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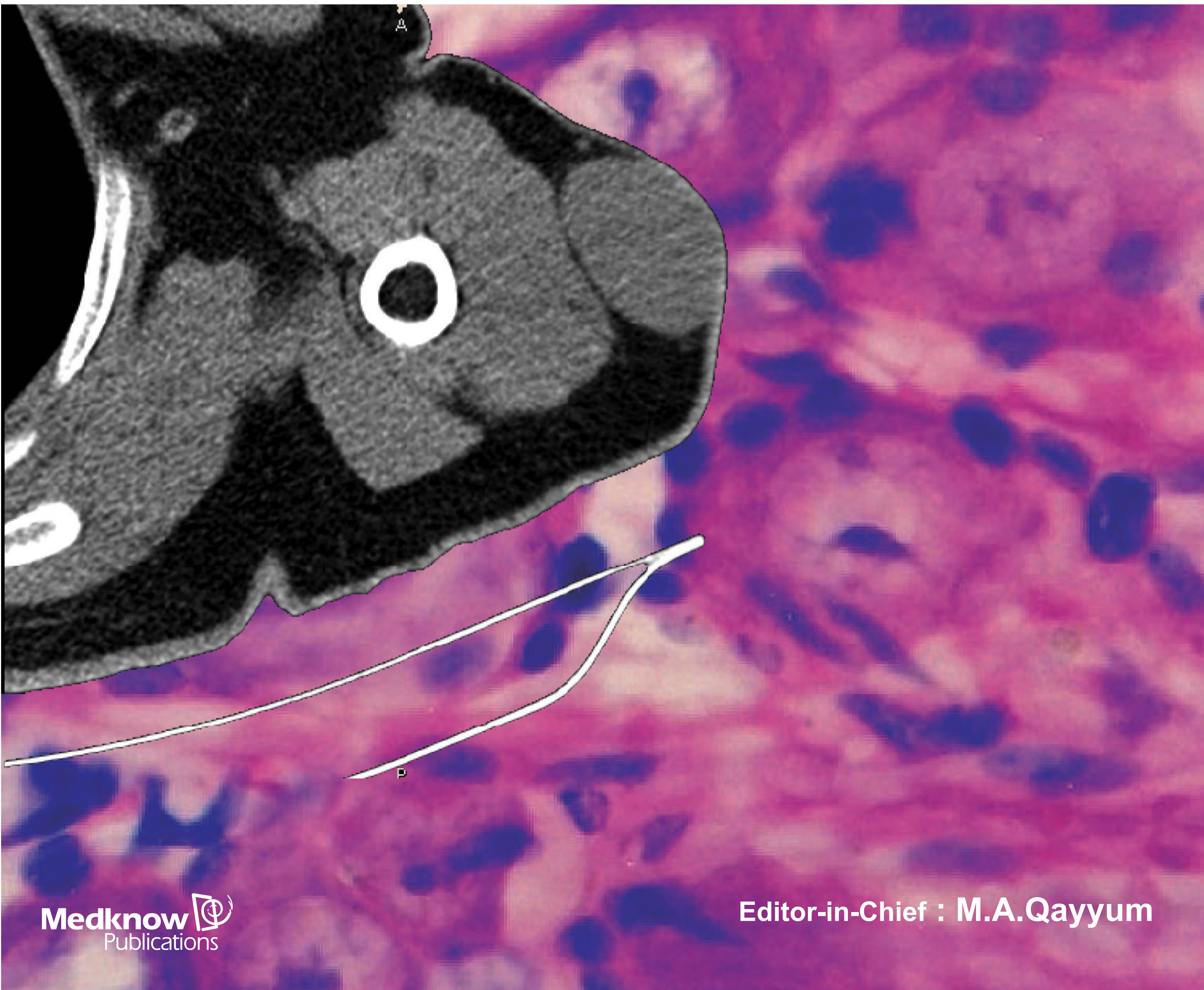

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Anterograde Synaptic Transport of Neuronal Tracer Enzyme (WGA-HRP): Further Studies with Rab3A-siRNA in Rats

