2 min mg/dl*	*5 min mg/dl*	*20 min mg/dl*	*50 min mg/dl*
I (SHAM)	114±1.98	270±2.02*	224±2.33*	142±1.65*	112±1.87
II (SHAM-Sitagliptin)	91±1.89	257±1.56*	210±2.26*	100±1.90*%	94±1.64
III (ischemia)	143±2.38%f&	405±2.81*%f&	331±2.33*%f&	270±1.87*%f&	205±1.83*%f&
IV (ischemia-Sitagliptin)	121±2.05f&†	322±2.37*%f&†	267±1.85*%f&†	131±2.20%f&†	127±1.73%f&†

* = significant difference from basal blood glucose within the same group; % = significant difference from group I; f& = significant difference from group II; † = significant difference from group III (P<0.05).

TABLE II : Effects of intestinal ischemia and sitagliptin on GLP-1 plasma level (pmol/l) and insulin (µ/ml) in different rat groups (mean±SEM).

GLP-1	*Insulin*	*Groups*
I Sham	9±0.6	23.3±1.13
II Sham + sitagliptin	13.22±0.7*	29±1.26*
III Intestinal ischemia	6.33±0.3*%	18.35±0.42*%
IV Intestinal ischemia + sitagliptin	8.76±0.3%f&	25.1±0.77%f&

*Significant difference from group I; % = significant difference from group II; f& = significant difference from group III (P<0.05).

3. Histological changes in the pancrease:

For both SHAM and ischemic groups; the islands of Langerhans were embedded within the acini forming the endocrine part of pancreas**.** The islets contain several cell types and were highly vascularized. They appeared as non-capsulated pale stained area inside the pancreatic lobules (Fig. 1). Insulin secreting beta-cells could be demonstrated in immune-histochemically stained sections with anti- insulin antibodies. They composed the major cell population of the islets that occupied mainly the central zone. The anti-insulin antibody reaction was seen as brown granules occupying the cytoplasm of the beta-cells. Positively stained beta-cells were seen among pancreatic acini. A positively stained immune reaction for insulin was also seen in the endothelial cells lining blood capillaries (Fig. 2). No differences were detected between different groups indicating that SMA partial ischemia did not affect insulin-secreting cells.

Discussion

The relationship between GLP-1 and Type II diabetes is somewhat complex. In general, meal-stimulated GLP-1 secretion in diabetic patients is impaired (7). Studies on first-degree relatives of patients with type II diabetes suggested that the GLP-1 secretion abnormality seen in diabetes may be acquired (8). A yet to be answered question is whether impairment of GLP-1 secretion is a contributing factor in the pathogenesis of diabetes or is just a consequence of diabetes itself.

The most important cause of chronic intestinal ischemia is atherosclerosis (11, 12). An important predisposing factor to such condition is diabetes mellitus (12). As the terminal ileum is an important source of GLP-1 (1), the present study investigated

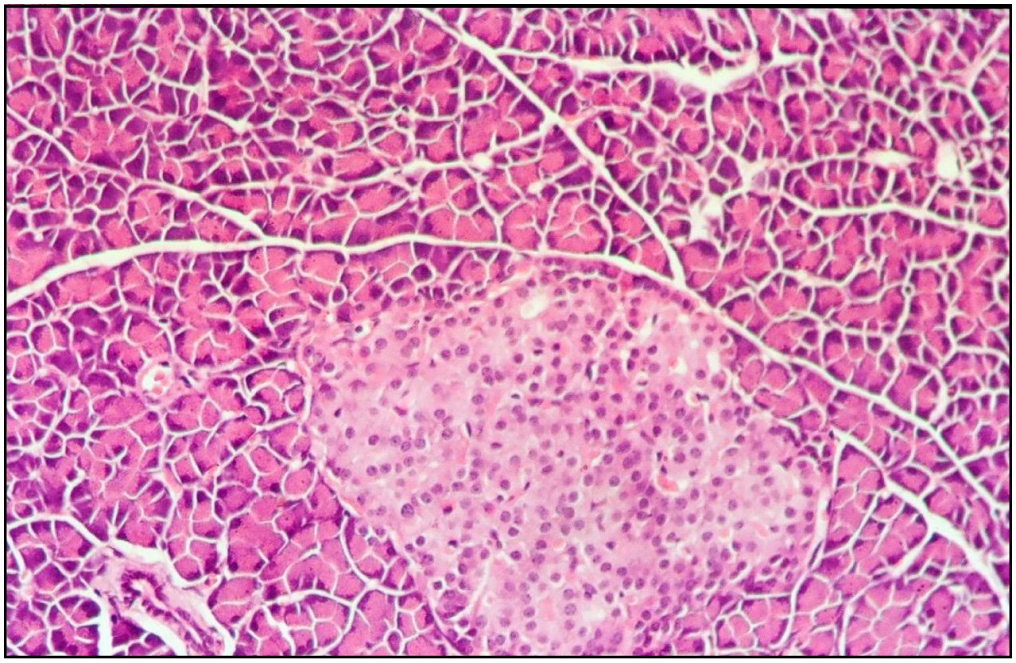

Fig. 1 : A photomicrograph of a section of pancreas from rats subjected to partial occlusion of the superior mesenteric artery (H & E). Lobules packed with acini and an islands of Langerhans situated in the center. Blood vessels and exocrine duct were seen among exocrine acini indicating that ischemia did not influence the pancreas. Magnification: x 200.

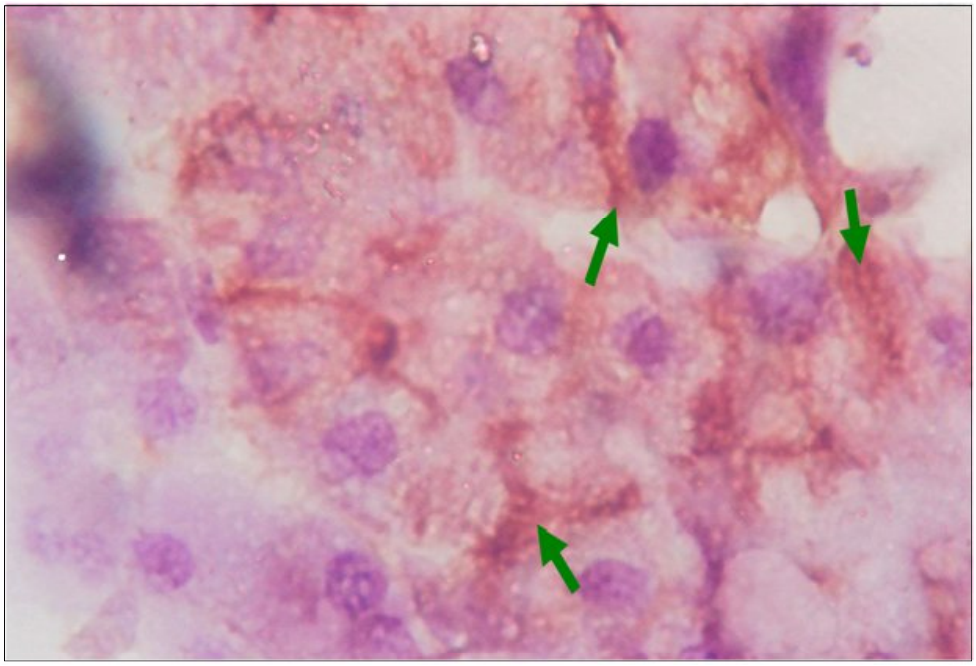

Fig. 2 : A photomicrograph of a section of pancreas from rats subjected to partial occlusion of the superior mesenteric artery stained with anti-insulin antibodies. It indicates that ischemia did not influence beta-cells. Magnification: x 400.

whether intestinal ischemia, by turn, could affect glucose homeostasis. We demonstrated that after 10 days of partial intestinal ischemia, IVGTT was negatively affected.

Abnormal glucose tolerance in case of mesenteric ischemia was reported only once by Sedlack and Bean (13). In a parallel way, resection of 5 cm of the small intestine 5 cm distal to the ligament of Treitz in adult rats resulted in reduced insulin release after IVGTT; an effect unrelated to corticosterone nor IL-6 changes (14). A simple explanation to the report of Sedlack and Bean (13) is that mesenteric ischemia affected the blood flow to the pancreas. To rule this out, we have intentionally constricted SMA below the origin of the middle colic artery to avoid affecting the blood supply of the inferior pancreatic-duodenal artery that supplies the pancreas. In addition, histological and immunohistochemical evaluation of pancreatic tissue of the rats subjected to intestinal ischemia showed no significant differences from the SHAM-operated rats. The present study indicated that beta-cells might be affected at the molecular or functional level rather than being directly injured by mesenteric ischemia. We demonstrated that reduced insulin levels in the ischemic group was secondary to reduced GLP-1 levels and that using a DPP-4 inhibitor partially restored GLP-1 and insulin levels and improved IVGTT. It was reported that, in diabetic patients, defects in the secretion of the incretin hormones are responsible for the reduced incretin effect and hence the inadequate insulin secretion but not responsible for development of diabetes (15).

Of note, it is well-known that GLP-1 is secreted mainly in response meals but not secondary to intravenous glucose (16). Intravenous glucose tolerance test rather than oral glucose tolerance test was done, as absorption of oral glucose from the intestine would be greatly affected by the intestinal ischemia. Nevertheless, it was found that there is a continuous secretion of GLP-1 from the intestine that contributes to measurable basal level of the hormone (5). Although no studies investigated the role of basal GLP-1 in insulin secretion, indirect evidence suggested that the basal hormone levels might be important. Using the GLP-1 analogue, exenatide, in diabetic patients was found to increase the already impaired first-phase insulin secretion (17); an event that occurs too early before the meal stimulates GLP-1 secretion. Such finding may suggest a role of basal GLP-1 in early insulin secretion. In another direction,

GLP-1 might have a direct protective mechanism against islet injury. A DPP-4 inhibitor was reported to suppress STZ-induced islets injury in monkeys; an effect which was dependent on activation of the IGFR/Akt/mTOR signalling pathways by GLP-1 (18). Although the histopathological evaluation did not reveal beta-cell injury that could be attributed to ischemia in this model, affection at the molecular level cannot be excluded. A third possibility that could explain the results of the present study is away from islet affection theory. It was found that GLP-1, through an islet-independent mechanism, can inhibit hepatic glucose production (19, 20). We believe that intestinal ischemia with subsequent impairment of portal drainage in this model might reduce the hepatic action of GLP-1. So, the amelioration of derangement in glucose homeostasis after using sitagliptin might be partially attributed to restoration of GLP-1 in the liver.

Of note, during early development of the model we used acute complete occlusion of the SMA. However, we could not exclude the effect of stress and surgical trauma from affecting glucose tolerance (data not published). Stress stimulates the hypothalamo-pituitary-adrenal axis activating both pituitary adrenocortical and the sympatho-adreno-medullary systems (21). By turn, catecholamines elevate the blood glucose due to stimulation of glycogenolysis and inhibition of insulin secretion (22). Partial ischemia was then used to avoid the effect of stress and as it is also more similar to atherosclerosis; where the blood flow is impaired rather than complete deprivation. One limitation in the present study that should be considered is that part of the L-cells that secret GLP-1 is localized in the rectum (23) and few are localized in the duodenum (1); both are not supplied by the SMA and could escape the effect of ischemia in this model.

Conclusion

In conclusion, chronic intestinal ischemia in rats resulted in significant elevation of basal blood glucose level and impaired IVGTT due to a decrease in basal GLP-1 secretion. Whether such effect of ischemia is a primary contributing factor in the pathophysiology of type II diabetes or just an add-on factor that worsen the disease is a point yet to be investigated.

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Original Article

Sildenafil Attenuates Vincristine-induced Early Onset Tactile Allodynia in Rats

Vikram Patil[1], Rachna Gupta[2] and Lalit Kumar Gupta[1]*

[1]Department of Pharmacology,
Lady Hardinge Medical College,
New Delhi, India 110001

[2]Department of Pharmacology,
University College of Medical Sciences & GTB Hospital,
Delhi, India 110095

Abstract

Cancer chemotherapies are often associated with painful neuropathies due to diverse causes. A deficit in nitric oxide is postulated to be involved in the pathogenesis. The purpose of the study was to evaluate the effect of sildenafil, a PDE-5 inhibitor, on nociception in neuropathy due to vincristine administration in rats.

Neuropathy was produced in male albino rats by vincristine administration. Animals received either of the following treatments on days 10-14: saline, gabapentin, sildenafil and sildenafil+L-NAME. The animals were assessed using 3 models of nociception viz., tactile allodynia using a monofilament, thermal allodynia by hot-plate method and tail flick test.

Significant tactile allodynia developed by day 5. There was no significant change in allodynia in the hot-plate test or tail flick test. Sildenafil (0.5, 1 and 2 mg/kg) showed a dose response effect in attenuating the vincristine-induced allodynia. Sildenafil (1 and 2 mg/kg) and gabapentin (50 mg/kg) attenuated tactile allodynia significantly ($p<0.05$) on all days whereas had no effect in thermal allodynia by hot-plate method and tail-flick test. Addition of L-NAME reversed sildenafil's attenuation of tactile allodynia.

The present study demonstrates the involvement of nitric-oxide in the pathogenesis of vincristine-induced early onset tactile allodynia which can be reversed by sildenafil.

Introduction

Neuropathic pain is a complex condition that may develop when nerve fibres are damaged or dysfunctional. A wide variety of insults including metabolic disorders like diabetes mellitus (DM), traumatic nerve injury, and neurotoxic drugs like vincristine result in peripheral neuropathy associated with loss of sensation and numbness in the feet, hands, and legs accompanied by painful tingling or burning sensation (1, 2). These neuropathies are characterized by hyperexcitability of the nociceptors and changes in central pathways that modulate

***Corresponding author :**
Lalit Kumar Gupta, Department of Pharmacology, Lady Hardinge Medical College, New Delhi – 110 001, India; E-mail address: lkg71@rediffmail.com

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sensory transmission (3-5).

Chemotherapy induced peripheral neuropathy (CIPN) has a high degree of similarity in pattern and spectrum of clinical manifestations caused by various chemotherapeutic agents (e.g. vincristine, platins, thalidomide), which includes length dependent, symmetrical glove and stocking distribution with predominantly sensory symptoms. The pathophysiology is poorly understood but interference with tubulin function and direct damage to sensory nerve cell bodies of dorsal root ganglia has been found (6). Administration of these chemotherapeutic agents induces neuropathic pain behaviour in rats which mimics that seen in patients (7).

Several compounds like nerve growth factor, amifostine, glutathione and glutamate have been tried to prevent the toxicities of chemotherapeutic agents (6). The symptomatic management of neuropathic pain due to chemotherapy mainly relies on the use of antidepressants, anticonvulsants, anaesthetics and opiates. However there is a clear medical need for new treatments to improve safety and efficacy (8).

There are reports suggesting the role of nitric oxide (NO) in pathogenesis of nerve conduction. Impaired NO production has been shown to be associated with the occurrence of various forms of neuropathic symptoms including hyperalgesia and spontaneous pain in animal models of CIPN (9-14). The effects of NO are mediated by cyclic guanosine monophosphate (cGMP), which is degraded by the enzyme phosphodiesterase-5 (PDE-5). Here, we aim to evaluate the role of sildenafil, which inhibits PDE-5 and thus potentiates NO, in alleviation of various parameters of peripheral neuropathy and in comparison to gabapentin in animal model of CIPN caused by vincristine.

Materials and Methods

Animals

Male albino rats weighing around 200-250 g were used. Animals were housed in polypropylene cages under standard laboratory conditions maintained at 25±2°C, humidity 65±5 and light and dark cycle of 12 hours. Animals had a free access to food and water.

The experiments were conducted between 0900-1600 hrs. The procurement, storage and experimentation of animals were done in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India (15) after approval from Institutional Animal Ethics Committee.

Drugs

Vincristine (Cipla, India), Sildenafil (Cipla, India) and Gabapentin (Sun Pharma, India) were purchased from the pharmacy. NG-nitro-l-arginine methyl ester (L-NAME) was purchased from Sigma chemicals (Switzerland). Sildenafil-citrate and gabapentin tablets were finely crushed into powder form. The drugs were then dissolved in 0.9% NaCl (saline) and solution was vigourously shaken using a test-tube shaker in a tube to make it homogeneous, immediately before loading in a syringe. It was administered by intraperitoneal (i.p.) route in a volume of 1ml.

Induction of neuropathic pain with vincristine

Vincristine sulphate was dissolved in 0.9% NaCl and rats received injections at a dose of 200 µg/kg i.v. (1 ml/kg) on days 1, 4 and 6 (cumulative dose 600 µg/kg) (16).

Procedure

The vincristine-treated rats were administered following drugs daily on days 10-14: saline, sildenafil (0.5, 1 and 2 mg/kg), gabapentin 50 mg/kg (17), sildenafil 2 mg/kg + L-NAME 20 mg/kg (18).

L-NAME, a nitric oxide synthase (NOS) inhibitor, was administered 30 min prior to sildenafil. For assessment of parameters, administration of drugs and observations were performed blind by different persons.

Nociceptive tests

Tactile Allodynia

Tactile allodynia was measured by assessing rat hind

paw withdrawal thresholds in response to mechanical stimulation by a modified technique using von Frey filament (INCO). The rats were acclimatized in individual clear Plexiglass boxes on an elevated wire mesh platform to allow access to the plantar surface of the hindpaws. The filament was pressed perpendicularly against the mid-plantar surface of the right hindpaw from below the mesh floor and held for 3-5 s with it slightly buckled. The experiment was started using the greatest length of the filament i.e. 6 cm and gradually the length of the filament was shortened with successive attempts. 3 attempts were done at each length at intervals of 5 s. The length of the filament that caused the animal to flinch or move the paw away from the stimulus 2 out of 3 times was determined to be the mechanical threshold. The shorter the length of the filament, the greater was the mechanical threshold. Testing was performed on days 0, 3, 5, 7, 10, 11, 12, 13, 14.

Thermal allodynia

Thermal allodynia was assessed by hot-plate method. Animals were placed on a metal plate adjusted to 38°C (19) The latency of the first reaction was recorded (licking, moving the paws, little leaps, or a jump to escape the heat, with a cut-off time of 30 s. Testing was done before and 2 hours after administration of drugs on day 0 and days 10-14.

Tail-flick latency

The animals were positioned on a tail-flick analgesiometer (Techno), so that their tail was subjected to radiant heat from a heated filament (20). The latency to the flicking of the tail was recorded. Baseline values were determined on day 0. Testing was done before and 2 hours after administration of drugs on day 0 and days 10-14.

Statistical analysis: All values were expressed as Mean±S.D. Tactile allodynia: In each group, repeated measures ANOVA (RMA) followed by Scheffe's test was used to compare the mean of day 0 values with that of other days. In total, forty-eight pair-wise combinations were tested (eight in each group). One way ANOVA followed by Dunnett's *t*-test (2-sided) was used to compare the mean values for tactile allodynia of different treatment groups from day 10 – day 14.

Data of thermal allodynia and tail flick test (pre and post-treatment) was compared using Student's *t* test; $p<0.05$ was considered statistically significant.

Results

Tactile allodynia

A progressive decrease in the threshold stimulus in comparison to day 0 values was noted in all animals which started building up from day 3 and fully established at day 7 [$F(8,45)= 31.3$ in saline group; 44.9 in gabapentin group; 12.8 in sildenafil 0.5 mg/kg group, 31.9 in sildenafil 1 mg/kg group 27.6 in sildenafil 2 mg/kg group, and 10.9 in sildenafil 2 mg/kg + L-NAME 20 mg/kg group ($p<0.001$ on day 7 in all the groups)]. It remained significantly low in comparison to day 0 values up to 8 days after the last injection of vincristine in vehicle-treated animals (days 7-14; $p<0.001$ for each comparison). Mean threshold stimulus was significantly higher in sildenafil 0.5 mg/kg than saline (control) on day 10 [$F(5,30) = 17.4$ ($p<0.05$ using Dunnett's test)] but not on days 11-14. Sildenafil 1 and 2 mg/kg and gabapentin 50 mg/kg significantly reversed vincristine-induced allodynia 2 h after i.p. injection from day 10 to day 14 compared with vehicle-treated animals {[$F(5,30) = 17.4$(day 10), 18.2 (day 11), 48.3 (day 12), 27.4 (day 13), 17.5 (day 14); ($p<0.001$ for each)]; $p<0.05$ for each using Dunnett's test}. Administration of L-NAME 20 mg/kg before sildenafil (2mg/kg) reversed its attenuation of vincristine-induced tactile allodynia (Fig. 1, 2).