Yoshiki Takeuchi, Yoshiki Matsumoto, Takanori Miki, Toshifumi Yokoyama[1], Katsuhiko Warita, Zhi-Yu Wang, Takashi Ueno, Tomiko Yakura, Mamoru Fujita[2]

Department of Anatomy and Neurobiology, Faculty of Medicine, Kagawa University, 1750-1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793, Japan, [1]Functional Morphology, Department of Animal Science, Graduate School of Agricultural Science, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan, [2]Graduate School of Health and Nutrition Sciences, Nakamura Gakuen University, 5-7-1 Befu, Jonan-ku, Fukuoka 814-0198, Japan

Abstract

Neuronal tracer enzyme of wheat germ agglutinin conjugated horseradish peroxidase (WGA-HRP) was employed to elucidate the detailed morphology of anterograde synaptic transport. After injection of WGA-HRP into the vagus nerve, sensory terminals in the nucleus of solitary tract (NST) were observed at the electron microscopic level using the tetramethyl benzidine and diaminobenzidine methods. In neuropil of the NST, electron-dense HRP-reaction product (HRP-RP) showed various types of lysosomal-like structures. The RP characterized by containing membranous substance crossed synapses forming a mass without membrane surrounding the RP. Additionally, phenomena of anterograde synaptic transport of the RP and exocytosis of synaptic vesicles never occurred simultaneously. These findings raised the possibility of inducing synaptic transport of WGA-HRP at the stage of no neurotransmitter release, i.e. no activation of neuron. Therefore, further experiments were performed after co-injection of Rab3A-siRNA with WGA-HRP into the vagus nerve. This co-injection frequently resulted in not only suppression of vesicle trafficking to active zones and docking to pre-synaptic membranes but also abnormal aggregation of synaptic vesicles at terminals. Furthermore, synaptic transport of WGA-HRP, including secretion, followed by endocytosis of post-synaptic neurons was better seen in the experiments.

Key words: Anterograde synaptic transport, Rab3A-siRNA, vagus nerve, WGA-HRP

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Introduction

Synaptic transmission on neuronal network is exclusively essential for the brain to function. Morphological analysis onto the functional implication at cellular and molecular levels is useful to understand the normal and pathological brain conditions. It is evident that synaptic vesicles move to axon terminals along microtubules under existence of kinesin superfamily [1-3] and chemical synaptic transmission based on exocytosis of synaptic vesicles is made at the pre-synaptic membrane with highly specialized structures forming contacts. However, some kind of protein, such as neuronal tracer enzyme of wheat germ agglutinin conjugated horseradish peroxidase (WGA-HRP), has also been well known to have anterograde synaptic transport [4-6]. It is believed that transport of WGA-HRP belongs to exocytosis as well as that of chemical synaptic transmission [7]. In this point, the present study was performed to investigate whether the manner of anterograde synaptic transport of WGA-HRP is in accordance with that of vesicular transport and, in addition, changes of chemical synaptic transmission exert influence on transport of WGA-HRP.

Recent genetic analysis of neuronal protein function has demonstrated that Rabs, GTP-binding proteins, are generally involved in regulating membrane traffic and function in neurotransmitter release. Particularly, Rab3A is associated with presynaptic vesicles regulating vesicle targeting to active zones [8-10]. Therefore, Rab3A related proteins are considered useful for investigating the influence on synaptic transport of WGA-HRP. In this study, Rab3A-siRNA inhibiting neurotransmitter release was injected into the vagus nerve with WGA-HRP. This co-injection makes it possible

to demonstrate the direct influence of Rab3A-siRNA *in vivo*.

Materials and Methods

The present experiments were performed on 24 male Wistar rats (SLC, Hamamatsu, Japan), weighing 180-236 g. Animals were housed in separate cages and maintained under standard laboratory conditions (23 plus/minus 1 °C, 12-h light: 12-h dark cycle, food and water *ad libitum*). Experimental procedures were conducted in accordance with National Institute of Health (NIH) for Care and Use of Laboratory Animals. The Kagawa University Animal Care and Use Committee approved the procedures, and all efforts were made to minimize the number of animals used and their suffering.

Animals were anesthetized with intra-peritoneal injection of chloral hydrate (490 mg/kg) for all surgical procedures. 0.4-2.0 µl of a solution of 4% WGA-HRP (Vector Laboratries, Inc) on 14 rats or working solution of 1 nM Rab3A-siRNA containing four per cent WGA-HRP on eight rats were injected into the vagus nerve on the right side or both sides using a 10-µl Hamilton microsyringe under the operation microscope. Rab3A-siRNA stock solutions (3.0nM) were diluted by double distilled RNAase free water and stored in aliquots at minus 80 °C. Working solution (1.0nM siRNA/5µl) was mixed with each aliquots (1:4 v/v) and four per cent WGA-HRP. Rab3A selective siRNA mixture (3 target-specific siRNA for Rho/Rab subfamily, santa cruz, sc-36343) did not show any sign of behavioral toxicity. After a survival period of 24, 48, 72 h in the case of injection of WGA-HRP or 12, 24, 48 h in the case of co-injection, the animals were sacrificed by perfusion through the ascending aorta with 0.1M phosphate buffer (pH 7.4) followed by a fixative of one per cent paraformaldehyde and 1.25-2.5% glutaraldehyde in 0.1M phosphate buffer.

The brain stem was removed from the skull and cut transversely into 200 µm-thick sections using a vibratome (Leica VT 1000S, Germany). The blocks containing the nucleus of solitary tract (NST) and dorsal motor nucleus of vagus nerve (DMV) were processed for demonstration of HRP-RP according to the TMB method [11] or heavy metal-intensified DAB method [12].

In experiments using the TMB method, the sections were immersed in two per cent ammonium molybdate, dissolved in a five per cent solution of 0.2M acetate buffer, pH 3.3, for 30 min. Then, the sections were postfixed in buffered one per cent osmium tetroxide for two hours, block-stained in saturated uranyl acetate for one hour, dehydrated in a graded acetone series and embedded in an epoxy resin mixture as reported previously [13]. In experiments using the DAB method, the sections were postfixed in buffered one per cent osmium tetroxide for two hours, block-stained in saturated uranyl acetate, dehydrated in alcohols and embedded in an epoxy resin mixture. As controls, two uninjected animals were used and blocks containing the NST region were taken. The blocks were reacted with TMB and DAB, and processed for electron microscopy in a manner similar to that described above. In all experiments, the NST region was identified by examination of toluidine blue-stained or unstained one µm-thick sections. Ultrathin sections of the region were cut and observed without further lead staining using a JEOL 200 CX electron microscope.

Results

In the case of injection of WGA-HRP into the vagus nerve, NST was confirmed easily by HRP-labeled fibers in the nucleus and labeled neurons in the DMV at 200 µm- and one µm-thick sections. Anterograde labeling was more dense in NST, with ipsilateral predominance, on the case of a survival period of 48 hours. HRP-RP was identified more clearly as electron-dense materials when lead citrate staining was omitted. Experiments using TMB method resulted in the RP showing lysosomal- or club-like structures without any membranes surrounding the RP in the axons and terminals.

The terminals were characterized by containing one or more products (Figures 1A-D) and clusters of the RP around synaptic contacts (Figure 1E). The RP was frequently observed from pre- to post-synaptic sites in continuity in the terminals participating in axosomatic (Figure 1B) and axodendritic synapses (Figures 1C-E). There were no remarkable changes of synaptic vesicles and membranes when the RP crossed the synapses. Furthermore, phenomena of exocytosis of synaptic vesicles and anterograde synaptic transport of the RP never occurred simultaneously (Figure 1A).

Experiments using DAB method represented a different configuration of RP which might result from the use of a different pH or chromogen. This method apparently provided a more detailed morphology for anterograde transport of WGA-HRP at synapses. The RP showed membrane-bound lysosomes or lysosomal-like structures in axons (Figure 2A) and terminals (Figures 2B, C). Interestingly, the RP frequently contained membranous substance and formed a large mass consisting of two to five of the RP without vacuoles at terminals. Contacts between RP and the presynaptic membranes suppressed the membrane surrounding the RP.