Thermal allodynia

No significant change in threshold to thermal stimulation was noted on days 10-14 following vincristine treatment. None of the drugs-sildenafil (2 mg/kg), gabapentin or sildenafil+L-NAME combination-showed any significant change in threshold to thermal stimulation by the hot plate test or tail-flick test after 2 h of i.p. injection (Table I, II).

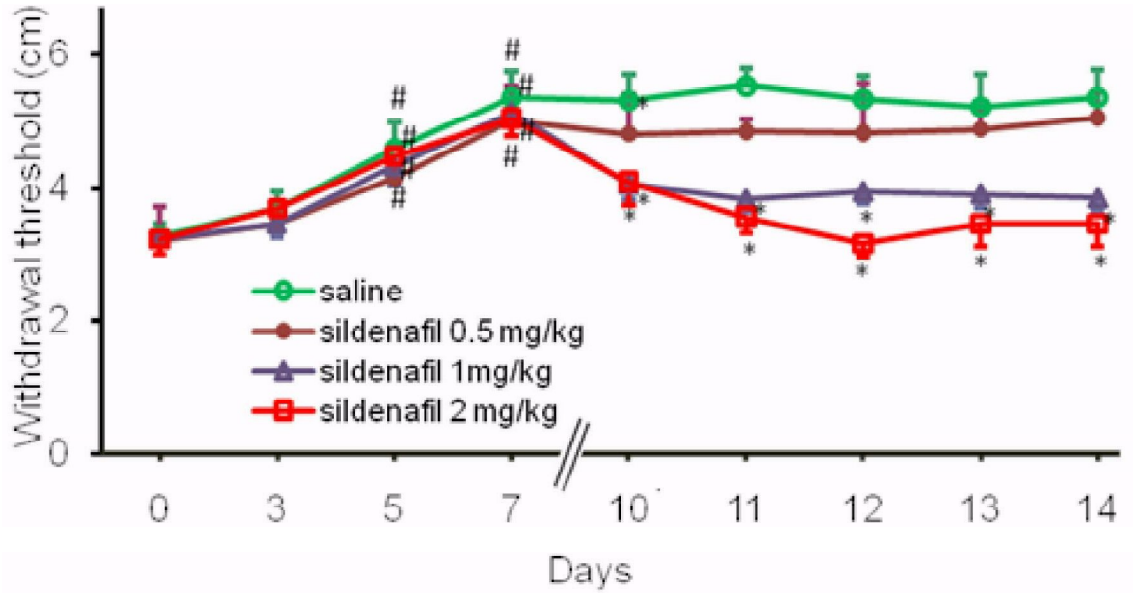

Fig. 1 : Effect of sildenafil (0.5, 1 and 2 mg/kg) on vincristine-induced tactile allodynia in rats. Day-0 represents values before administration of vincristine. Data are expressed as Mean±SD, (n=6 in each group). #p<0.05 compared versus day-0; *p<0.05 compared with vehicle-treated rats on same day. A progressive decrease in the threshold stimulus in comparison to day-0 values was noted in all animals which fully established after the third injection on day-7 and remained significantly low up to day-14 in vehicle-treated animals (repeated measures ANOVA followed by Scheffe's test). 0.5 mg/kg dose of sildenafil significantly reversed vincristine-induced allodynia 2 h after i.p. injection on day-10 while 1 and 2 mg/kg dose significantly reversed the allodynia on days 10-14 compared with vehicle-treated animals (one way ANOVA followed by Dunnett's t-test).

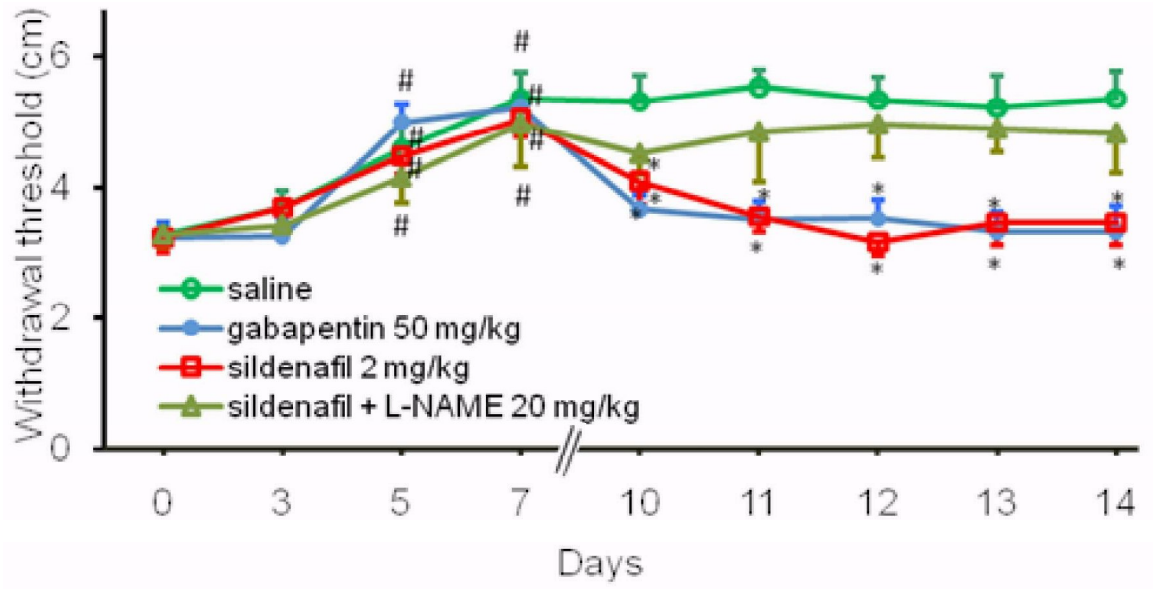

Fig. 2 : Effect of saline, sildenafil, gabapentin, sildenafil+L-NAME on vincristine-induced tactile allodynia in rats. Day-0 represents values before administration of vincristine. Data are expressed as Mean±SD, (n=6 in each group). #p<0.05 compared versus day-0; *p<0.05 compared with vehicle-treated rats on same day. A progressive decrease in the threshold stimulus in comparison to day-0 values was noted in all animals which fully established after the third injection on day-7 and remained significantly low up to day-14 (repeated measures ANOVA followed by Scheffe's test). Both sildenafil and gabapentin significantly reversed vincristine-induced allodynia 2 h after i.p. injection from days 10-14 compared with vehicle-treated animals; sildenafil+L-NAME significantly reversed tactile allodynia only on day 10 (one way ANOVA followed by Dunnett's t-test).

TABLE I: Thermal allodynia in vincristine-treated rats.

	Day 0	*Day 10 before drug*	*Day 10* after drug
Group I (0.9% NaCl)	30±0	29.56±0.19	29.21±0.07
Group II (Gabapentin)	30±0	29.43±0.26	28.95±0.19
Group III (Sildenafil)	30±0	29.73±0.18	29.41±0.48
Group IV (Sildenafil + L-NAME)	30±0	29.58±0.19	29.55±0.30

Values are expressed in seconds as Mean±S.E.M. (n=6)
*p<0.05, significant in comparison to day 0.
[a]p<0.05, significant in comparison to Group I.

TABLE II: Tail-flick test in vincristine-treated rats.

	Day 0	*Day 10 before drug*	*Day 10* after drug
Group I (0.9% NaCl)	5.91±0.34	6.08±0.29	6.03±0.25
Group II (Gabapentin)	6.01±0.21	5.93±0.36	5.78±0.31
Group III (Sildenafil)	5.96±0.28	5.93±0.27	6.03±0.24
Group IV (Sildenafil + L-NAME)	5.58±0.41	6.16±0.32	5.92±0.36

Values are expressed in seconds as Mean±S.E.M. (n=6)
*p<0.05, significant in comparison to day 0.
[a]p<0.05, significant in comparison to Group I.

Discussion

Administration of vincristine, along with other antimitotic drugs produces neuropathy that can manifest as painful paresthesia. Models of vincristine-induced neuropathic pain have been developed in rats using daily i.v. injections of vincristine (16). Our results demonstrate that rats treated with vincristine in a dose of 600 µg/kg over a period of three days developed significant tactile allodynia without thermal allodynia. The decreased threshold to the mechanical stimulus was evident from day 3 itself as previously shown by Bordet et al. which remained significant till day 14 (16). This model thus would be useful for assessing treatment effects in early onset vincristine-induced neuropathic pain. Similar results have been observed in models developed by Aley et al., 1996 and Nozaki-Taguchi et al., 2001 whereby the animals displayed significant tactile allodynia without thermal allodynia (21,22). However rodent models described by Kamei et al., 2005, whereby, vincristine is administered in a dose of 0.05-0.125 mg/kg twice a week for 6 weeks, have shown to induce thermal hyperalgesia (13). Although the detailed reason is not clear, this discrepancy could be due to the differences between the dose and the treatment schedule of vincristine administration and longer duration of observation.

In the current study, it was demonstrated that tactile allodynia in rodents can be assessed using a single von Frey filament by altering its length - shorter length of the filament indicates greater mechanical threshold. Sildenafil (0.5, 1 and 2 mg/kg) showed a dose-response effect in attenuating the vincristine-induced allodynia. In a recent study, sildenafil given as a loading dose and continuous intravenous infusion did not show any beneficial effect in a chronic constriction injury animal model of neuropathic pain (23). In another study performed in the same model, sildenafil (100 and 200 µg, intrathecal) caused worsening of neuropathic hyperalgesia but in lower dose did not alter the nociceptive threshold (24). Possible explanation for this difference could be activation of different pathophysiologic events in the two models of neuropathic pain. A deficit in NO is shown to be involved in vincristine neuropathy (13). Aley and Levine (2002) suggested that increased activity of Protein Kinase C (PKC) might trigger vincristine-induced hyperalgesia, since PKC inhibitor reversed the vincristine-induced hyperalgesia in rats. Furthermore, it was also reported that activity of PKC lead to the reduction of neuronal NOS activity (25, 26). Sildenafil (1 and 2 mg/kg) and gabapentin significantly reversed tactile allodynia on all days from day 10 to day 14 in the present study. The effects of sildenafil (2 mg/kg) and gabapentin were comparable. The effects of sildenafil could have been mediated by NO-cGMP pathway. Sildenafil, as stated earlier, acts by inhibiting hydrolysis of NO and facilitates the NO-cGMP pathway. Several studies support the beneficial role of NO in allodynia due to neuropathic and central mechanisms (9-14).

Kamei et al., 2005 demonstrated that pre-treatment with L-arginine, an NO donor, dose-dependently reversed vincristine-induced thermal hyperalgesia in mice which was tested 6 weeks later (13). Our study shows that even early manifestations of vincristine-induced neuropathy such as tactile allodynia can be significantly attenuated by sildenafil. This beneficial

effect of sildenafil was antagonized by L-NAME, a NOS inhibitor, suggesting that a decrease in *de novo* neuronal NO synthesis could play a role in vincristine-induced early onset tactile allodynia. In an ex vivo study using human chorionic plate arterial rings, it was shown that vasodilatory effects of sildenafil citrate in the feto-placental circulation were not reversed by L-NAME, indicating that *de novo* nitric oxide generation is not required to produce sildenafil citrate mediated vasorelaxation in this circulation (27). Our results are in line with an earlier study in which beneficial effect of PDE-5 inhibitor tadalafil were shown in carrageenan- and diabetes-induced hyperalgesia also, which was reversed by L-NAME, suggesting a role of NO-cGMP pathway. Further, in a mouse model of diabetic peripheral neuropathy it was shown that hyperglycemia substantially upregulated PDE-5 in Schwann cells and sildenafil showed beneficial effects by activating the cGMP/PKG signaling pathway (28).

In conclusion, this study shows that vincristine-induced early onset tactile allodynia can be significantly attenuated with sildenafil. Addition of L-NAME reversed sildenafil's attenuation of tactile allodynia indicating a decrease in *de novo* neuronal NO synthesis could play a role in vincristine-induced early onset tactile allodynia

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Original Article

Topical Losartan Reduces IOP by Altering TM Morphology in Rats with Steroid-induced Ocular Hypertension

Renu Agarwal[1]*, Anna Krasilnikova[1,3], Siti Nur Laili Mohamed[1], Puneet Agarwal[2], Nor Salmah Bakar[1], Lily Diana Binti Zainudin[1], Igor Iezhitsa[1], Nafeeza Mohd Ismail[1]

[1]Universiti Teknologi MARA,
Faculty of Medicine, Sungai Buloh Campus,
Selangor, Malaysia

[2]International Medical University,
IMU Clinical School, Department of Ophthalmology,
Jalan Rasah, Seremban, Malaysia

[3]Volgograd State Medical University,
Department of Clinical Pharmacology & Intensive Care,
Volgograd, Russia

Abstract

Purpose: Elevated intraocular pressure (IOP) in primary open angle glaucoma patients is associated with extracellular matrix (ECM) remodeling in trabecular meshwork (TM). Prevention of ECM remodeling may be of great benefit in attenuating long-term deterioration of TM morphology. Since, inhibitors of renin-angiotensin system are known to prevent ECM remodeling in tissues such as heart, we investigated if the IOP lowering effect of angiotensin receptor blocker, losartan, is associated with altered ECM remodeling in TM.

Methods: Effect of single drop and 3-week long multiple drop application of losartan potassium 2% on IOP was evaluated in steroid-induced oculohypertensive rats. Secondly, the effect of topical losartan on aqueous humor matrix metalloproteinase (MMP) -2 and -9 levels and TM morphology was studied. A comparison was made with latanoprost 0.005%.

Results: Single drop treatment with losartan resulted in 26.86% reduction in IOP from baseline, 8 hours post-instillation. The peak IOP lowering effect of losartan was comparable to latanoprost, however, the duration of this effect was 12 hours compared to 24 hours with latanoprost. Twice daily instillation of losartan over 3 weeks caused sustained IOP lowering which was comparable to once daily latanoprost. The IOP lowering effect of losartan was associated with significantly elevated aqueous humor MMP-2 and -9 levels, significantly increased TM cellularity and significantly reduced TM thickness compared to vehicle treated rats.

***Corresponding author :**

Dr. Renu Agarwal, Associate Professor, Universiti Teknologi MARA, Faculty of Medicine, Sungai Buloh Campus, Selangor, Malaysia; E-mail: renuag02@gmail.com

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Conclusions: Topical application of losartan reduces IOP in steroid-induced ocular hypertensive rats due to alteration in TM tissue remodeling, which could be attributed to increased MMP-2 and -9 secretion in the aqueous humor.

Introduction

Glaucoma, the leading cause of irreversible blindness, is often associated with elevated intraocular pressure (IOP). IOP is determined by the critical balance between the rate of secretion of aqueous humor and rate of its outflow mainly through trabecular meshwork (TM) and Schlemm's canal and to a little extent through uveoscleral pathway. Elevated IOP in patients with primary open angle glaucoma (POAG) is often attributed to increased resistance in TM due to increased deposition of extracellular matrix (ECM). The ECM in TM undergoes continuous remodeling by matrix metalloproteinases (MMPs), which are constitutively secreted by TM cells (Alexander et al. 1991; Bradley et al. 2003; Kelley et al. 2007). Reduced MMP levels favour deposition of ECM and decreased MMP levels have been reported in glaucomatous eyes (Nga et al. 2014; Määttä et al. 2006). IOP elevation in steroid-induced glaucoma is also associated with increased ECM deposition in TM (Razali et al. 2015a; Razali et al. 2015b; Razali et al. 2016). Snyder et al. (1993) have shown that corticosteroid treatment of TM organ and cell cultures causes increased ECM deposition. Hence, steroid-induced glaucoma in animals is considered a close representation of TM changes in glaucomatous human eyes (Agarwal and Agarwal 2017).

Several drug classes are currently used in clinical practice and majority of these drugs reduce IOP by improving the aqueous outflow facility. IOP reduction, however, is often suboptimal and is associated with several adverse effects. Furthermore, most of the current medications are not known to prevent TM remodeling. The ability of drugs to prevent TM remodeling could be of great significance particularly in view of controlling or preventing underlying pathological changes and hence providing long term control of IOP. Prostaglandin analogs such as latanoprost have been studied for their possible effects on MMPs and tissue inhibitors of metalloproteinases (TIMPs) in the TM. However, the studies have not shown a significant effect of this class of drugs on MMPs (el-Shabrawi et al. 2000; Oh et al. 2006; Pradhan et al. 2015). Over the past decade inhibitors of renin–angiotensin system (RAS) have become the drugs of interest as studies have shown the IOP lowering and neuroprotective effects of inhibitors of RAS (Shah et al. 2000; Costagliola et al. 2000; Yang et al. 2009; Mehta et al. 2010; Loftsson et al. 2010). Several components of RAS have been detected in ocular tissue (Vaajanen and Vapaatalo 2011). Our previous studies also showed the IOP lowering effects of angiotensin converting enzyme inhibitor (ACEI), enalaprilat, and angiotensin receptor blocker (ARB), losartan, in oculonormotensive rats (Agarwal et al. 2013). Although, IOP lowering effects of inhibitors of RAS are widely known in human and animals, it remains unclear if the IOP lowering effect of inhibitors of RAS involves increased MMP secretion and prevention of TM remodeling.

Hence, for the first time, we investigated the effects of 3 week long topical administration of losartan on aqueous humor MMP levels and TM morphology and evaluated the association of these changes with those in the IOP of rats with steroid-induced ocular hypertension.

Methods

Sprague Dawley rats weighing 100-120 g of either sex were housed under standard laboratory conditions of 12-hour light/12-hour dark cycle and free access to food and water. All the methods used in this study complied with ARVO statement of Use of Animals in Ophthalmic and Vision Research. Losartan potassium (ChemoLab) 2% solution was prepared in HPMC 1%.

Commercially available Latanoprost 0.005% (Xalatan®) was used as reference standard. HPMC1% was used as vehicle.

IOP estimation: IOP was estimated using a calibrated tonometer (TonoLab, Icare®, Finland) in conscious animals without topical anesthesia. Before starting the experiment, ten normotensive rats weighing 120-140 g were subjected to IOP measurements using TonoLab by two independent observers, twice at one week interval. Each observer was blinded to the IOP recorded by the other observer and to their own recordings on previous occasion. All IOP estimations were carried out between 8.00 and 10.00 AM. The mean value of 6 consecutive readings at all time points was considered as final estimation. Subsequently, mean intraobserver and interobserver differences were calculated.

Experimental model: The steroid-induced model of ocular hypertension was developed as described previously (Razali et al. 2015b). Briefly, after recording the baseline IOP, rats were treated bilaterally and topically with 5 µl of dexamethasone 0.1%, twice a day for 36 days. The IOP was measured twice a week during 36 days of treatment. At the end of 36 days, rats with IOP elevation of at least 25% above baseline were considered to be oculohypertensive and were included in the study.

Study 1: The oculohypertensive rats were randomly divided into 2 groups of 6 animals each. After estimation of baseline IOP, the animals in group 1 were topically treated with losartan 2% in one of the randomly chosen eyes (Test eye). Similarly, group 2 received latanoprost 0.005% in the test eye. The contralateral eyes in both the groups received single drop of vehicle. Single drops of vehicle as well as losartan/latanoprost were of 5 µl volume. After instillation of drug/vehicle, IOP was measured at regular intervals using Tonolab.