Furthermore, it was likely that indentation structure at synapses is formed to pass through the synapses (Figure

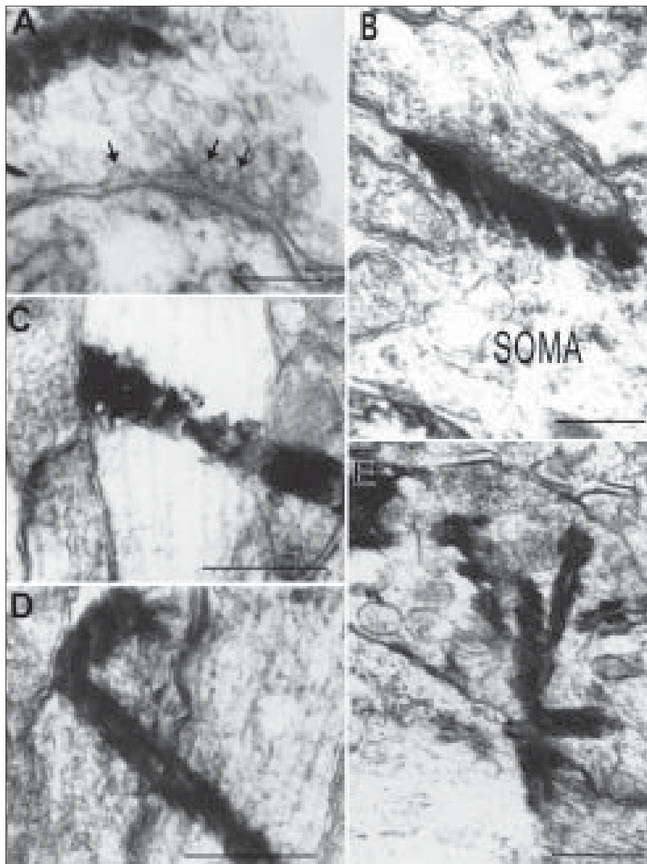

Figure 1: *Electron micrographs of terminals containing one or more electron-dense HRP-RP. The RP was located separately from synaptic site in terminal characterized by exocytosis (arrows) of synaptic vesicles (A)*

3A). Round or irregular shaped RP was observed to form synaptic transport (Figures 3B-D). Exocytosis of synaptic vesicles never occurred when the RP made contacts with

However, the RP was frequently observed from pre- to post-synaptic sites in continuity in the terminals participating in axosomatic (B) and axodendritic synapses (C-E) without any changes of synaptic membranes and vesicles. TMB method: Calibration bar is equal to 0.2 μm in A and B, 0.5 μm in C and D, one μm in E.

Presynaptic membranes or crossed synapses (Figures 3A and B). The RP transported to postsynaptic neurons was not surrounded by membrane (Figure 2D).

Co-injection of Rab3A-siRNA with WGA-HRP into the vagus nerve resulted in similar structure of RP to that identified in the case of WGA-HRP injection using the DAB method. In the present experiments, the RP was also observed to cross synapses at a survival period of 24 and 48 h with a slightly high frequency (Figure 4B). Many terminals containing the RP revealed not only suppression of vesicle trafficking to active zones and docking to pre-