Study 2: The oculohypertensive rats were randomly divided into 3 groups of 6 animals each. After estimation of baseline IOP, animals in one of the groups received single drop of vehicle in both eyes. The 2 other groups were bilaterally treated with losartan or latanoprost. Losartan 2% was applied topically twice daily, while the latanoprost 0.005% was administered once daily. Another group consisted of normotensive rats that received treatment with HPMC 1% for 36 days instead of dexamethasone. Bilateral topical administration of respective drugs or vehicle in all groups was done daily in a volume of 5 µl for a total of 21 days. IOP was estimated twice a week between 8.00 and 10.00 AM and the drug instillation in the morning was done after IOP measurement to allow assessment of IOP reduction corresponding to trough concentrations of drugs.

At the end of three weeks treatment period, the aqueous humor was collected before the rats were sacrificed. The rats were anaesthetized by subcutaneous injection of ketamine and xylazine solution. Once the rats were unconscious, the eyeballs were washed using phosphate buffer saline (PBS) and wiped clean. The cornea of the eye was then pricked by a sterile needle. The aqueous humor was immediately collected using a 20-µl micropipette into a siliconized microcentrifuge tube. The aqueous humor sample was stored at –80°C till further processing for estimation of MMP levels. Pooled aqueous humor from 2 eyes of same animals was used as one sample. Commercially available Rat MMP-2 and MMP-9 Elisa Kits (Abnova, Taiwan) were used to quantify MMP-2 and MMP-9 in the aqueous humor.

After aqueous humor drainage, rats were sacrificed by overdose of ketamine and xylazine, the eyes were enucleated and the eyeballs were fixed in 10% formalin solution. The enucleated eyes were paraffin embedded, sectioned and subjected to hematoxylin and eosin staining for histopathological examination of TM.

Statistical methods

All data is presented as Mean±SD. Comparison of IOP between two eyes in the same group was done using paired student t-test. Intergroup comparisons for study 2 were done using one-way Anova. $P<0.05$ was considered significant.

Results

Firstly, IOP lowering effect of unilateral single drop application of losartan potassium was studied using a calibrated tonometer (TonoLab, Icare®, Finland). Subsequently, effect of bilateral topical application of losartan over 3 weeks on IOP, aqueous humor MMP-2 and -9 levels and TM morphology was studied. For both studies Sprague Dawley rats were used in which ocular hypertension was induced by topical application of dexamethasone for 36 days.

IOP measurements and effect of dexamethasone of IOP

Before starting the experiment, technique of IOP measurement was standardized to ensure repeatability and reproducibility of the method. The IOP estimation by first observer was 13.4±1.27 and 13.35±1.27 mmHg on the first and second occasion, respectively. Mean IOP estimation by second observer was 13.7±1.26 and 13.75±1.37 mmHg on first and second occasion, respectively. The mean differences were 0.05±1.10 and 0.05±1.28 mmHg for first observer and second observer respectively. All intraobserver and interobserver differences of IOP estimation were within 2 SD from mean, which indicates good repeatability and reproducibility of IOP estimation by Tonolab.

After starting the topical treatment with dexamethasone, IOP elevation in both eyes was observed from day 8 until day 36 ($p<0.05$). The IOP reached plateau after 32 days. There was no significant difference between the mean IOP of right and left eyes at any time point. At the end of 36 days, the mean IOP elevation achieved was 42.29 and 46.13% for right and left eyes respectively. Fig. 1 and 2 show the progressive increase in IOP from baseline over 36 days of steroid treatment.

IOP lowering effect of single drop application of losartan potassium versus latanoprost

Topical treatment with single drop of losartan caused significant reduction of IOP compared to baseline starting from the first hour until the 10th hour post-treatment. Whereas in latanoprost treated group, IOP reduction started 3 hour post-instillation and lasted until 24th hour post-treatment. Maximum mean IOP reduction of 26.86% was observed at 8 hour post-instillation in losartan treated group but the same was 28.40% at 10th hour post-instillation in latanoprost group. The maximum IOP reduction by losartan potassium was comparable to latanaprost ($p>0.05$). However, it was observed that the duration of significant IOP reduction in losartan group was 10 hours compared to 24 hours in latanaprost group (Table I, Fig. 1).

TABLE I: IOP lowering effect of single drop application of losartan potassium 2% versus latanoprost 0.005% in rats with steroid–induced ocular hypertension. All values represent group mean.

Time post–instillation (hour)	*Losartan % of reduction from baseline*	*Latanoprost % of reduction from baseline*
0	–	–
1	–5.07	0.36
2	–9.49	–4.31
3	–15.53	–4.48
4	–17.58	–8.62
6	–20.82	–13.01
8	–26.86	–17.80
10	–13.59	–28.40
12	–3.02	–15.49
16	0.22	–5.47
20	–0.65	–5.47
24	–0.43	–4.39

IOP lowering effect of bilateral multiple drop application of losartan potassium versus latanoprost over 3 weeks

Bilateral treatment with losartan significantly ($p<0.01$) reduced IOP of steroid-induced oculohypertensive rats compared to vehicle treated oculohypertensive rats. The reduction in IOP was sustained over the treatment period without significant fluctuations. A similar observation was made in latanoprost treated group (Table II, Fig. 2).

Effect of losartan on aqueous humor concentration of MMP-2 and MMP-9

MMP-2 and -9 concentrations in oculohypertensive vehicle treated group were 6.29 and 2.10 fold lower, respectively, compared to normotensive group. In losartan treated group, there was 3.32 and 1.89 fold increase in the concentration of MMP-2 and -9,

TABLE II: IOP lowering effect of daily topical application of losartan 2% versus latanoprost 0.005% over 3 weeks in rats with steroid-induced ocular hypertension. All values represent group mean.

Time (Days post–treatment)	*IOP Reduction from baseline (%) after 36 days of steroid/vehicle treatment*							
	Normotensive		*Oculohypertensive*					
			Vehicle		*Losartan 2%*		*Latanoprost 0.005%*	
	Right eye	*Left eye*	*Right eye*	*Left eye*	*Right eye*	*Left eye*	*Right eye*	*Left eye*
3	–0.35	0.10	–0.67	–0.88	–14	–14	–22.76	–24.99
7	–0.89	–0.25	0.40	0.38	–15	–14	–20.41	–21.44
11	–1.33	–0.94	–0.98	–1.70	–18	–17	–21.86	–21.79
14	–0.89	0.35	–0.41	–0.31	–17	–16	–29.61	–29.66
18	–0.80	0.67	–1.10	–1.07	–21	–21	–29.34	–29.38
21	–2.04	0.87	–0.81	–0.63	–19	–18	–21.90	–23.65

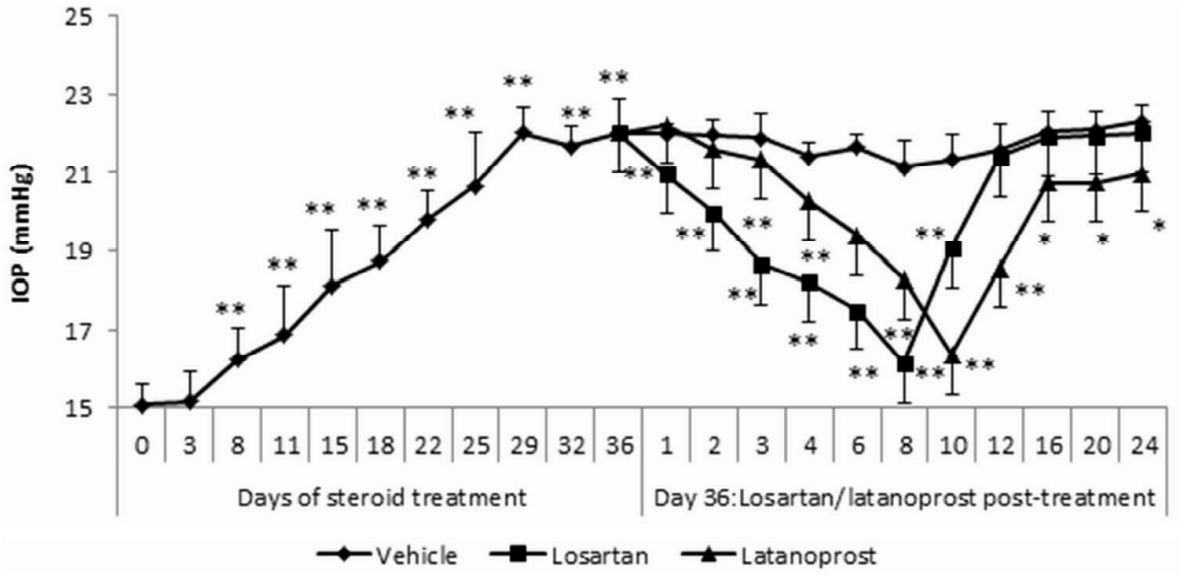

Fig. 1: Effect of unilateral single drop application of losartan 2% after 36 days of treatment with dexamethasone in rats. Up to day 36 (Days of steroid treatment): *p<0.05; * *p<0.01 vs. corresponding baseline. On Day 36: *p<0.05; * *p<0.01 test eye vs. control eye. Since control eyes of both the latanoprost and losartan treated groups showed no differences at any time point, single data series from control eye is shown on day 36.

respectively, compared to oculohypertensive vehicle treated group. Although MMP-2 concentration in losartan treated group remained lower than normotensive group ($p<0.05$), both MMP-2 and -9 levels were higher in this group compared to latanoprost treated group ($p<0.01$ and <0.05, respectively). In latanoprost treated group, MMP-2 levels were higher than oculohypertensive vehicle treated group ($p<0.05$) but no difference was observed for MMP-9 (Table III).

Effect of losartan on TM morphology

The H&E stained sections were examined for TM morphology. Thickness of TM in four groups was measured at 4 sites as shown in Figure 3A. The average of 4 measurements was taken as the final estimate. The vehicle treated oculohypertensive rats showed significantly greater mean TM thickness compared to normotensive group ($p<0.01$). Losartan-treated group showed significantly lower ($p<0.01$) TM thickness compared to the vehicle-treated oculohypertensive rats. Latanoprost treated group also showed a significantly thinner ($p<0.01$) TM compared to the oculohypertensive vehicle-treated group (Table IV).

We also did a cell count in the 100 µm

TABLE III : Mean aqueous humor MMP-2 and -9 concentrations among 4 groups.

	Normotensive	*Oculohypertensive*		
		Vehicle	*Losartan 2%*	*Latanoprost 0.005%*
MMP-2 (ng/ml) (N=6)	21.85±4.92	3.47±1.33**	11.55±0.60*$^{\#\#\$\$}$	6.34±0.28**$^{\#}$
MMP-9 (pg/ml) (N=6)	12.32±3.50	5.87±0.35*	11.09±2.46$^{\#\#\$}$	6.99±2.14*

All values are expressed as Mean±SD. *(p<0.05); **(p<0.01) vs. normotensive group, $^{\#}$(p<0.05); $^{\#\#}$(p<0.001) vs oculohypertensive vehicle treated group. $^{\$}$p<0.05; $^{\$\$}$p<0.01 versus oculohypertensive latanoprost treated group.

TABLE IV : Mean TM thickness and number of cells among 4 groups.

	Normotensive	*Oculohypertensive*		
		Vehicle	*Losartan 2%*	*Latanoprost 0.005%*
Trabecular meshwork thickness (µm) (N=6)	9.98±1.57	13.62±1.45**	9.29±1.88 $^{\#\#}$	9.53±1.42$^{\#\#}$
Number of Cells (N=6)	23.61±4.63	15.86±6.61***	22.05±6.12$^{\#\#\$}$	18.57±4.53**

All values are expressed as Mean±SD. *p<0.05, **p<0.01, ***p<0.001 vs normotensive group; #p<0.05 and ## p<0.01 vs oculohypertensive vehicle treated group; $^{\$}$p<0.05 vs oculohypertensive latanoprost treated group.

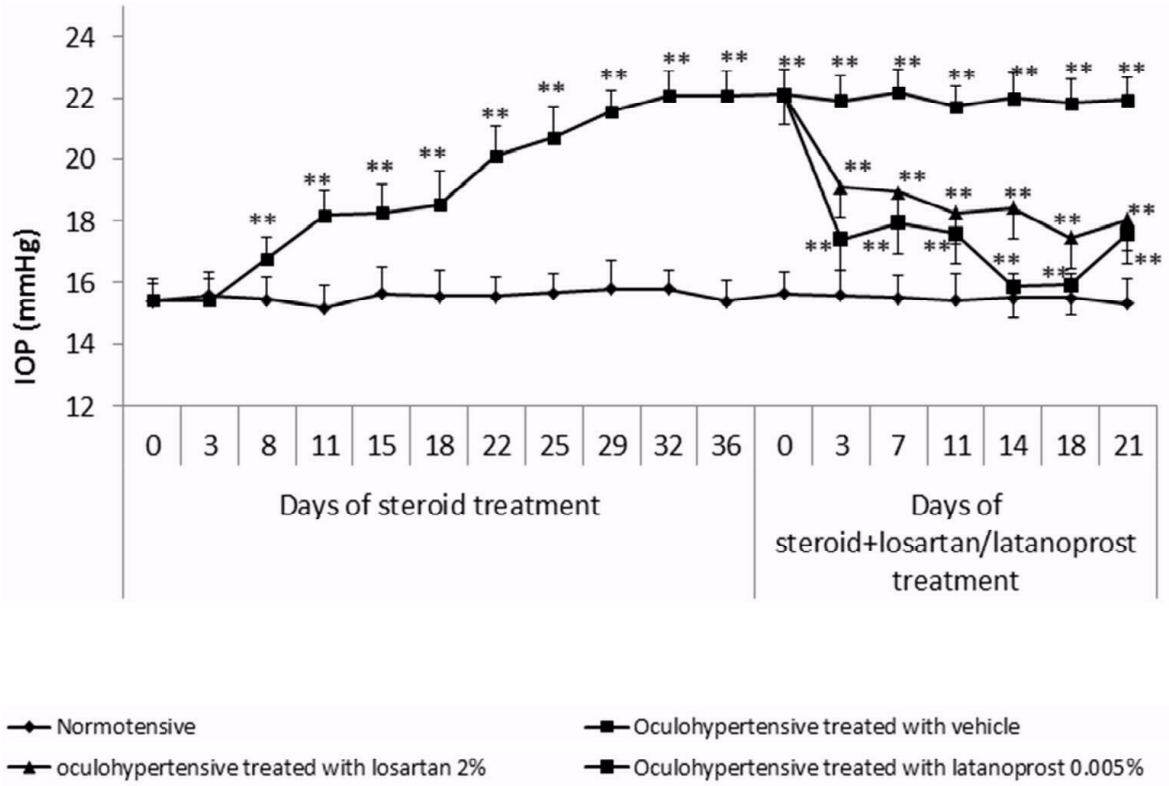

Fig. 2 : Effect of repeated dose application of losartan 2% over 3 weeks in rats with steroid-induced ocular hypertension. Each data series represents average IOP of right and left eyes. Up to day 36 (Days of steroid treatment): **p<0.01 vs. corresponding baseline. From day 0 to day 21 (Days of steroid + losartan/latanoprost treatment): **p<0.01 versus oculohypertensive vehicle treated group.

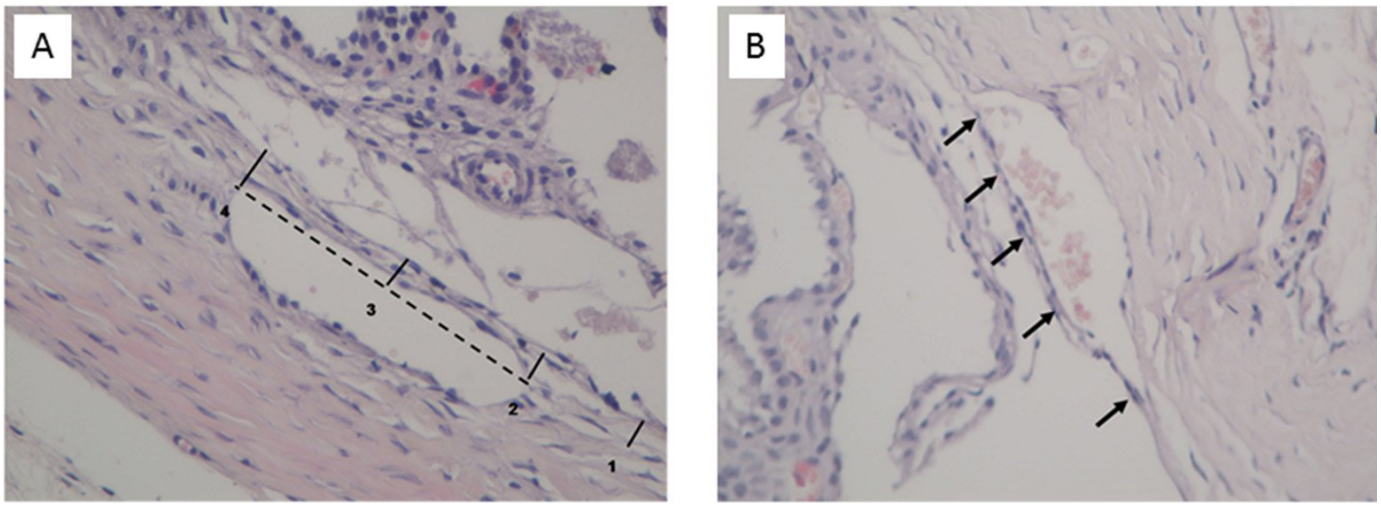

Fig. 3 : 3A: Photomicrograph showing the anterior chamber angle of a normal Sprague–Dawley rat. Lines 1–4 indicate the positions at which TM thickness was measured. 3B: The cells were counted in the 100 µm paracanalicular area as indicated by arrows.

paracanalicular area as indicated in Fig. 3B. The spindle shaped nuclei of fibrous connective tissue cells were counted in the predefined trabecular area as indicated in Fig. 3B. There was a significant difference ($p<0.05$) between the number of cells between normotensive and oculohypertensive vehicle-treated group, with significantly higher mean number in normotensive group. The cell count in losartan-treated group was significantly higher than oculohypertensive vehicle-treated as well as latanoprost treated groups and was comparable to that in normotensive rats. In latanoprost-treated group, the cell count remained significantly lower than the normotensive group (Table IV).

An average of both the TM thickness and TM cell count from 2 eyes of each animal was considered as representative of one sample. Both morphological estimations were done by two masked observers independently and the average of two was taken as the final estimate.

Discussion

Systemic RAS is known to play a significant role in blood pressure homeostasis. It is now also considered to play an important role in tissue-specific regulatory functions, hence producing local effects and long-term changes in tissues. Presence of the components of RAS in ocular tissue has been described by several researchers (Danser et al. 1994; Savaskan et al. 2004; Paul et al. 2006; White et al. 2015). Accordingly, besides angiotensin II type 1 receptor blockers, IOP lowering effects of angiotensin converting enzyme inhibitors and Mas receptor agonist have been investigated in few studies (Loftsson et al. 2010; Vaajanen et al. 2008). Among angiotensin receptor blockers, CGP 48933, losartan and CS-088, were shown to reduce IOP when applied topically to rabbit eyes (Kaiser et al. 1997; Shah et al. 2000; Inoue et al. 2001) or CS-088 in monkey eyes (Wang et al. 2005). Kaiser et al. also studied the effects of topical losartan in human, however, no significant effect on IOP was observed. It is noteworthy that this human study involved only 5 patients. None of these studies have described if angiotensin receptor blockers can produce sustained IOP reduction on prolonged administration and particularly so in an animal model such as steroid-induced ocular hypertension that could be considered closest to POAG in human (Agarwal and Agarwal 2017).

The current study showed that the single drop application of losartan produces significant IOP reduction in rats with steroid-induced oculohypertension. The peak IOP lowering by single drop of losartan was comparable to that produced by latanoprost, however, the duration of significant IOP lowering was longer with latanoprost. This study

for the first time has demonstrated that repeated dose administration of losartan over 3 weeks produces sustained IOP reduction which is comparable to that produced by latanoprost. The frequency of administration of losartan, however, was twice daily compared to once daily administration of latanoprost due to its longer duration of action. Since very few studies have been done to investigate the IOP lowering effects of angiotensin receptor blockers, the mechanisms underlying these effects remain unclear.