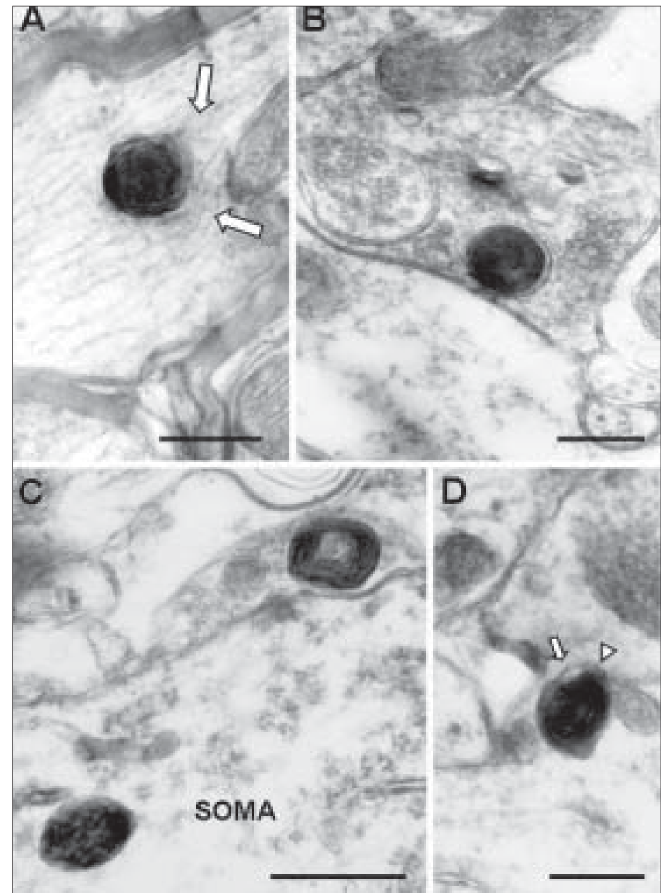

Figure 2: *Electron micrographs of HRP-RP identified clearly as electron-dense materials. The RP revealed membrane-bound lysosomes or lysosomal-like structures containing membranous substance in axon (A) and terminals (B, C). Note that the RP located in postsynaptic sites of dendrite (D) was not surrounded by membrane in contrast to the RP in presynaptic sites. Large arrows in A show bending of the microtubules caused by RP in axon. Small arrow and arrowhead in D indicate that a part of the RP and membrane remain in the presynaptic site, respectively. DAB method. Calibration bars = 0.5 μm in A and C , 0.3 μm in B and D*

synaptic membranes but also central concentration or weak aggregation of synaptic vesicles (Figures 4A and B).

This co-injection also resulted in secretion of terminals followed by adsorptive endocytosis of postsynaptic dendrites.

The RP in secretion was characterized by electron-dense homogeneous substance in contrast to that containing membranous substance (Figures 4C and D). In control rats, sections reacted with TMB and DAB showed no similar structure of RP at terminals in the neuropil of the NST to that described above.

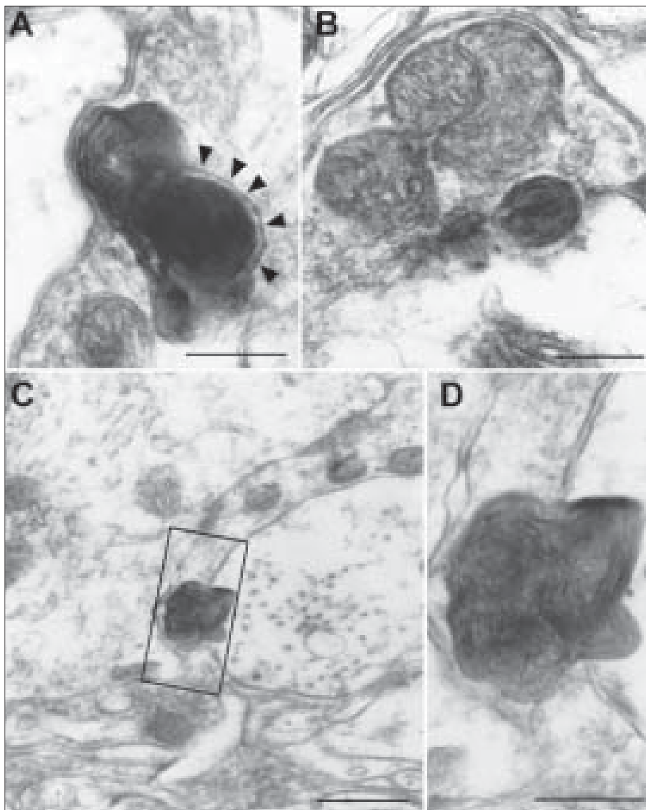

Figure 3*: Electron micrographs of anterograde transport of WGA-HRP at synapse. The RP frequently forms a large mass containing membranous substance. Note that contacts of the RP with the pre-synaptic membrane locally suppressed the membrane surrounding the RP (arrowheads) and the indentation-like structure was also observed (A). A small round (B) and large irregular shaped mass (C, D) of the RP are passing through synapses. Framed area in C is magnified in D. DAB method. Calibration bar is equal to 0.3 µm in A and B, 0.5 µm in C, 1 µm in D*