One of the recent studies has shown that the IOP lowering effect of systemically administered losartan in CD1 mice is associated with prevention of RGC loss (Quigley et al. 2015). Instead of possible vascular effects of losartan in retina leading to neuroprotective effects, this study provides evidence that these effects of losartan are attributed to reduced thickness of sclera which normalized the biomechanical scleral response to elevated IOP. The scleral thinning in response to losartan treatment involved inhibition of increase in pERK and reduced scleral fibroblast activation. Losartan was also shown to produce similar responses in mouse model of Marfan syndrome in the presence of intact angiotensin II signaling (Habashi et al. 2011). Both of these studies demonstrated higher effectiveness of losartan compared to enalaril for the possible reason that losartan maintains or even enhances signaling through angiotensin II type 2 receptors whereas enalapril limits signaling through both angiotensin II type 1 and type 2 receptors. Angiotensin receptor blockers have also been shown to attenuate cardiac remodeling (Zornoff et al. 2000), however, it is not known if similar responses could be observed in TM. Within TM, the major site of increased resistance is the juxtacanalicular tissue (JCT). Continuous remodeling of ECM in JCT is critical in preserving aqueous outflow channels in open state by releasing trapped debris and associated ECM fragments from the outflow pathways (Keller and Acott 2013). Studies have shown that in POAG eyes, there is significant reduction in the number of TM cells and significant increase in the ECM deposition in JCT (Alvarado et al. 1984; Grierson and Howes 1987; Lütjen-Drecoll et al. 1981). TM cells are responsible for detecting mechanical stimulus of elevated IOP and therefore, their reduced number limits the ability of tissue to respond to elevated IOP (Keller and Acott 2013). Similar TM changes have been reported earlier in rat eyes with steroid-induced glaucoma (Tektas and Lütjen-Drecoll 2009). Our previous studies have also shown that treatment of human TM cells with dexamethasone results in increased expression of collagen type I, III, IV, fibronectin and alpha smooth muscle actin (Hassan et al. 2016). Hence, reduced TM thickness indicating reduced ECM deposition and increased TM cellularity observed in the current study clearly demonstrate that losartan positively alters TM tissue composition resulting in IOP lowering.

MMPs have been described as important modulators of aqueous humor outflow as they continuously remodel ECM composition in TM and maintain a stable outflow resistance and IOP (De Groef et al. 2013). The current study for the first time shows that topical treatment with losartan results in increased aqueous humor MMP-2 and -9 levels. In this study we measured total MMP levels and not the active MMPs. However, it could be assumed that even if increased MMP level are due to proMMPs there is larger readily available pool for production of active MMPs after treatment with losartan. In line with our observation, one of the studies showed that the treatment of vascular smooth muscle cells with angiotensin II reduced MMP-2 secretion but treatment with losartan inhibited the effect of angiotensin II (Papakonstantinou et al. 2001). Varo et al. also showed that chronic treatment of spontaneously hypertensive rats with losartan reverses myocardial fibrosis and this effect of losartan involves increased collagenase activity along with reduced expression of TIMP1. Similarly both the short and long-term treatment of rat aortic smooth muscle cells with angiotensin II were shown to increase the TMIP-1 expression (Castoldi et al. 2003). TIMPs are inhibitors of MMPs and hence an altered ratio of MMP/TIMP in response to losartan has possibly contributed to reduced TM thickness in the current study.

It can be concluded from the results of this study that IOP reduction in response to topical losartan in rats with steroid-induced ocular hypertension is associated with increased TM cellularity and reduced

ECM deposition and these morphological changes could be attributed to increased MMP-2 and -9 secretion. It is also noteworthy that transforming growth factor-β is involved in increased ECM deposition in the TM of glaucomatous eyes (Agarwal and Agarwal 2010; Agarwal and Agarwal 2015) and angiotensin II stimulates collagen production via TGF-β-dependent pathways. Hence, inhibitors of RAS may also reduce ECM deposition by inhibiting TGF-β signaling (Diop-Frimpong et al. 2011). Therefore, the effects of losartan on TM morphology observed in the current study may involve not only the pathways that enhance ECM degradation involving increased MMP secretion but also those that inhibit ECM deposition. Additionally, angiotensin II acts as a secretagogue in human ciliary body non-pigmented epithelium and increases aqueous humor secretion via angiotensin II type 1 receptors. Treatment with losartan inhibits this activity of angiotensin II (Cullinane et al. 2002). Hence, reduced rate of aqueous humor secretion may also be a contributory factor in the IOP lowering effect of losartan in the current study. Moreover, the contractile effects of angiotensin II on vascular smooth muscles are countered by losartan and since TM cells possess smooth muscle like properties, losartan may cause TM relaxation, an effect that contributes to reduced outflow pathway resistance (Abu-Hassan et al. 2014).

In conclusion, the current study showed that topical application of losartan lowers IOP. This lowering of IOP is associated with increased cellularity and decreased ECM deposition in TM. The morphological changes in TM in response to losartan could be attributed to increased aqueous humor levels of MMP-2 and -9. Further studies are needed to explore the molecular pathways involved in losartan-induced increase in MMP levels, effects of losartan on pathways involved in ECM biosynthesis in TM and other mechanisms that may contribute to changes in aqueous humor dynamics.

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All authors have substantially contributed to conception, designing, drafting the article and in final approval of the manuscript version to be submitted. All authors have jointly decided to designate Assoc Prof Dr Renu Agarwal to be responsible for taking decision regarding the presence of authors and the order of their presence in the manuscript. Assoc Prof Dr Renu Agarwal has also been selected by all authors to be responsible for all future communication with the journal regarding this manuscript.

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Original Article

Treatment With Saffron Extract of the Diabetogenic Rats Induced by the Food Colorant Tartrazine

Iliass Lahmass[1]*, Assia Sabouni[1], Ali Berraouan[2], Kaoutar Zoheir[2], Safae Belakbir[2], Mohammed Elyoubi[1], Redouane Benabbes[1], Imane Himri[1], Slimane Mokhtari[3], Hassan Mekhfi[2], Mohammed Bnouham[2] and Ennouamane Saalaoui[1]

[1]University Mohamed Ist,
Faculty of Sciences, Department of Biology,
Laboratory of Biochemistry, Oujda, Morocco

[2]University Mohamed Ist, Faculty of Sciences,
Department of Biology, Laboratory of Physiology and Ethnopharmacology
URAC-40, Oujda, Morocco

[3]Regional Laboratory of Medical Analysis,
Hospital Al Farabi, Oujda, Morocco

Abstract

This study evaluated the antidiabetic and antidiabetogenic effect of saffron against diabetes induced by artificial dye Tartrazine on normal male rats. Rats were divided into 5 groups consisted of six rats and treatment was performed daily and orally. Levels of blood glucose and body weight have been evaluated every 10 days and clinical demonstrations and metabolic parameters were evaluated at the end of experiment. Results showed that treatment with Tartrazine and saffron did not affect body weights, metabolic parameters but changed the blood glucose levels after 105 days of administration. The levels of glucose and creatinine were significantly increased in group2 and group3 compared to control group. Treatment with saffron decrease creatinine level. The outcomes suggest that saffron has curative (antidiabetic) and protective (antidiabetogenic) effect against diabetes induced by Tartrazine via reducing blood glucose level and creatinine. Therefore, it should be considered in future therapeutic researches.

Introduction

Diabetes mellitus is a metabolic disorder characterized by a persistent hyperglycaemia and considered as a major health risk in the world. The estimation of number of diabetic adults in the world will increase to 300 million by the year 2025 (1, 2). Diabetes influences the quality of life of the patients as well as forcing them to undergo lifestyle changes such as regular monitoring of their blood glucose levels (3). As a public health problem, T2DM is an important endocrine and metabolic disorder, and the

***Corresponding author :**
University Mohamed Ist, Faculty of Sciences, Department of Biology, Laboratory of Biochemistry, Oujda, Morocco

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incidence is on growth all over the world, especially in China (4). Treatment by chemical medicines such as Acarbose or Metformin is extensively used to treat this disease, but there have many negative effects can cause great damage to the health of patients. Several studies showed that elements extracted from plants do well in treatment of T2DM such as *Crocus sativus* (5, 6) *Alluim sativum* (7) and *Trigonella foenum-graecum* (8).

Tartrazine is an orangecoloured, water soluble powder used worldwide as food additives to colour several foods, drugs and cosmetics. It has been added in cooking with a principal aim to give a colour to a foodstuff. Tartrazine has been widely used as a food additive for the yellow colour and is often responsible for allergic reactions in humans (9, 10). The results of some studies showed that Tartrazine had the carcinogenetic and mutagenetic effects (11-15). Tartrazine also increase blood glucose level and plasma creatinine, cholesterol, triglyceride (16).

Saffron is a spice derived from the flower of *Crocus sativus* Linn., it's a genus in the family Iridaceae. Saffron is one of the highest priced and the most used spices around the world for flavouring and colouring food (17). To treat diseases traditionally, a crude extract of pistils of *Crocus sativus* in water was administered orally alone or with other medicinal plants (18). For a long time, the use of the plant of *Crocus sativus* was interested only in the part of the red stigmata. Egyptians used saffron by mixing with tea or associated with food in the kitchen for its stimulating and euphoric effects (19). The ancient Romans had hoped to benefit from its reputed ability to prevent hangovers by scraping stigma into their wine (20).

As a medicinal plant, saffron is used in traditional Persian medicine, for throat problems, depression, menstrual disorders and inflammation (21). In Ayurvedic medicine, saffron was used to treat asthma, arthritis, colds and as an aphrodisiac, adaptogenic, antispasmodic, carminative, expectorant and sedative (22). It has been used in the remedy against eye diseases, scarlet fever, asthma, smallpox, colds and heart disease (23, 24). Numerous studies have demonstrated that saffron have anti-oxidant (25, 26), and crocin have beneficial effects in the treatment of neurodegenerative disorders such as Alzheimer's disease (27), also saffron or its active constituents has demonstrated an antinociceptive effect, as well as acute and/or chronic anti-inflammatory activity (28). Aqueous extract of saffron and its constituent showed an aphrodisiac activity in normal male rats (29). Recently, it was found that saffron extract, exhibited significant decreased of blood glucose level, cholesterol (30), anti-hyperglycemic effects (6, 31).

Many people use traditionally the macerate of saffron in water (crude extract) to treat diseases, the present study aims to assess the curative and protective effects of crude extract of saffron on Tartrazine induced diabetic rats. For the first time we investigated the model of diabetes induced by Tartrazine in healthy male rats.

Materials and Methods

Plant materials

Crocus sativus L. or saffron was obtained from Taliouine (Taroudant Province, Souss-Massa-Drâa, Morocco), local name: zaâfran. Three specimens of the plant have been deposited at the plant section of Herbarium University Mohammed Premier Oujda Morocco (HUMPOM), under the voucher number (HUMPOM210). The identification of the plant has been done and confirmed by a professional botanist, Professor Fennane Mohammed from Scientific Institute in Rabat, Morocco. Dried milled powder of stigmas of *Crocus sativus* L. was macerated for 12 hours in distilled water before usage and crude extract was used to treat male rats.

Chemicals

Tartrazine (CAS 1934210, Purity 86.7%), was purchased from Alfa Aesar (Germany), SigmaAldrich (Japon) and was dissolved in distilled water 12 hours before use.

HPLC analysis of crude extract of stigma from *Crocus sativus*

One hundred µL of extracts samples were injected into a liquid chromatography (HPLC) to determine the chemical compounds of the saffron extract at 440 nm. A Waters Symetry® C18 (4,6 µm x 250 mm) column. A linear gradient of methanol (10–100%) in water (15% of acetonitrile) was used as a mobile phase with a flow-rate of 1 ml/min for a maximum elution time of 60 min at room temperature. The sample size was 20 µl of the test solution (32). The analyses were triplicated for each sample.

Animals

Maintenance and handling of rats were in accordance to the internationally conventional standard guidelines and with the Helsinki declaration for use of laboratory animals. 30 male Wistar rats weighting 150-200 g were housed in individual cages under standard laboratory conditions in a 12 h/12 h light/dark cycle and at a temperature of 21-25°C (animal house of the department of biology, faculty of sciences, Oujda, Morocco) and were given free access to water and dry rat pellets feeds (SONABETAIL Society, Oujda, Morocco).

Experimental design

Animals were arbitrarily separated to five groups of equal number and weight (six animals each). All animals were treated by daily oral gavage for 105 days with a volume of 10 ml/kg.

Group 1 (normal group): Rats were given distilled water.

Group 2 (Tartrazine-saffron group): Animals were treated with Tartrazine (10 mg/kg) for 60 days and then administered with saffron (120 mg/kg) until the last day of treatment.

Group 3 (Tartrazine group): Rats were administered only with Tartrazine (10 mg/kg) for all period of treatment.

Group 4 (saffron-Tartrazine group): Animals were treated with saffron (120 mg/kg) for 60 days and then administered with Tartrazine (10 mg/kg) until the last day of treatment.

Group 5 (saffron group) : Rats were administered only with saffron (120 mg/kg) for all period of treatment.

Determination of blood glucose levels during experimental period (105 days) was done once every 10 days by using a One Touch Ultra 2 Glucometer based on glucose oxidase. Blood samples were collected from tail veins of the rats after the animals had been fasted for 12 hours. Body weight was evaluated once every 10 days during experimental period.

On the day of necropsy, blood samples were collected via the abdominal aorta for measurements of biochemical parameters. Glucose concentration and creatinine levels in plasma were estimated by enzymatic colorimetric method according to Trinder and Fabiny successively (33, 34). Plasma concentrations of ALT and AST were determined by the method of (35) and biochemistry determinations were performed by using ILab 300 (Instrumentation Laboratory Corporate Headquarters, Barcelona, Spain).

Statistical analysis

All data were expressed as Means±SEM. Significant differences among control and experimental groups was determined by one-way analysis of variance (ANOVA) followed by Tukey post-test using Graph Pad Prism 5.

Results

Compared to water control group, treatment with Tartrazine and saffron did not affect body weights (Fig. 1), but it influenced the blood glucose levels after 105 days of administration (Fig. 2).

As shown in the Table I, treatment with Tartrazine and saffron did not affect metabolic parameters like pH and urine volume and the difference was

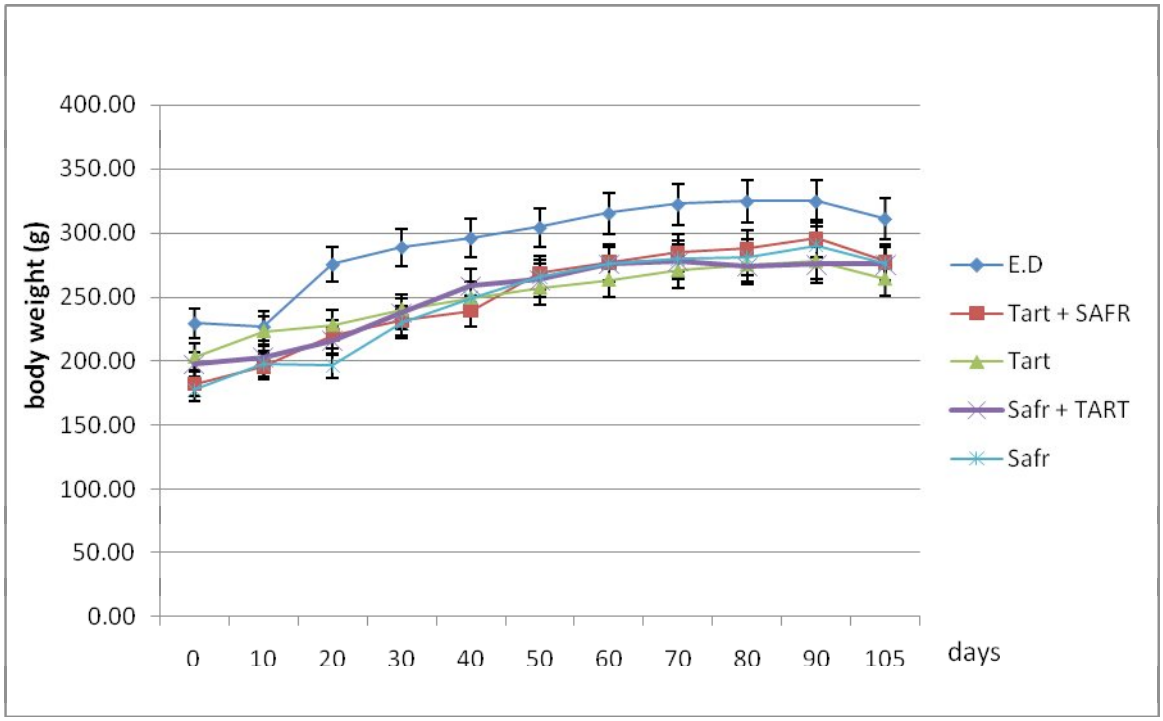

Fig. 1 : Body weights of Wistar rats for 105 days treated orally with Tartrazine and Saffron. ED: Rats treated with distilled water, Tart: Rats treated at the dose of 10 mg/kg Tartrazine, Tart + safr: Rats treated at the dose of 10 mg/kg Tartrazine + 120 mg/kg saffron, Safr + Tart: Rats treated at the dose of 120 mg/kg saffron + 10 mg/kg Tartrazine and Safr: Rats treated at the dose of 120 mg/kg saffron.

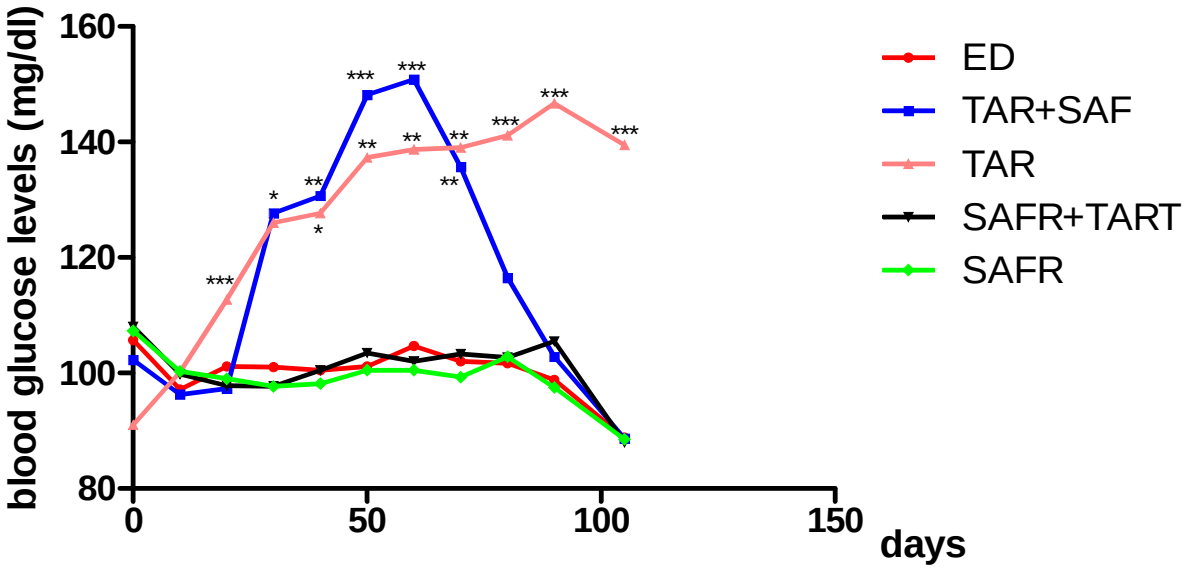

Fig. 2 : Variation of blood glucose levels during experimental period (105 days). ED: Rats treated with distilled water, Tart: Rats treated at the dose of 10 mg/kg Tartrazine, Tart + safr: Rats treated at the dose of 10 mg/kg Tartrazine + 120 mg/kg saffron, Safr + Tart: Rats treated at the dose of 120 mg/kg saffron + 10 mg/kg Tartrazine and Safr: Rats treated at the dose of 120 mg/kg saffron.