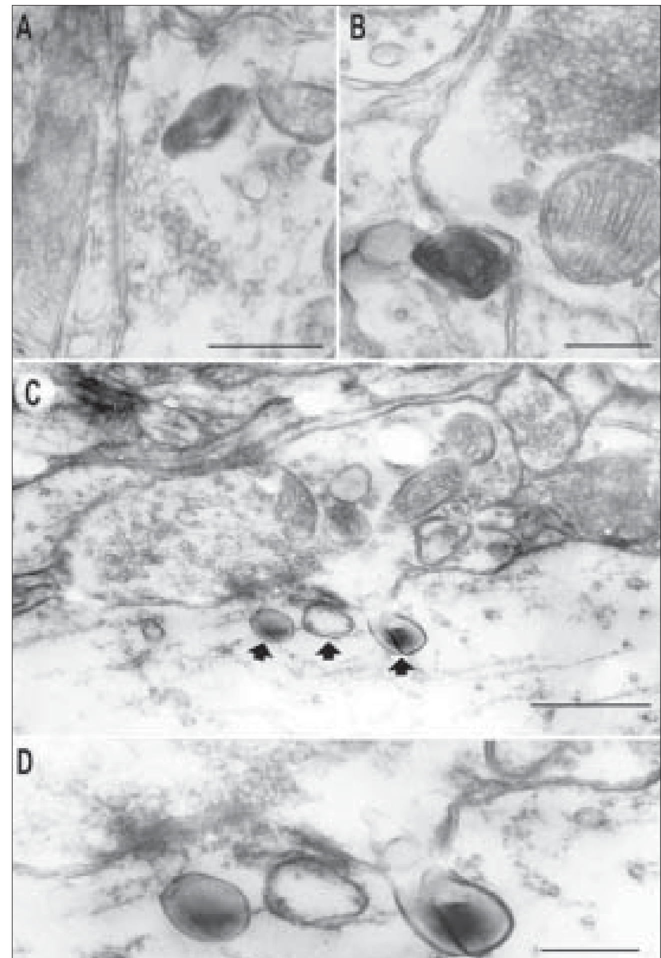

Figure 4*: Electeron micrographs of HRP-RP in terminals after co-injection of Rab3A-siRNA with WGA-HRP. This co-injection frequently resulted in not only suppression of vesicle trafficking to active zones and docking to presynaptic membranes but also central concentration or weak aggregation of synaptic vesicles (A—C). Note that secretion followed by endocytosis of dendrite is better seen in terminal (C and D). Endocytosed structures (arrows) in C are magnified in D. DAB method. Calibration bars is equal to 0.5 µm in A, 0.3 µm in B, 0.5 µm in C and 0.2 µmi*

Discussion

WGA-HRP has been employed for ultra-structural investigation of anterograde synaptic transport. Particularly, further observation in the present study was made to demonstrate the direct influence of Rab3A-siRNA at terminals *in vivo*. Experiments using TMB method resulted in elongated RP passing through synapses which was also identified in our previous study in the central nervous system [13]. Although the RP located from pre- to post-synaptic sites in continuity,might be induced by leakage of WGA-HRP, there is no possibility of leakage because of any normal synaptic membrane structure and membrane bound RP demonstrated using DAB method. However, the possibility cannot be excluded that the elongated structure is TMB crystal growth across membranes.