Note: values represent the Mean±SEM of six rats; ***p<0.001 highly significantly different from controls. **p<0.01 highly significantly different from controls. *p<0.05 significantly different to group control.

TABLE I: Metabolic parameters of Wistar rats feeding with Tartrazine and saffron and sacrificed after 105 days of treatment.

Metabolic parameters	*Control group*	*Tartrazine (10 mg/kg)*	*Tartrazine (10 mg) + saffron (120 mg)*	*Saffron (120 mg) + Tartrazine (10 mg)*	*Saffron (120 mg/kg)*
Water consumption	37.5±1.71	46.00±3.65*	37.5±4.79	36.67±6.15	30.00±5.63
Food consumption	32.21±1.56	20.34±2.04*	25.72±4.91	16.51±2.68*	35.28±3.39
pH	8.71±0.07	8.69±0.09	8.64±0.1	8.44±0.13	8.38±0.27
Urine volume	13.0±1.63	15.16±1.46	12.17±0.87	15.17±3.91	11.33±1.2

Note: values represent the Mean±SEM of six rats;* p<0.05. Significantly different from controls.

significant on consumption of food and water; also, the difference between liver, right kidney and heart weight is not significant (Table II).

The levels of glucose and creatinine were significantly increased in all groups treated with 10 mg/kg b.w of Tartrazine compared to control group. The level of creatinine was significantly increased in group treated with 10 mg/kg b.w of Tartrazine + 120 mg/ kg b.w of saffron. There was no significant difference in the level of glucose and creatinine, among all groups treated with 120 mg/kg b.w of saffron + 10 mg/kg b.w of Tartrazine. Treatment with 120 mg/kg b.w of saffron did not have any significant effects on the level of glucose, but it influnced on creatinine levels.

After 105 days of treatment with Tartrazine and saffron, significant difference was observed on plasma glucose (Fig. 3) between control group compared to group treated with 10 mg of Tartrazine (Group 1) and there was no significant difference between groups treated with distilled water (control Group), treated with 10 mg of Tartrazine + 120 mg of saffron (Group 2), treated with 120 mg of saffron + 10 mg of Tartrazine (Group 3) and Group 4 treated with 120 mg of saffron. Significant difference in the level of plasma glucose was observed between group 1 compared to group 2 and group 2 compared to group 4. In contrast, the difference between group 3 and group 4 was statistically not significant.

The results presented in Fig. 4 revealed that level of plasma creatinine was significantly increased ($p<0.05$) in group 1 and group 2 as compared to control group. Oral administration of saffron did not cause any significant difference on plasma creatinine of group 3 and 4 compared to untreated group. The level of plasma creatinine on the group 1 significantly

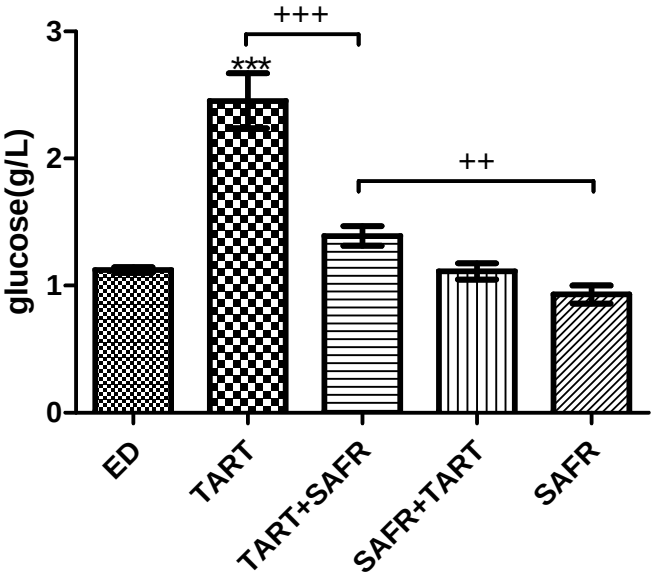

Fig. 3: Effects of Tartrazine and saffron on plasma glucose level. ED: Rats treated with distilled water, Tart: Rats treated at the dose of 10 mg/kg Tartrazine, Tart + safr: Rats treated at the dose of 10 mg/kg Tartrazine + 120 mg/kg saffron, Safr + Tart: Rats treated at the dose of 120 mg/kg saffron + 10 mg/kg Tartrazine and Safr: Rats treated at the dose of 120 mg/kg saffron.

Note: values represent the Mean±SEM of six rats; $^{+++}p<0.001$ highly significantly different from group 2. $^{++}p<0.01$ highly significantly different from controls. ***$p<0.001$ highly significantly different from controls. (+ symbol of comparison with other groups; * symbol of comparison with control group)

decreased compared to group 3 and there was significant difference observed between the group 2 and group 4.

Plasma concentrations of AST and ALT as indicator of liver functions are recorded in table 3. Data revealed that significant difference was observed on ALT between control group, groups treated with Tartrazine only and saffron followed by Tartrazine and on AST between groups treated with Tartrazine followed by saffron and saffron followed by Tartrazine compared

TABLE II : Organ weight of Wistar rats sacrificed on day 105 of subchronic treatment and feeding with Tartrazine and saffron.

Metabolic parameters	*Control group*	*Tartrazine (10 mg/kg)*	*Tartrazine (10 mg) + saffron (120 mg)*	*Saffron (120 mg) + Tartrazine (10 mg)*	*Saffron (120 mg/kg)*
Liver	6.63±0.15	6.68±0.21	6.9±0.18	6.25±0.58	6.51±0.29
Heart	1.00±0.03	0.96±0.04	0.98±0.04	0.85±0.05	0.79±0.03
Right Kidney	0.92±0.03	0.92±0.05	0.92±0.04	0.91±0.04	0.91±0.05

Note: values represent the Mean±SEM of six rats.

TABLE III : Effects of Tartrazine and saffron on plasma AST and ALT. Control group treated with distilled water, Group 1 treated at the dose of 10 mg/kg Tartrazine, Group 2 treated at the dose of 10 mg/kg Tartrazine + 120 mg/kg saffron, Group 3 treated at the dose of 120 mg/kg saffron + 10 mg/kg Tartrazine and Group 4 treated at the dose of 120 mg/kg saffron.

Metabolic parameters	*Control group*	*Tartrazine (10 mg/kg)*	*Tartrazine (10 mg) + saffron (120 mg)*	*Saffron (120 mg) + Tartrazine (10 mg)*	*Saffron (120 mg/kg)*
ALT (U/L)	32,833±1.75	40,75±1.02*	36,5±4.35	42,667±3.40*	37.833±7.36
AST (U/L)	82,333±3.76	85,5±1.22	113,167±21.15*	115,5±5.17*	107.167±11.9

Note: values represent the Mean±SEM of six rats;* p<0.05. Significantly different from controls.

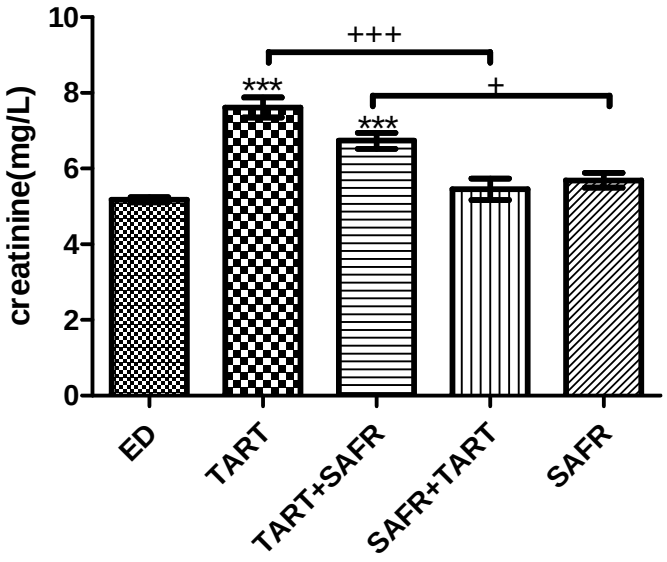

Fig. 4 : Effects of Tartrazine and saffron on plasma creatinine level. ED: Rats treated with distilled water, Tart: Rats treated at the dose of 10 mg/kg Tartrazine, Tart + safr: Rats treated at the dose of 10 mg/kg Tartrazine + 120 mg/kg saffron, Safr + Tart: Rats treated at the dose of 120 mg/kg saffron + 10 mg/kg Tartrazine and Safr: Rats treated at the dose of 120 mg/kg saffron.

Note: values represent the Mean±SEM of six rats; .***p<0.001 highly significantly different to group 3; +p<0.05 significantly different from controls. ***p<0.001 highly significantly different from controls. (+ symbol of comparison with other groups; * symbol of comparison with control group).

to group treated with distilled water.

The chemical composition of crude extract of stigma was determined using HPLC analysis. The chromatographic conditions employed permitted the identification of major components in saffron sample. The compound was identified by comparison of its retention time as previously described in the literature (32). The figure 5 depicts the HPLC chromatogram of the saffron extract at 440 nm. We identified four major peaks of carotenoids:

- Peak 1: crocin-1-*trans* with a retention time of 16,86 and 110,68±0.16 mg/g dry extract;
- Peak 2: crocin-3-*trans* with a retention time of 20,4 and 23.6±0.001 mg/g dry extract;
- Peak 3: crocin-4-*trans* with a retention time of 28,66 and 32,13±0.02 mg/g dry extract;
- Peak 4: crocin-3-*cis* with a retention time of 29,52 and 33,04±0.09 mg/g dry extract;

Discussion

The differences in mean body weight, organ weights and metabolic parameters like pH and urine volume, between control and groups treated with Tartrazine and saffron were not significant. The difference was significant on consumption of food between control group and groups treated with only Tartrazine and

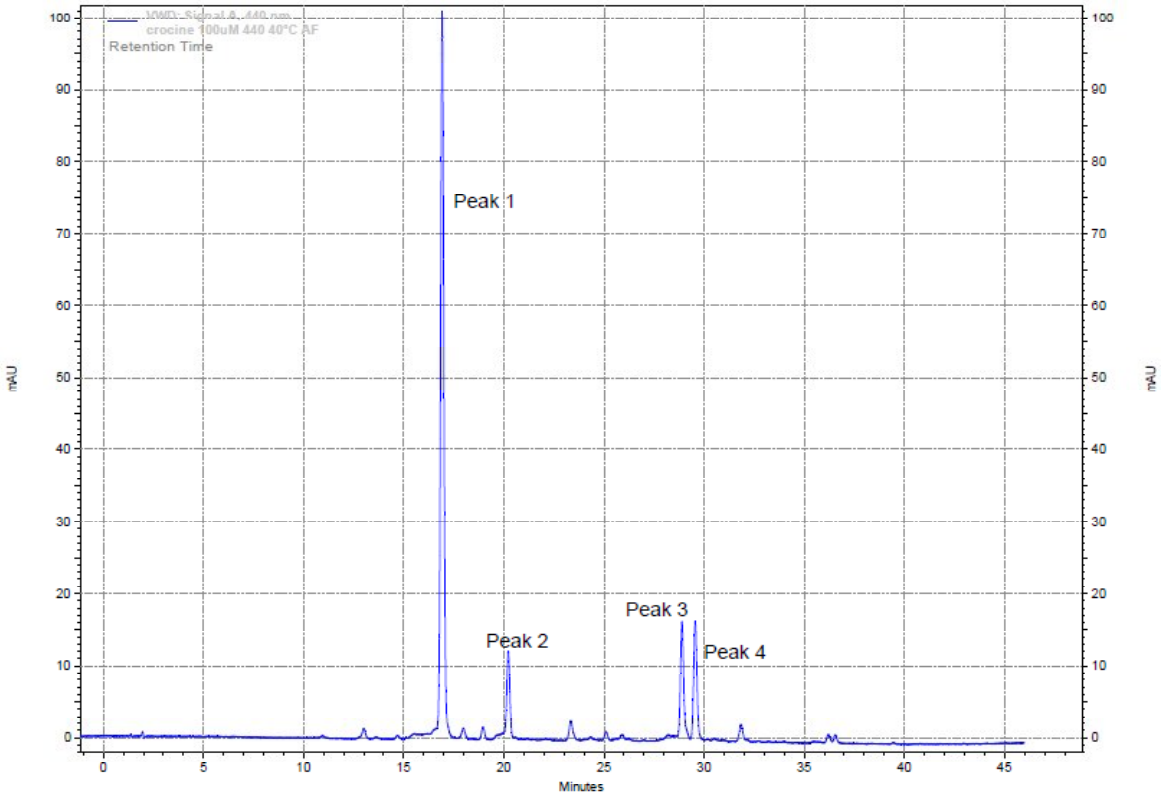

Fig. 5 : HPLC chromatogram of extract of saffron with different peaks of various components of the stigma at 440 nm. A Waters Symetry® C18 column, a linear gradient of methanol (10–100%) in water (15% of acetonitrile), and a flow rate of 1 ml/min were used for qualitative determinations.

saffron + Tartrazine (Group 3). For the water consumption, the difference between control group and group treated only by Tartrazine.

The present study showed that the daily administration of Tartrazine for 105 days induce a significant increase in serum glucose concentration when compared with control rats. These results were alike to Himri's study (16) who observed a significant increase in the serum glucose in rats treated with Tartrazine.

The treatment with saffron showed no significant difference in serum glucose concentration with few diminutions compared to control group, this results was in accordance with Mohajeri (36) who suggest that oral administration and intraperitoneal injection of saffron at different doses reduce the blood glucose levels in healthy rats.

Our work revealed that rats which consumed 10 mg/kg b.w of Tartrazine and followed by 120 mg/kg b.w of saffron and the other way around showed no significant difference in serum glucose concentration when compared to control rats. This outcome prove that consummation of saffron can protect the body from elevation of blood glucose levels and keep it stable, furthermore orally administration of crude extract of saffron decrease the concentration of glucose in blood because this extract contains carotenoids which has antioxidant effect especially crocine who is responsible for these protective effects. Mohajeri (5) exhibited that the ethanolic extract of saffron has significantly decreased blood glucose levels and increased serum insulin in diabetic rats, also Arasteh (30) indicated that the saffron extract and its active constituent significantly decreased serum glucose. The active constituent of *Crocus sativus* L. has antioxidants properties which may be very helpful to reduce defects in insulin secretion hence it prevents diabetes complications (37). In a recent study, Mohajeri showed that saffron extract augmented insulin secretion in diabetic rats (36). This data was in accordance with Hemmati who

demonstrated that hydroalcoholic extracts of saffron increased adiponectin levels therefore the decrease of diabetes by carotenoids crocin, the active ingredients of saffron (38).

Our data indicate that oral administration of saffron caused no significant difference on plasma creatinine of group treated with saffron compared to untreated group. This result was similar to Kianbakht's study who showed that extract of saffron did not have any significant effects on the blood creatinine levels in the diabetic rats after 6 weeks of administration (31).

This study suggests that the level of plasma creatinine was significantly increased in the group treated with Tartrazine and the group of Tartrazine followed by saffron as compared to the control group. Moreover, these results are in accordance with study reported by Himri who observed a significant rise of serum creatinine in rats treated with Tartrazine orally for 90 days and with Ashour who concluded that creatinine level of rats treated by gavage with fast green for 35 days had a significant rise (16, 39). Furthermore our data is also in accordance with the data reported by Amin who observed that when rats treated with high or low dose of Tartrazine (500 mg/kg b.w, 15 mg/kg b.w respectively) a rising level of creatinine (40).

The level of plasma creatinine on rats which consumed Tartrazine significantly decrease compared to rats treated with saffron and followed by Tartrazine. This result showed the protective effect of saffron against elevation of plasma creatinine. Jorns indicated that during interaction with â-cell many substances act with free radicals formed from alloxan and can prevent radical formation or improve diabetogenic effect of alloxan in animals and Assimoupoulo reported that saffron and its active constituents (41) has shown significant radical scavenging activity and good antioxidant activity against free radicals and our finding confirms that consumption of aqueous extract of saffron had a major role including protective effect of vital tissues (liver, pancreas, kidney) (2, 41, 42). This effect could be attributing firstly to scavenging activity of crocine and safranal and to regenerative properties of the extract.

One hundred µL of extracts samples were injected into a liquid chromatography (HPLC) to determine the chemical compounds of the saffron extract. The carotenoid compounds were identified based on their retention times and quantified according to the respective standard calibration curves (Fig. 5). The HPLC chromatogram of the saffron extract indicated crocin and its isomers as the major compound present in the extract with a percentage of safranal.

The peak identification is as follows: number 1 was crocin-1-trans, peak 2 was crocin-3-trans, peak 3 was crocin-4-trans and peak 4 was crocin-3-cis. According to this analysis, different form of crocins were detected in our saffron samples. The HPLC analysis shows that the trans-crocin is the most abundant carotenoid compound in the extract. The finding agrees with previous study reporting the same carotenoids profile in saffron sample (32). This result led as to suggest that crocin might be the principal compound responsible of the antidiabetic, diabetogenic effect and antioxidant activities demonstrated previously.

From this study, we can conclude that oral administration of crude extract of stigmas from *Crocus sativus* Linn. has a significant beneficial effect. In fact, the consumption of this extract reduces blood glucose level and creatinine. These results showed the curative (antidiabetic) and protective (antidiabetogenic) effect of saffron against diabetes induced by Tartrazine. While it has the potential to give therapeutic effect in diabetes. Further studies are necessary to elucidate in detail the mechanism of action of this medicinal plant at the cellular and molecular level. Therefore, saffron may be regarded as a useful therapy for diabetes mellitus.

Conflicts of Interest

The authors declare no conflict of interest.

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Original Article

Evaluation of Antitumor Activity of *Crotalaria Burhia* Buch.-Ham. Roots Against Ehrlich's Ascites Carcinoma Treated Mice

Praful A. Talaviya[1], Bhavesh M. Vyas*[2], Shaival K. Rao[3], Vimal Patel[4] and Sneha Ghadiya[5]

[1]Department of Pharmacology,
Faculty of Pharmaceutical Sciences,
Pacific University, Udaipur, India.

[2]Department of Pharmacology,
AMC-MET Medical College, Ahmedabad India.

[3]Department of Pharmacognosy,
Shree Samanvay Institute of Pharmaceutical Education and Research,
Botad, India.

[4]Department of Pharmacology,
KB Institute of Pharmaceutical Education and Research,
Gandhinagar, India.

[5]Lecturer, Rai University, Saroda, India.

Abstract

The study was aimed to evaluate antitumor activity of methanolic extract of *Crotalaria burhia* Buch.-Ham. roots (MECB) in Swiss Albino mice against Ehrlich's Ascites Carcinoma (EAC). The mice were divided into seven groups (n=20) in which Group I was treated with Sterile Physiological Saline (SPS) (Normal mice), Group II served as EAC control and Group III, IV, V or VI were treated with MECB at 100, 150, 300 or 400 mg/kg body weight i.p., respectively. Group VII were treated with standard drug 5-Fluorouracil (20 mg/kg i.p). The MECB was administered for 9 consecutive days. After 24 hours of last dose and 18 h of fasting, ten mice from each group were sacrificed and remaining mice from each group were kept to evaluate mean survival time. Antitumor activity of MECB was assessed by determining tumor volume, tumor weight, viable cells and nonviable cells count, hematological parameters, biochemical parameters and liver antioxidant status of EAC bearing host. The MECB was shown potent dose dependent antitumor activity. MECB at different doses level showed significant (P<0.05) reduction in tumor volume, tumor weight, and viable cells count with increased the life span of EAC mice. Interestingly, no mortality was observed during nine consecutive days of treatment. In further, the imbalanced hematological parameters of tumor bearing mice was significantly (P<0.05) regained after treatment of MECB. Moreover, altered biochemical parameters such as SGOT, SGPT, ALT, albumin and total protein in EAC mice were regained after treatment of MECB. Fascinatingly, MECB was showed significant antioxidant activity and which was characterized by reducing

***Corresponding author :**

Dr. Bhavesh M. Vyas, Department of Pharmacology, AMC-MET Medical College, Ahmedabad India; Contact No. +919898622896 / +91 9825059149; E-mail: drbmvyas@yahoo.com

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level of lipid peroxidation with increasing the level of catalase and reduced-glutathione. Results of present study revealed that the MECB had significant dose dependent antitumor and antioxidant activity that is comparable to 5-FU. In further, *C. burhia* roots can be a good source as antitumor and antioxidant in future.

Introduction

Plants are a potential and important source for discovery and development of newer pharmacological agents for chemotherapy (1). Numerous plants have been studied for their anticancer activity using various experimental models and this was resulted in availability of nearly 30 effective anticancer molecules (2). Since the ancient times herbs are recognized as a source of remedies. India is a richest source of medicinal plants and known as botanical garden of the world. Different types of plants or plant extracts are used in various systems of medicine (Ayurveda, Unani, and Siddha) for treatment of various diseases. Only a few of them are scientifically evaluated and still many more medicinal plants are left behind to be evaluated. Secondary metabolites of medicinal plants such as flavonoids, terpenes, alkaloids have received considerable attention in recent scenario due to their various pharmacological properties (3, 4).

C. burhia Buch.-Ham. also known as *Khip*, is an under shrub and fibrous plant, commonly found in the arid parts of India, West Pakistan and Afghanistan. In ancient Indian medicinal system of ayurveda, *khip* has been recognized as a potential medicinal plant. The leaves, roots and branches of *C. burhia* is use as a cooling and antitumor medicine, while fresh plant juice is useful for eczema, gout, hydrophobia, pain and swelling. Roots extract with sugar is use to cure chronic kidney pain and roots decoction is use for treatment of typhoid (5, 6).

Phytochemical screening revealed the presence of pyrrolizidine alkaloids as main compounds in *C. burhia*. In addition, flavonoid (quercetin) and steroid (β-sitosterol) were isolated from this plant. Anticancer, anti-inflammatory, analgesic, antimicrobial and antibacterial activities of *C. burhia* have been reported in various literatures (7, 8).

In the present investigation the roots of *C. burhia* was selected for assessment of their antitumor activity, because as per traditional medicine system of India and other Asian countries *C. burhia* roots are commonly use for treatment of tumors, pain, swelling and fever. However, till date there is no report of either ethnopharmacological or pharmacological study to evaluate the antitumor activity. Therefore, the present study was carried out to evaluate antitumor activity of *C. burhia* and establish the scientific basis to supports their traditionally claimed uses.

Materials & Methodology

Materials

Plant material

C. burhia Buch.-Ham. roots were collected from Rajasthan University campus Jaipur, Rajasthan (India), during the months of Oct-Nov 2010. The plant was identified by Mr. P.J. Parmar, Joint Director in Botanical Survey of India (BSI), Jodhpur (Rajasthan, India). Authenticated voucher specimen (JNU/JPR/PC/SK-1) has been deposited at Botanical Survey of India, Jodhpur, India.

Chemical

All the required chemicals were purchased from Merck Ltd., Mumbai, India and standard for biochemical and antioxidant were purchased from Sisco Research Laboratory Mumbai, India. All other chemical and reagent were used of analytical grade.

Animal care and handling

The animal care and handling was done according to guidelines set by the WHO (World Health Organization), Geneva, Switzerland, INSA (Indian

National Science Academy, New Delhi, India) and the "Guide for the care and use of Laboratory Animals" (9). Female Swiss Albino mice weighing 20-22 g was selected from departmental animal house. The mice were divided into groups and housed in polyacrylic cages under standard laboratory conditions (temperature 25±2°C; dark-light cycle [14-10 h]). Mice were allowed free access to standard dry pellet diet (Hindustan Lever, Kolkata, India) and water *ad libitum*. All the described procedures were reviewed and approved by Institutional Animal Ethical Committee of Gyan Vihar School of Pharmacy, Jaipur, India.

Tumor model

EAC cells were procured from the Division of Radiobiology and Toxicology, Manipal Life Science Centre, Manipal University, Manipal, India. The EAC cells were maintained in *Swiss Albino* mice (*in vivo*) by intraperitoneal innoculation of 2×10^6 cells/mice after every 10 days in an aseptic environment. Ascitic fluid was drawn out from EAC tumor bearing mice at the log phase (days 7-8 of tumor bearing) of the tumor cells. Tumor was produced by intraperitoneal injection of 0.1 ml of tumor cell suspension containing 2×10^6 tumor cells.

Methodology

Preparation of methanolic extract

The air and shade dried powdered roots of *C. burhia* was exhaustively extracted with methanol by Soxhlet continuous extraction method. The final extract (MECB) was concentrated under reduced pressure, on a rotary evaporator at 40-45°C and percentage yield of crude extract was 2.1% w/w.

Preparation of drug and treatment mode

The MECB solution was prepared according to method described by Jagetia and Rao (2006) with minor modifications (10). Briefly, MECB and 5-Flurouracil (5-FU) were suspended in Sterile Physiological Saline (SPS) containing 0.5% Carboxy Methyl Cellulose (CMC). All animal were treated via intraperitoneal (i.p.) route of administration.

Acute toxicity study

The acute toxicity study was performed according to method described by Litchfield and Wilcoxon (11). The oral LD_{50} value of MECB in Swiss Albino mice was determined and found to be 2 g/kg body weight.

Treatment schedule

The mice were divided into the following groups (n=20) (12).

All mice in each group except group-I received EAC cell (2×10^6 cell/mouse i.p). This was taken as day '0'.

Group I (Normal Control): mice treated with 0.5 ml/kg SPS i.p, once daily, consecutively for 9 days.

Group II (EAC Control): EAC inoculated mice, served as EAC control (2×10^6 cell/mouse i.p).

Group III (MECB 100): 24 hrs after transplantation of EAC, mice were treated with MECB at the dose of 100 mg/kg body weight i.p, once daily and consecutively for 9 days.

Group IV (MECB 150): 24 hrs after transplantation of EAC, mice were treated with MECB at the dose of 150 mg/kg body weight i.p, once daily and consecutively for 9 days.

Group V (MECB 300): 24 hrs after transplantation of EAC, mice were treated with MECB at the dose of 300 mg/kg body weight i.p, once daily and consecutively for 9 days.

Group VI (MECB 400): 24 hrs after transplantation of EAC, mice were treated with MECB at the dose of 400 mg/kg body weight i.p, once daily and consecutively for 9 days.

Group VII (Standard 5-FU): 24 hrs after transplantation of EAC, mice were treated with 5-flurouracil at the dose of 20 mg/kg body weight, i.p, consecutively for 9 days.

After 24 hrs of last dose and 18 hrs of fasting, 10

mice from each group were sacrificed to determine antitumor and antioxidant activity along with biochemical and hematological parameters. The remaining mice were kept under observation to evaluate life span of the tumor host.

Tumor volume

The ascitic fluid was aspirated aseptically from peritoneal cavity. The volume was measured by taking it in a sterile graduated centrifuge tube (13).

Tumor weight

Tumor weight was measured by evaluating mice weight before and after the collection of ascitic fluid from peritoneal cavity.

Percentage increase in life span

The effect of MECB on percentage increases in life span was calculated on the basis of mortality of experimental mice (14).

$$MST = \frac{\text{First death} + \text{last death in the group}}{2}$$

(MST: mean survival time and time is denoted in days)

The increase in life span (% ILS) was calculated by following formula:

$$ILS(\%) = \frac{\text{MST of treated mice} - \text{MST of control}\ X100}{\text{MST of control}}$$

Tumor cell count

The ascitic fluid was collected by WBC pipette and diluted 100 times. A drop of collected cells suspension was placed on the Neubauer's counting chamber and the numbers of cells in 64 small squares were counted.

Viable and nonviable tumor cell count

Viabile and non viabile EAC cells were counted by trypan blue dye assay. The cells were stained with trypan blue dye (0.4% in normal saline). The cells that did not take up the dye were noted as viable and those are took the dye were noted as nonviable. These viable and nonviable cells were counted by following formula.

$$\text{Cell count} = \frac{\text{Number of cell} \times \text{Dilution factor}}{\text{Area} \times \text{Thickness of film}}$$

Hematological and biochemical parameters

At end of the experimental period, the next day after an overnight fasting blood samples were collected from tail vein and used for estimation of hematological (hemoglobin (Hb) content, red blood cell (RBCs) count, white blood cell (WBCs) count & lymphocyte count) and biochemical parameters (serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), albumin, alkaline phosphatase and total protein) by standard procedures. Further, the mice were sacrificed and liver were taken out for evaluation of antioxidant activity.

Antioxidant activity

Ten percent of liver tissue homogenate was prepared in Tris-HCl buffer (0.1M, pH 7.4) for estimation of lipid peroxidation (15), catalase (16) and reduced glutathione (17) by following standard procedures.

Statistical analysis

All the data are expressed as Mean±S.E.M. (n=10 mice per group). The data were analyzed by one-way ANOVA between the treated group and the EAC control followed by dunnett's post hoc test. The $P<0.05$ and $P<0.01$ were considered significant and highly significant, respectively.

Results

Intraperitoneal administration of methanolic extract of *C. burhia* (MECB) was shown dose depended antitumor activity in EAC bearing mice. No mortality was observed for nine consecutive days. Antitumor activity of MECB against EAC was assessed by evaluating tumor volume, tumor weight, viable and

non-viable cells count, MST and %ILS (Table I). Administration of MECB at different doses level caused significant (P<0.05) reduction in tumor volume, tumor weight and viable cells count, and increased MST, %ILS and non-viable cells count when compared to EAC control group (Table I). High dose of MECB (400 mg/kg) was found with higher antitumor activity than 300 mg/kg and followed by 150 mg/kg. Lower dose of MECB (100 mg/kg) was shown negligible antitumor activity (Table I).

Moreover, MST of EAC control group was noted 20 days, while it was significantly (P<0.05) increased by 1 day (MST was 21 days; increased 10% ILS), 9.5 days (MST was 29.5 days; increased 47.5% ILS), 17 days (MST was 37 days; increased 85% ILS), and 19.5 days (MST was 39.5 days; increased 97.5% ILS) with treatment of MECB at the dose of 100 mg/kg, 150 mg/kg, 300 mg/kg and 400 mg/kg, respectively, when compared to EAC control group. MECB at the low dose (100 mg/kg) had negligible impact on MST and %ILS.

On 10th day, after the completion of study period, hematological parameters of EAC bearing mice were found significantly altered when compared to normal control group (Table II). The total WBCs count was increased and RBCs count was decreased in EAC bearing mice when compared normal group. In differential count, WBCs and the percentage of lymphocytes were found elevated in EAC bearing mice when compared to normal mice. Intraperitoneal administration of MECB at different doses were shown significant (P<0.05) effect to regain altered hematological parameters towards saline control in dose depended manners (Table II).

Effect of MECB on biochemical parameters of tumor bearing mice is shown in Table III. The biochemical parameters such as SGOT, SGPT and ALT level were increased, whereas albumin level and total protein content were significantly decreased in tumor bearing mice when compared to saline treated mice. In MECB treated mice, the biochemical parameters showed significant restoration toward saline control (Table III).

TABLE I: Effect of the MECB on tumor volume, tumor weight, mean survival time (MST), percentage increase life span (%ILS), viable cells and nonviable tumor cells count in EAC bearing mice.

Parameters	*EAC control*	*100 mg/kg*	*150 mg/kg*	*300 mg/kg*	*400 mg/kg*	*5-FU*
Tumor volume (ml)	3.27±0.1	3.1±0.15^{NS}	1.53±0.11*	1.13±0.11*	0.68±0.17*	0.46±0.03*
Tumor weight (g)	3.5±0.13	2.93±0.25*	1.17±0.11*	1.18±0.16*	0.71±0.16*	0.54±0.04*
MST (day)	20	21	29.5	37	39.5	42
%ILS	0	10	47.5	85	97.5	107.5
Viable cell	8.21×10^7±0.33	7.11×10^7±0.3	3.62×10^7±0.29*	2.44×10^7±0.22**	1.4×10^7±0.1**	0.84×10^7±0.1**
Nonviable cell	0.35×10^7±0.06	0.67×10^7±0.2	1.13×10^7±0.11**	2.13×10^7±0.19*	3.09×10^7±0.11*	3.6×10^7±0.15**
Total cell	8.56×107	7.78×10^7	4.75×10^7	4.57×10^7	4.49×10^7	4.44×10^7
Viable %	95.91	91.38	79.38	53.39	31.18	19.14
Non viable %	4.08	8.61	20.61	46.6	68.81	81.08

Values are expressed as Mean±S.E.M. (n=10).
*P<0.05, **P<0.01, Values are significantly different when compared with EAC control

TABLE II: Effect of the MECB on hematological parameters in EAC bearing mice.

Parameters	*WBC (cell×10^6/µl)*	*RBC (cell×10^6/µl)*	*Lymphocytes (%)*	*Hemoglobin (g/dl)*
Normal	6.2±0.54	6.6±0.47	75.5±0.3	12.46±0.42
EAC	15.9±0.27**	3.9±0.23*	86.3±0.36**	4.97±0.03**
100 mg/kg	14.7±0.3^a	4.06±1.2^b	85.7±0.5^b	5.39±0.05^a
150 mg/kg	12.4±0.28^b	4.5±0.22^a	78.9±0.3^b	6.22±0.16^b
300 mg/kg	10.2±0.8^a	5.1±0.23^a	76.6±0.4^a	7.62±0.17^a
400 mg/kg	8.63±0.2^a	5.54±0.31^b	75.93±0.1^b	8.71±0.3^b
5-FU	7.7±.0.17^a	6.1±0.17^a	74.1±0.4^b	10.6±0.17^b

Value are expressed as Mean±S.E.M. (n=10).
*P<0.05, **P<0.01, Values are significantly different when with control (Normal).
^{a}P<0.05, ^{b}P<0.01, Values are significantly different when with EAC control.

TABLE III: Effect of the MECB on biochemical parameter in EAC bearing mice.

Treatment	*Total protein (gm/dl)*	*Albumin (gm/dl)*	*SGOT (IU/L)*	*SGPT (IU/L)*	*ALP (IU/L)*
Normal	5.65±0.29	1.6±0.09	34.08±1.16	28.17±1.89	77.44±3.47
EAC control	2.9±0.18*	1.17±0.1**	64.97±1.72**	59.14±1.82**	122±2.55*
100 mg/kg	3.14±0.2[a]	1.2±0.1[b]	61.17±1.32[a]	54.37±0.82[b]	116.1±1.9[a]
150 mg/kg	3.54±0.27[b]	1.25±0.08[a]	56.75±2.42[b]	50.14±0.59[b]	106.57±1.98[a]
300 mg/kg	4.4±0.5[a]	1.34±0.02[a]	49.52±1.04[a]	40.32±1.83[a]	94.65±2.81[b]
400 mg/kg	4.98±3.8[a]	1.43±2.61[b]	43.97±1.63[a]	34.17±2.25[a]	87±2.75[a]

Value are expressed as Mean±S.E.M. (n=10).
*P<0.05, **P<0.01, Values are significantly different when compared with control (Normal).
[a]P<0.05, [b]P<0.01, Values are significantly different when compared with EAC control.

TABLE IV: Effect of MECB on lipid peroxidation and antioxidant parameters in EAC bearing mice.

Treatment	*Lipid peroxidation (nMol/mg protein)*	*Catalase (Unit/mg/protein/min)*	*Reduced-glutathione (mMol/gm)*
Normal	162.28±0.54	4.36±0.22	28.87±0.56
EAC control	438.48±0.3**	1.3±0.19**	7.46±0.2**
100 mg/kg	389.68±0.23[a]	1.96±0.25[b]	9.16±0.2[b]
150 mg/kg	325.19±0.2[a]	2.52±0.07[a]	12.92±0.2[a]
300 mg/kg)	234.96±0.18[a]	3.2±0.5[b]	22.63±0.21[a]
400 mg/kg	197.3±0.4[a]	3.97±0.09[b]	24.73±0.1[a]

Value are expressed as Mean±S.E.M. (n=10).
*P<0.05, **P<0.01, Values are significantly different compared with control (Normal).
[a]P<0.05, [b]P<0.01, Values are significantly different when compared with EAC control.

As shown in Table IV, significant elevation in lipid peroxidation were observed in tumor bearing mice when compared to normal mice (P<0.05). MECB treatment at different tested dose level caused significant reduction in lipid peroxidation. In addition, the level of reduced-glutathione and catalase were significantly decreased in EAC mice when compared to EAC treated mice. Administration of MECB at different doses level was able to restore the level of reduced-glutathione and catalase toward normal in tumor bearing mice (Table IV). Finally, results clearly indicate that the MECB has remarkable capacity to inhibit the growth of EAC tumor in a dose dependent manner.

DISCUSSION

Preliminary phytochemical investigation was indicated that the MECB is rich in alkaloids, flavanoids and steroids. Previously alkaloids, flavanoids and steroids are reported with antitumor activity. Flavanoid have been shown to possess antimutagenic and antimalignant effect (18). In present investigation, MECB was shown potent dose depended antitumor and antioxidant activity in EAC bearing mice.

Prolongation of life span and reduction in WBCs count are the reliable criteria for judging antitumor activity of any medicinal agents. In this study, reduction in tumor volume and increased mean survival time of EAC bearing mice imply the delaying impact of MECB on EAC cell division (14). In present study, MECB has shown antitumor activity in dose depended manner, which is amusingly supported by increased life span and reduced viable cells count further supported by reduced tumor volume and tumor weight.

A regular and rapid raised in ascites tumor volume were noted in EAC bearing mice. An ascitic fluid is direct nutritional source for tumor cell and rapid raise in ascitic fluid with growth of tumor would be a means to meet nutritional requirement of EAC cell.

In the present study, reduction in tumor volume, tumor weight, and viable tumor cells count, and prolongation of life span and increased number of nonviable cells were noted in tumor bearing mice after treatment of

MECB when compared to EAC control mice. So this event is suggesting that the MECB had promising effect to prolong life span of EAC bearing mice by diminishing nutritional fluid volume with arresting tumor growth. In further, MECB decreased the numbers of viable cells count and increased number of nonviable cells in tumor bearing mice. These exploit indicates that the MECB has direct association with tumor cell as tumor cell directly absorbed drug from peritoneal cavity and this antitumor agent produces lysis action on cell wall by direct cytotoxic mechanism (19).

Myelosuppression and anemia are major complication during cancer chemotherapy. The anemia encountered in tumor bearing mice is mainly due to reduction in RBC or hemoglobin content. This may occur either due to iron deficiency or due to hemolytic or myelopathic condition (20). Treatment with MECB brought back the hemoglobin content, RBCs and WBCs count more or less towards normal levels in EAC bearing mice. This indicates that the MECB possess non toxic or protective effect on the hemopoietic system of tumor bearing mice (21).

Superoxide dismutase and catalase enzyme in serum are markers for early indication or detection of neoplasia and also helps to judge progression and regression of disease (22). Hepatotoxicity may occur due to cytotoxic agent itself or due to its toxic metabolites. In certain circumstances they can be carcinogenic (i.e. they may themselves cause cancer). Rapid cell destruction with extensive purine catabolism can be characterize with precipitation of ureates in renal tubules and which is responsible for kidney damage. Elevated level of biochemical parameters such as SGOT, SGPT and ALP is reported, whereas the levels of total protein and albumin were decreased in tumor bearing mice (22). In present investigation the MECB showed promising effect to restore these biochemical parameters toward normal level that means the MECB did not produce toxicity on liver or kidney.

The Ehrlich's tumor growth induces an inhibition of superoxide dismutase and catalase enzymes (23) which are fundamental in the elimination of free radicals as superoxide and hydrogen peroxide (24). In Ehrlich's tumor-bearing mice the antioxidant acts by modulating lipid peroxidation and augmenting antioxidant defense system (25).