The membranous substance in the RP seems to be formed by endocytosis of lysosome for WGA-HRP which has an association with Golgi apparatus[14] and similar to autophagic-like structure which shows degeneration of cytoplasmic components [15,16]. Although this structure , often containing vacuoles, has been reported in the previous study using WGA-HRP method, there is no description about synaptic transport [17]. In the present study it is not concluded that the membranous substance is completely in accordance with autophagy. However, the membranous substance possibly including lipids might play some important role in passing through synapses.

With respect to lectin, immunoblot analysis demonstrated that the 62-kDa lectin (WGA)-HRP conjugate (WGA: 22-kDa, HRP: 40-kDa) binds to specific carbohydrate moieties attached to glycoproteins and glycolipids expressed on surface plasma membranes [18]. WGA has been used as an effective tracer in neuronal systems among a number of lectins because of common expression of WGA receptors on surface plasma membranes of most neurons [19]. These findings suggest that synaptic transport of WGA-HRP is highly dependent upon lectin activity. Recent studies also have indicated a relationship between lectin activity and proteins of the annexin family. In particular, annexin IV binding to glycosaminoglycants in the presence of calcium ions is identified to be a lectin [20]. Annexin V also has same lectin activity and is suggested to play certain roles in the central nervous system as a neurotrophic factor [21].

The relationship between synaptic transport of RP and vesicular transport raised the possibility that synaptic transport of WGA-HRP is induced at the stage of no neurotransmitter release, i.e. no activation of neuron. In this point, Rab3A-siRNA injection into the vagus nerve is useful for investigating the phenomena. The present experiments revealed not only a loss of docked synaptic vesicles adjacent to the postsynaptic density but also abnormal aggregation of synaptic vesicles. These findings are well consistent with those reported in previous Rab3A knock-out mice [10,17,22,23]. Furthermore, in the present study, synaptic transport of WGA-HRP including secretion followed by endocytosis of dendrites was better seen after administration of Rab3A-siRNA. In secretion, the RP showed homogeneous structure which seemed to have no association with lysosome in contrast to membranous substance. Although more detailed molecular analysis for the relationship between the synaptic membranes and protein endocytosed by lysosome should be studied in further experiments, co-injection of Rab3A-siRNA with WGA-HRP apparently makes it possible to demonstrate the direct influence of Rab3A-siRNA in HRP-labeled terminals *in vivo*. Indeed more studies are required to establish more details about the structures. In the previous study investigating synaptic transport of WGA-HRP in the olfactory bulb after intranasal administration, Broadwell and Balin[7] suggested that the RP is exocytosed from axon terminals for adsorptive endocytosis and possibly fluid-phase endocytosis by postsynaptic neurons. The present findings of secretion are considered to be in agreement with their proposal.

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Correspondence:
Yoshiki Takeuchi,
Department of Anatomy and Neurobiology,
Faculty of Medicine, Kagawa University,
1750-1 Ikenobe, Miki-cho, Kita-gun,
Kagawa 761 0793, Japan.
E-mail: takeuchi@med.kagawa-u.ac.jp

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Characteristics, Severity and Management of Insect-stung Patients

Shirina Alsowaidi, Abdulrahman Mohamed[1], Roos Bernsen[3], Abdishakur M Abdulle, Abdulbari Bener[2]

Department of Internal Medicine, [3]Department Community Medicine, Faculty of Medicine, UAE University; UAE, [1]Department of Medicine, Jimi Hospital, UAE, [2]Department of Medical Statistics & Epidemiology, Hamad Medical Corporation, and Weill Cornell Medical College, Qatar

Abstract

We aim to assess the demographic, clinical manifestation and management of insect-stung patients. About 783 (46% male; 95% expatriate workers) records of Al-Ain hospital patients were analyzed. According to Muller classification, class I severity was common in the youngest age group. Majority (56.9%) of the subjects had class III severity. Multi-variate analysis confirmed that significantly less severe cases were present in the youngest age group (0-10 years) only (*P* less than 0.0005) and in male patients (*P* less than 0.0005). Anti-histamine and corticosteroids, but not epinephrine, were commonly administered. Where data was available (32.6%), the site of the sting was mostly on legs (16.6%). Insect stings were incompletely reported, often with severe symptoms (class III), and under-treated. The severity of allergic reactions was associated with older age and female gender. Emphasis on training of healthcare providers could potentially improve the current level of diagnosis and management.