Oxidative stress which is one of the key factor in cancer pathology and that is evident in the present study by reduced levels of glutathione in tumor bearing mice. Elevated lipid peroxidation is producing degeneration effect on tissues as the consequences of excessive oxidation is also reported in the present study lipid peroxide aggravate the damage by propagating process of lipid peroxidations (26). Malondialdehyd (MDA), the end product of lipid peroxidation was reported too higher in carcinomatous tissue than in non disease tissue (27), and their levels can be correlated with tumor progression (28). Moreover, MDA is reported as a tumor promoter and co-carcinogenic agent because of its high cytotoxicity and inhibitory action on protective enzymes (26, 29). Glutathione is a potent inhibitor of neoplastic process, which plays an important role as an endogenous antioxidant system (30). The potent reduction in lipid peroxidation and elevation in glutathione (GSH) and catalase (CAT) level were observed in MECB treated mice.

Conclusion

In conclusion, the present study illustrates that the MECB reduces tumor volume and tumor weight, increases the mean survival time and life span of EAC bearing mice. Decreasing lipid peroxidation and thereby augmented the endogenous live antioxidant enzymes in liver. Improvement in hematological pofile and all other biochemical parameters suggests that the methanolic extract of *C. burhia* roots exhibits potent antitumor and antioxidant activity. Further pharmacological and phytochemical investigations are required to elucidate exact mechanism and active chemical constituents which are responsible for their antitumor and antioxidant activity. Thus our present study suggests that the MECB possess potent dose depended antitumor and antioxidant activity against EAC tumor. In future, *C. burhia* can be a good source for cancer treatment.

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Medical Education / Original Article

Motivating but Not Labelling the Students: A Qualitative Study on the Preferences of Learning Styles Among Undergraduate Medical Students

Raja TAR[1] and R. Priyadharsini[2]*

[1]Professor, Department of Pharmacology,
Melmaruvathur Adhiparasakthi Institute of Medical Sciences (MAPIMS),
Affiliated to MGR Medical University, Tamil Nadu – 603 319, India.

[2]Assistant Professor, Department of Pharmacology,
Indira Gandhi Medical College and Research Institute (IGMC&RI),
Puducherry – 605 009, India.

Abstract

Introduction: Every individual has his/her learning style preferences. A student should not be labelled as a particular type of learner rather he/she may be empowered with the different learning style preferences. Visual-aural-read/write-kinaesthetic (VARK) is a sixteen-item questionnaire which defines the preference of learning based on sensory modalities.

Aims: The aim of this study was to assess preferences in learning style among undergraduate medical students and to compare their relation to gender.

Methods and Material: This was a descriptive cross-sectional questionnaire based study. Institute Ethics Committee approval and informed consent were obtained prior to start of this study. Preferred modes of learning of 300 undergraduate medical students were assessed by means of self-administered VARK questionnaire. A p value < 0.05 was considered statistically significant.

Results: Among 300 students, 172 (57%) were females and 128 (43%) were males. Nearly 228 (76%) had unimodal learning style and 72 (23.9%) preferred multimodal learning. Among unimodal learners, a majority (37%) were of auditory learners followed by kinaesthetic (25.3%). There was a significant difference ($p<0.008$) between the learning style preferences among males and females.

Conclusions: A majority of students had unimodal learning style. Male and female students differed significantly in their learning style preferences. Understanding different concepts of learning preferences and incorporation of appropriate unimodal and multimodal teaching methods will be beneficial in helping teachers in facilitating student's learning. This paves way for improving quality of teaching and learning attitudes among medical professionals. Thus, a student should not be labelled as a particular type of learner rather he/she may be empowered with the different learning style preferences.

***Corresponding author :**

Dr. R. Priyadharsini, Assistant Professor, Department of Pharmacology, Indira Gandhi Medical College and Research Institute (IGMCRI), Puducherry – 605 009, India; Phone: +91-9943491252; Email: shalipriya85@gmail.com

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Introduction

The art of learning differs from individual to individual. Each and every individual has his/her learning style preferences. Interestingly the different learning styles are neither superior nor inferior to one another, but they differ from each other with their own advantages and disadvantages (1). Thus, these styles makes the learning process in an easier way and thereby the processed information can be retained in the memory for a longer period. The majority of the preferences in learning varies with the type of information to be processed and the situation in which it really occurs. There are different theories and models pertaining to learning styles (2). These can be classified in general into personality, information processing, social interaction and instructional preferences models. 'Personality model' classifies a person as an introvert or an extrovert in relation to the manner of student's reaction to the information being processed. The 'information processing model' concentrates on the processing of acquired information to be stored for a longer period. 'Social interaction model' focuses on the behaviour and the interaction of a student in a classroom. Instructional preferences model categorises a person on the basis of sensory modality of perceptions (3). Visual, aural, reading/writing and kinaesthetic (VARK) is one of the instruments which can be classified under instructional preferences model of learning. The VARK questionnaire was developed based on three principles. Everyone can learn through their own style of preference. Different preferences in learning style make them get motivated and empowered. By this way, students learn the education process by experience, projection, contemplation, and accomplishment (4, 5). Even though we practise the multimodal way of teaching in institutes nowadays, we still were not able to find whether they adequately address the different type of learners among the male and female students. Further a student should not be labelled as a particular type of learner rather he/she may be empowered with the different learning style preferences. Moreover studies have shown controversial results on the gender differences in learning style preferences also (1, 6–8). Hence, we assessed the preferences in learning styles among undergraduate medical students and compared their relation to gender.

Materials and Methods

This was a descriptive cross-sectional questionnaire-based study conducted during regular working hours in the college premises between February and April 2016. Institute Ethics Committee approval and informed consent were obtained verbally before initiation of the study. Preferred modes of learning of 300 prefinal and final year undergraduate medical students were assessed using self-administered VARK questionnaire (© Copyright Version 7.8 (2014) held by VARK Learn Limited, Christchurch, New Zealand with prior permission obtained from the developer). Convenient sampling method was used. Specific instructions for answering the questionnaire were provided to all the medical students and were asked to return the completed questionnaire to the investigator. The questionnaire consisted of sixteen multiple choice questions with four responses, and the students were asked to select either single or multiple responses. VARK questionnaire assesses the students' preferred mode of learning by giving a scenario (real life situations) with four responses and each response will represent one of the learning styles (V/A/R/K) or a particular preferred sensory modality. The sensory modality with the highest score will be the overall preference of that particular student. All the students participated voluntarily and were free to clarify their questions regarding filling up of questionnaire. They were asked to mention their gender and steps were taken to ensure that no name or initials be recorded. The obtained data were entered in the Microsoft Excel spread sheet. Variables or individual VARK components were expressed as mean±standard deviation and frequencies in percentages of students. The statistical analysis was performed using SPSS for Windows version 16.0 software (SPSS, Chicago, USA). Chi-square and independent samples t-test was used wherever it is appropriate. A p-value < 0.05 was considered statistically significant.

Results

At the end of the study period, a total of 300 medical

students participated in the study and completed the questionnaire. Among the 300 students, 172 were females (57%) and 128 were males (43%). Nearly 228 (76%) students had unimodal learning style and remaining 72 (23.9%) preferred multimodal learning. Thus, majority of students preferred a single sensory modality of learning. Among the unimodal learners, a majority, nearly 111 students (37%) preferred Aural (A) type or were auditory learners followed by 25.3% kinaesthetic learners (n=76). The multimodal learners include 20.3% bimodal (n=61), 3.3% trimodal (n=10) and 0.3% quadrimodal (n=1) (Fig. 1). In the bimodal learners, most of them preferred AK, a combination of auditory and kinaesthetic styles (10%) followed by VA contributing to 3.3%. However, in the trimodal learners, VAK and ARK styles contributed equally (1.3%) when compared to VAR (0.7%). The quadrimodal learners (VARK) were around 0.3% (Fig. 2). The mean scores for individual VARK components were also assessed. The auditory learners had a higher mean score of around 7.01±2.658 followed by kinaesthetic learners 6.26±2.447. The visual learners had a score of 4.88±2.648 and the Read/Write learners had the lowest mean score of 4.22±2.451. Fig. 3 shows the pattern of gender differences among the different modes of learners. There was a significant difference ($p<0.008$) between the learning style preferences of males and females (Fig. 4). The female students had a higher preference for unimodal and quadrimodal learning while the male students preferred bimodal and trimodal learning. Table I

TABLE I: Mean scores of individual VARK components based on sex.

VARK Component	*Mean score*	*P value*
Visual		
Men	4.43±2.616	0.011
Women	5.21±2.630	
Auditory		
Men	6.88±2.718	0.438
Women	7.12±2.615	
Read/Write		
Men	4.05±2.364	0.283
Women	4.35±2.512	
Kinaesthetic		
Men	5.66±2.138	0.000
Women	6.71±2.570	

Values are expressed as Mean±SD. Mean scores compared using Independent samples t-test. p value <0.05 considered statistically significant.

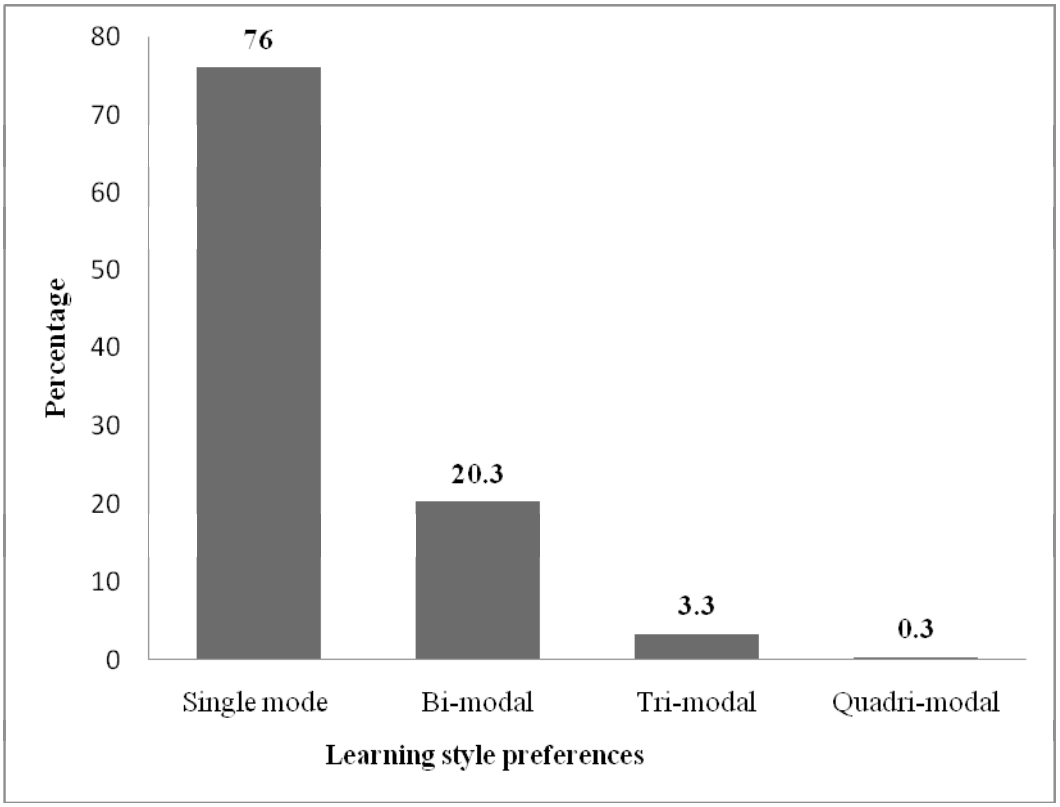

Fig. 1: Distribution of single and multiple learning preferences among medical students (n=300).

depicts the mean scores of different learning style preferences based on sex among the individual VARK components. It was found that there was a significant difference between the males and females among the visual (P<0.011) and kinaesthetic learners when compared to the other modalities of learning.

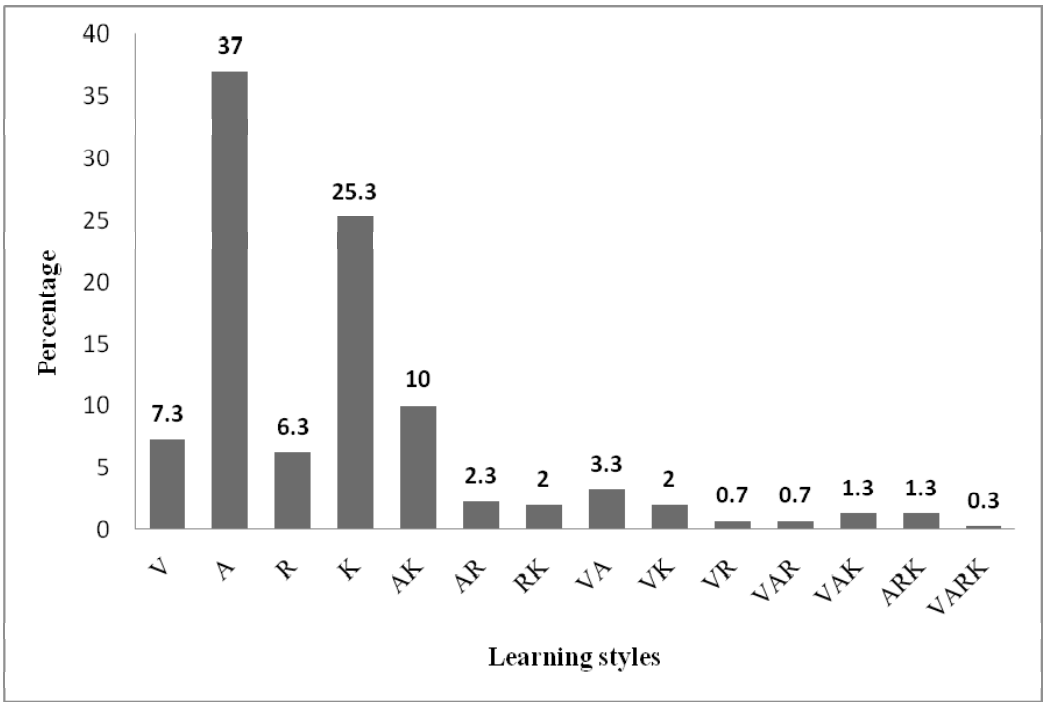

Fig. 2 : Pattern of different learning style preferences among medical students (n=300).

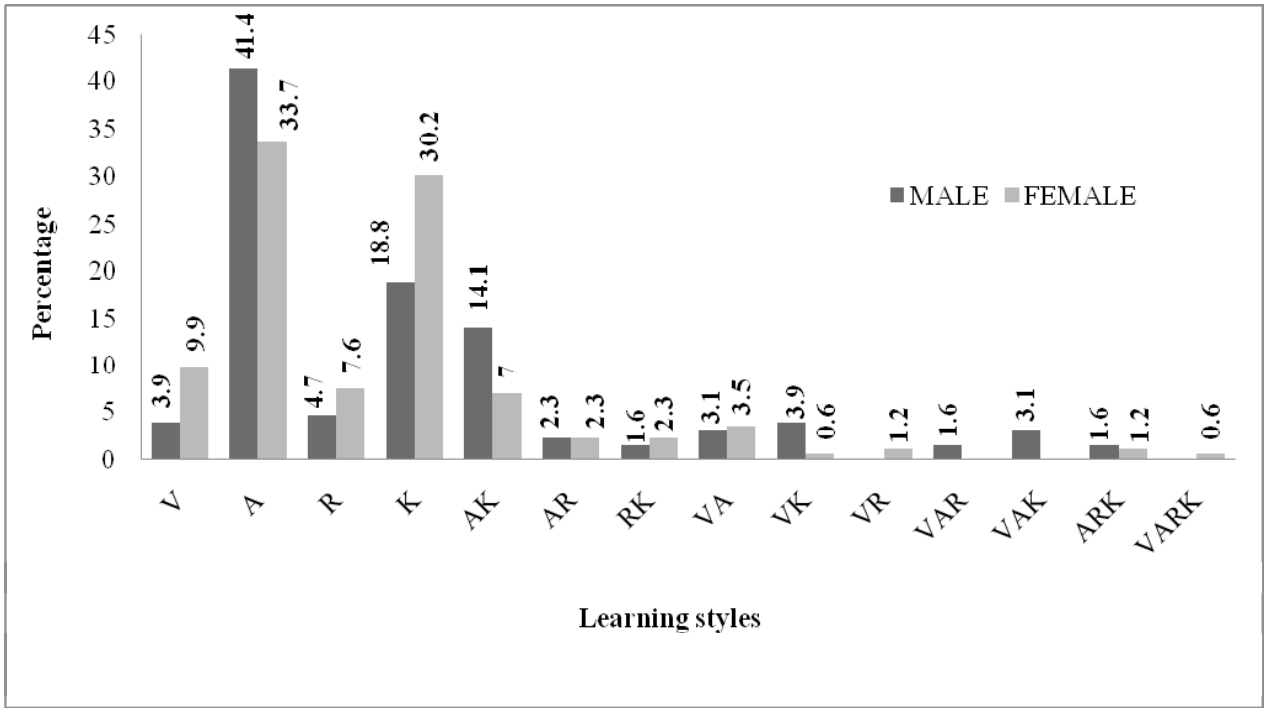

Fig. 3 : Pattern of gender differences among individual VARK components (M=128; F=172).

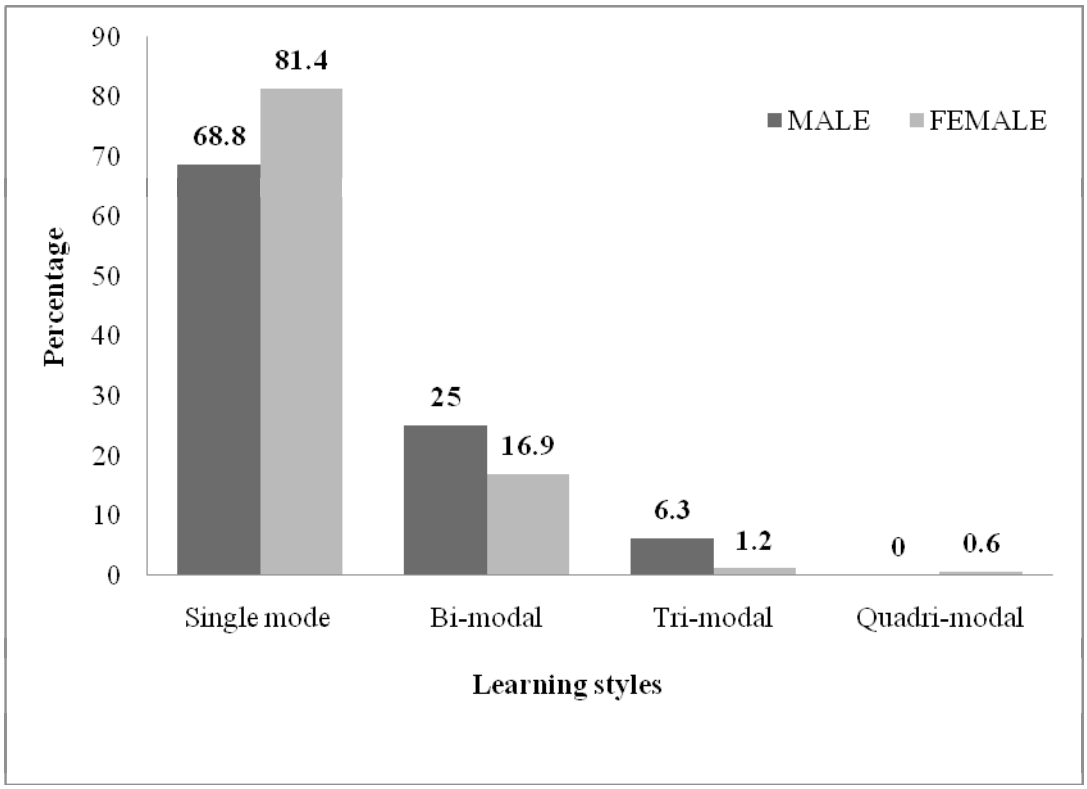

Fig. 4 : Gender differences among learning style preferences (M=128; F=172).

Discussion

Learning should be an active process. All students should acquire deep understanding of the subject through the process of preferential learning (9). Bhagat *et al.* (2015) have stated that the awareness of learning styles, among students, motivated them to adapt to other learning strategies (10). Thus incorporating appropriate learning style model is essential for better and in-depth understanding of the subjects. There are several inventories which can be used for assessing learning style preferences. This study was undertaken with the intention to assess the preferences in learning style among the prefinal and final year undergraduate medical students using the VARK questionnaire which falls under the instructional preferences model. In the present study nearly 76% of students were unimodal learners (Aural-37%, Kinaesthetic-25.3, Read/write-6.3%, visual-7.3%) and remaining 23.9% were multimodal learners. These findings were almost similar to the study done by Marwaha *et al.* (2015) in North India. In their study 51% students preferred unimodal learning style with Kinesthetic (27%), Aural (15%), read/write (6%) and visual mode (3%) followed by 49% students preferring multiple modes (bi-modal (23%), tri-modal (17%), quad-modal preference (9%). V score of females was significantly higher when compared to males (p=0.004) (11). However, in contrary to the present study, in majority of the studies done in different parts of the world, multi-modal learning style (quadri-modal being the major) was the dominant learning style of students (6–8,12–23). The reason behind these may be due to the fact that the learning style preferences may vary among individuals based on the socio-economic status, culture, the level of preschool education etc (3). Hence both unimodal, as well as multimodality of teaching, should be offered to the students as the learning preferences may vary depending on the situational need. There should be a blend of activities to the students which may stimulate all the four sensory modalities apart from the regular didactic lectures. Thus, visual learners may be benefitted from using demonstrations, models, charts and simulations. The auditory learners may be satisfied with discussions during role play, seminars, debates

etc. The read/write and kinaesthetic learners may assimilate information through printed words and practical applications. Our study also depicted that majority of the students were auditory learners followed by kinaesthetics. These results were almost similar to that shown by the other studies (1, 6, 21). Thus, the current strategy of teaching should incorporate the use of multimedia technology approaches which can provide opportunities for auditory and kinaesthetic learners.

It has been found that gender differences also play a significant role in learning process. A study done by cheong *et al.* in 2004 has shown that male students have a preference for logical and rational evaluation, and the female students have preferences over elaborative processing (tend to look for personal connections and relevance with learning material). Moreover, male students are more achievement oriented whereas the female students are social and performance oriented (24). Thus in the present study, the female students had a higher preference for unimodal and quadrimodal learning while the male students preferred bimodal and trimodal learning. There was also a significant difference in gender among the visual ($P<0.011$) and kinaesthetic learners when compared to the other modalities of learning which is almost similar to the other studies (1, 6, 19, 25). But in contrary to this scenario some studies have shown that learning styles did not differ much between the male and the female students (7, 8, 23, 26).

In the past, in our institution, the theoretical lectures in pharmacology were delivered through chalk & board teaching and by using PowerPoint lectures. Practical classes were delivered through demonstrations through charts and discussions through PowerPoint presentations. Based on the findings of our study, since the modalities of learning differ from student to student, the modalities of teaching should also be changed depending on the need. Thus, at present, in our institution, brainstorming sessions, computer simulations, small and large group discussions, role plays, debates were introduced and an appropriate feedback from the learners was obtained. In this way, the lectures and practicals in pharmacology may be best assimilated in a better way by all the four sensory modalities of learners. Thus the selection of appropriate T-L methods with a better understanding of learning style preferences may help us to refine the teaching curriculum in Pharmacology for a better academic performance.

Our study attempted to explore the possibility of describing the learning style preferences of undergraduate medical students in our institute. However, we did not compare the learning style preferences with academic achievements of the students which may be considered as one of the limitations of our study. The other limitation was that we did not compare the questionnaire based learning style preferences with the self-perceived learning style preferences of every individual student. Further, the comparison between the preferences in the learning style between the prefinal and the final year students was not made. We also did not consider the confounding factors such as the age, socioeconomic status, culture, etc. which may have a substantial effect on the study. Thus, based on the preferences in learning styles, a student should not be labelled as a particular type of learner rather he/she may be empowered with the different learning style preferences.

Conclusion

In the present study majority of students are unimodal learners. Male and female students differed from each other significantly in their learning style preferences. Hence, incorporation of appropriate unimodal and multimodal teaching-learning methods plays an essential role in strengthening the quality of medical education depending on the needs of the students. Further, a better understanding of different concepts of learning preferences will be beneficial in helping teachers in facilitating student's learning. Thus, the knowledge of learning style preferences should act as a catalyst for student learning rather than labelling them as a particular type of learner. This understanding paves way for improving the quality of teaching and learning attitudes among medical professionals.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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Obituary

Professor Narayanji Krishnaji Bhide (1928-2017) : Exceptional, Eccentric and Extraordinary

Prof. Narayanji Krishnaji Bhide
(1928-2017)

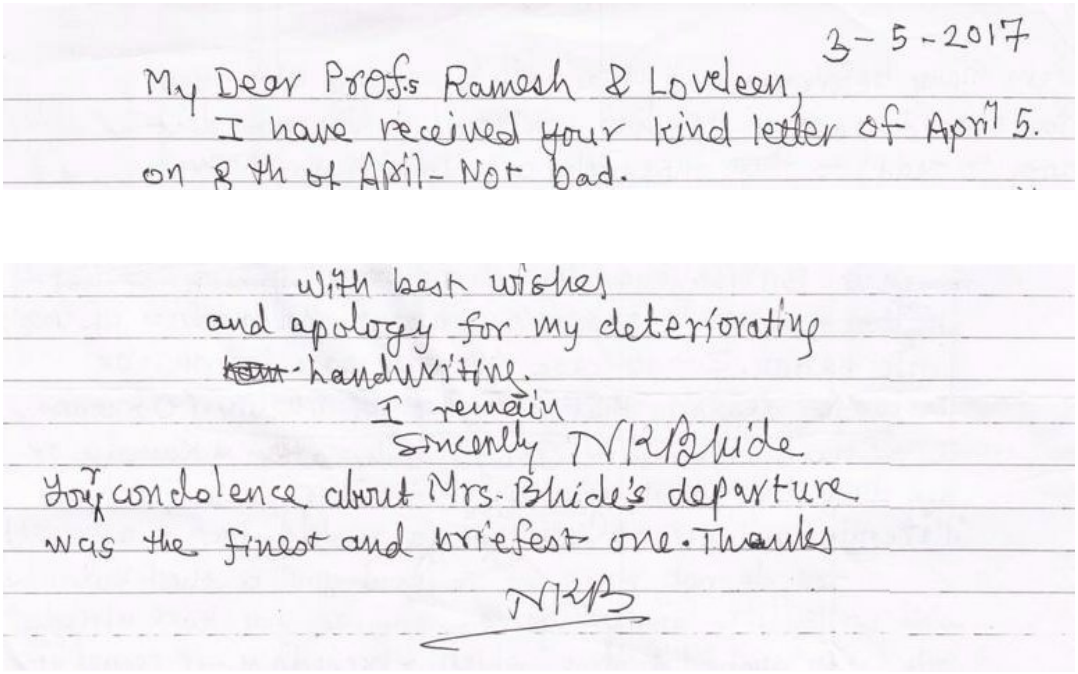
The year was 1966. Imagine a first year medical student absorbed in reading something on a notice board in the department of Physiology on the second floor. He receives a light touch on his back. He turns around and discovers that that the hand that had touched him belongs to a middle aged man. The man asks him, “Young man, are you aware of the fact that your right shoulder is at a slightly lower level than the left?” The boy, still recovering from the surprise, just manages to say “No sir”. And soon the man starts walking away to climb the staircase. I was the medical student, and the man who had touched me was Dr. N.K. Bhide, as I discovered after passing my first MBBS examination when he started teaching me Pharmacology. A few years later, after finishing my MBBS, once I reminded him about this episode and asked him about the significance of the lower level of the right shoulder. He said, “It means your right leg is probably shorter than the left. It won’t do you any harm now, but gradually the spine may start curving, and by old age there could be symptoms due to pressure on the nerve roots.” I asked him, “Sir, what can be done about it?” He said, “You may need a special shoe, in which the right heel is higher than the left heel”. This episode tells many things about Professor Narayanji Krishnaji Bhide (1928-2017). He was a very good observer. Nobody else has ever pointed out to me the difference in the levels of my shoulders. His knowledge went far beyond Pharmacology. The treatment that he suggested was based on commonsense, an uncommon commodity, of which he had plenty. And it taught me three general things about the art and science of medicine: the importance of good observation, the place of logic and commonsense in diagnosis and treatment, and the limitations of medicine as a science.

Dr. Bhide was a remarkable man, who might have given me at the most 20 classes in Pharmacology in 1967-68, but the impression that he left on me led to a lifelong intimate relationship. In one of his classes, he asked “What is a poison?” I replied, “It is a substance that kills even when consumed in small amounts”. He said, “That is what the popular view about a poison is, and I am glad you articulated it. But, broadly speaking, a poison is a substance which has an adverse effect on protoplasm. And, in that sense, almost anything can be a poison in certain circumstances. A food can be a poison and so can water be in some situations.” His way of looking at a poison was not based on a textbook definition. It reflected his originality and breadth of vision, which he inculcated through such interactions. The same applies to his definition of research: “Research is original and critical intellectual activity”. I have yet to come across a more comprehensive definition, one that can encompass even research in languages or literature. It was Dr. Bhide who in his classes sometimes dwelt upon George Bernard Shaw and his play *The Doctor’s Dilemma*, or Paul de Kruif’s *Microbe Hunters*, and students like me would go and hunt for these books in the library. In his class on antihistaminics, he said, “You students have an enormous capacity to misunderstand the teacher”, and then went on to justify the allegation. He said, “I asked a full question on antihistaminics in an exam given to one of your senior batches. The students came and complained, ‘Sir, in your class, you had said that antihistaminics are not important, and then you have asked a full question on antihistaminics’. What I had said in the class was that the antihistaminics had not fulfilled the high expectations that had built up

soon after their discovery regarding their value in the treatment of allergies. But that does not mean that you cannot get a question on antihistaminics in the exam. There is a big difference between the two". This was a lesson in precision in the use of language, and clear thinking.

After finishing MBBS, I joined physiology but my interactions with Professor Bhide continued. I went to him whenever I needed the advice of a wise man, and such occasions were by no means rare. On his side, he passed on to me material on a wide range of subjects including, but not limited to, history of medicine, medical education, public speaking and scientific writing. He gifted me a set of books on the philosophy of science, as well as Charles Dickens' *Pickwick Papers* and Alexandre Dumas' *The Three Musketeers*. After his retirement, he moved to Pune but continued to write to me and send me material by post, as well as recommend books that I may read. In the last such letter he offered to send me a biography of Pythagoras, which he said he would get photocopied and post it to me if I wanted it. He wrote that Pythagoras was not just a mathematician but also a musician and a philosopher with a mystic bent of mind, and that many of his ideas resembled Vedanta. I did a Google search on Pythagoras, and just on Wikipedia I found more than I could read, but was happy to discover, thanks to Prof. Bhide, more about a person whom I had so far identified with just the Pythagoras' theorem. I was amazed that at almost 90, Professor Bhide still had the stamina to get a book photocopied, pack it in an envelope and post it to me. So, very politely, I told him that I would never have the time to read the book because now I am also 70 and read very little, and so he need not take the trouble. Further I told him that times have changed. Now in one second I can find out a lot about Pythagoras by turning to Professor Google. But he did not give up, and wrote again on 3 May 2017 that I must accept his offer, to which I again replied politely and at length saying why I did not want him to go through all the trouble of posting the material to me. These were the last letters we exchanged, and I regret that I disappointed him. I do hope he will forgive me wherever he is.

Prof. Narayanji Krishnaji Bhide
(1928-2017)



3-5-2017

My Dear Profs Ramesh & Loveleen,
I have received your kind letter of April 5. on 8th of April - Not bad.

With best wishes
and apology for my deteriorating
~~Hand~~ handwriting.
I remain
Sincerely NKBhide

Your condolence about Mrs. Bhide's departure was the finest and briefest one. Thanks

NKB

The beginning and the end of the letter dated 3 May 2017 from Prof. N.K. Bhide to the author.

After his retirement, while he was at Pune, I visited him several times during my visits to Kaivalyadhama, Lonavala – my last visit was probably towards the end of 2015. But my most lengthy interactions with him were in December 2001 when I had a few days to spare between two conferences that I was attending in Pune. I had gone prepared to spend those days on collecting material on Professor B.B. Dikshit, the first Director of AIIMS, for an editorial in the *Indian Journal of Physiology and Pharmacology (IJPP)* because 2002 was the centenary year of Professor Dikshit. And, I knew that Prof. Bhide was an ardent admirer of Prof. Dikshit. So, I brought the journalist in me to the front. I went prepared to spend a few hours with Prof. Bhide every day for three consecutive days to collect the material: a delicious lunch prepared by Dr (Mrs.) Vimal Bhide and Kamala, and served with great love, was an added bonus. The first day, Prof. Bhide spoke, and I took notes. The second day, I carried with me something that I wrote on the basis of the first day's notes, made corrections in my draft, and took some more notes. The third day, I carried a manuscript that had a clearer contour, read it out to Prof. Bhide, made some more corrections, and took still more notes. After coming back to Delhi, naturally there was some more writing and rewriting. But finally I had a decent biography of Prof. Dikshit, which appeared in *IJPP* (1). I am glad I did it, because a Google search shows that that article is the only article available on the internet that has something substantial about Prof. Dikshit, who continues to be remembered with great reverence by his students to this day (2). But for Prof. Bhide, this article would not have been there.

Prof. Bhide was a perfect synthesis of the East and the West, which he had achieved through a process that Sri Aurobindo calls 'critical assimilation' – a process in which you if you accept something, it is because you understand it; and if you reject something, that is also because you understand it. One could discuss with Dr. Bhide with great profit the systems of Indian philosophy and Ayurveda, or Greek mythology and the European Renaissance – he was equally at home with all these. He had all the volumes of *Charaka Samhita*, and he had read them. He was a great admirer of the British qualities of "profound scholarship, sound commonsense and philosophical equanimity".

Prof. Bhide was simple, original, and had the courage of conviction to swim against the current. He never bought a car; he had no air-conditioner at home or in his office, and had simple home-made vegetarian food, seldom going anywhere outside to eat. He was extremely reluctant to add to his possessions, much to the annoyance of his family. He travelled very little even in India, leave aside going abroad. His only trip abroad worth the name was a one-year stay in the US on a TCM Fellowship in the 1960s. After his return, he had submitted a detailed report, of which he had many extra cyclostyled copies. A copy of that highly educative report has been one of my proud possessions for about 50 years. But once he did confide in me that now he regretted having restricted his travel to that extent. That denied him exposure in scientific circles, which perhaps curtailed his scientific progress; much worse is the fact that his attitude deprived many other scientists in the world from learning more from this exemplary man. But even with his restrictive ways, he has a sizeable number of research publications, most of them in collaboration with pediatricians and dermatologists (3).

He had an extremely good command over the English language, and he wrote accurate, unambiguous and elegant prose, often with a touch of subtle typically British humour. I learnt a lot about writing by reading what he had written, and by sitting with him a couple of times while he edited research articles, I learnt a lot about editing. But sometimes he went to extremes. He wanted everything to be read twenty times before letting it go. The product at the end no doubt read as smooth as silk, but such obsessions restricted his output. If he had not gone to such extremes, he could have written a lot more from which the world would have benefited. He could have written books on a variety of subjects, but did not write even one. If he had chosen to write a textbook of pharmacology, he could have easily given the world an Indian book comparable in style to Laurence's *Clinical Pharmacology*.

He spent the last 26 years of his life in his house in Pune, where he lived with his wife, Vimal, and daughter, Kamala, her family, and a few cats. It was his ancestral home, and even some of the furniture was more than a hundred years old. His wife passed on about a year before he left his body on 28 October 2017 at about 6 pm, just a few weeks short of his ninetieth birthday. Not one for aggressive medical treatment, he would have liked to end this life peacefully at home. But his family did take him to the hospital, where the end came after a short stay. He is survived by his daughters Kamala and Kumud, and son Vasudev, and a large number of students and admirers scattered all over the world. May his soul be at peace in a place full of unmixed love and joy; and if it gets embodied again, let it get on earth conditions that are the most favourable for spectacular growth of consciousness.

Ramesh Bijlani

Sri Aurobindo Ashram – Delhi Branch
New Delhi – 110 016
Email: rambij@gmail.com

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Indian Journal of Physiology and Pharmacology,
Department of Physiology,
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Executive Editor :

Dr. K. K. Deepak
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Editor (Physiology)
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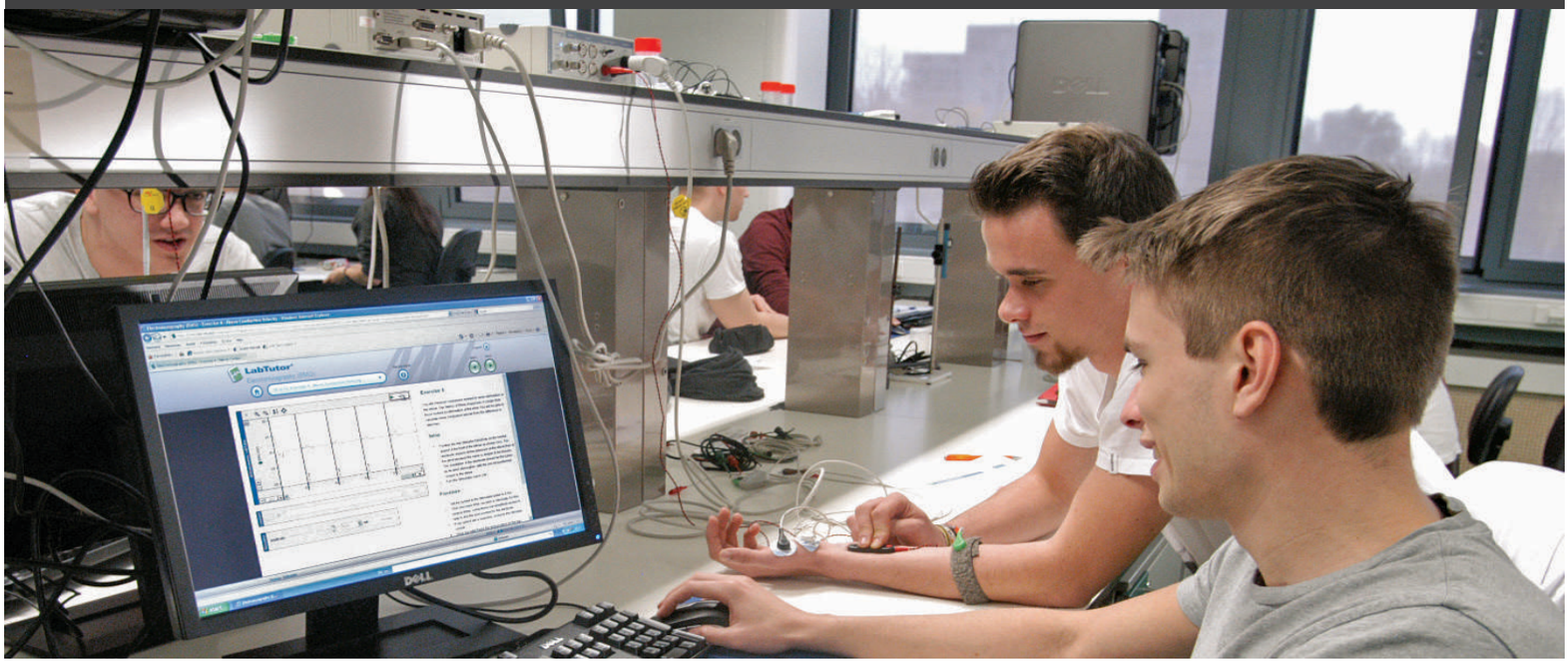

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