Key words: Antihistamine, allergic, characteristics, insect stings

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Introduction

Allergic reactions resulting from insect stings, predominantly hymenoptera, are one of the most common causes of anaphylaxis worldwide [1,2]. In the United Arab Emirates (UAE), limited information is available with regard to patient characteristics, risk factors, and current management guiltiness.

In general, the causes of allergic reactions include insect stings, food allergens, and other environmental risk factors. Moreover, the severity of allergic reactions is often related to older age, gender and ethnicity. Although allergic reactions may vary in magnitude, the consequences of under-diagnosed and under-treated allergic reactions may lead to life-threatening anaphylaxis [3]. Thus, appropriate prevention strategies, diagnosis, and often aggressive treatment approaches are necessary. Currently, the treatment of choice in the emergency unit includes parenteral epinephrine, cardiovascular stabilization, systemic anti-histamines and corticosteroids as well as inhaled agonist.

In the UAE, among other factors, intensive plantation projects, over the past two decades or so, have helped increase the number of insects in wild areas as well as recreational areas. This has led to an increase in incidence of morbidity from insect stings and subsequent allergic reactions. In this study, we aim to investigate the demographic characteristics, clinical manifestation, and management of allergy reactions due to common insect stings among emergency department patients in Al-Ain hospital, UAE.

Aim

To assess the demographic characteristics, clinical manifestation and management of allergy reactions due to common insect sting among emergency department patients in Al-Ain hospital, United Arab Emirates (UAE).

Materials and Methods

In this retrospective observational study, we analyzed a total of 783 records of insect stung patients who visited

the emergency department (ED) of Al-Ain hospital, in the period between February 1993 to September 1995. Demographic data, clinical manifestation and treatment regimens were collected and analyzed.

Statistical analysis

Statistical Package for Social Sciences (SPSS) version 15.0 software for Windows was used. Cross tabulation was used to present the descriptive values of the combined population. Cross tabulation was used to present the distribution of severity by age group.

We assessed the independent relationship of age (5 categories) and sex with severity (0=class 1-2; 1=class 3-4) by means of logistic regression. A P-value < 0.05 will be considered statistically significant.

Results

Table 1 shows the demographic data of the patients. The overwhelming majority of patients were none UAE nationals (95%) of heterogeneous expatriate workers, 46% of subjects were male. More than 50% of all subjects were children and young adults.

Cross tabulation of age and severity of reaction, according to Muller classification, are shown in Table 2. Unlike class I severity found in the youngest age group, majority of the population (56.9%) suffered from allergic reaction of class III severity. Multivariate Logistic regression analysis confirmed that significantly less severe cases were present in the youngest age group (0-10 years) only (P less than 0.0005) and in male patients (less than 0.0005).

Table 1: The demographic variables of patients

Patients (N=783)	Number (%)
Males (%)	358 (45.7)
Nationals (%)	39 (5.0)
Median age (years)	30
Range (years)	(1-78)

With regard to treatment, anti-histamine and corticosteroids, but not epinephrine, were administered to most of the patients and regardless of severity (Table 3).

The site of the sting was mostly on legs (16.6%), arms (8.9%), head (2.7%), trunk (2.7%), and the neck (1.5%). Nevertheless, majority of the patients (67.4%) had no available data with regard to the site of sting.

Discussion

The overwhelming majority of cases seen at the emergency department was young people and may possibly represent the general population. Consistent with the literature, allergic reactions due to insect stings are seemingly more severe among adults as compared to children and among males as compared to females in this population [6-8]. The less severe findings among children may indicate appropriate parental awareness to early treatment even with mild symptoms. Occupational exposure, particularly among adult males, could not be explained by the current data. Contrary to current practice, epinephrine was not commonly used as a favorable first line emergency treatment for allergic reactions possibly due to under diagnosis. However, cardiovascular stabilization, systemic antihistamines and corticosteroids as well as inhaled volterin seem to have been adequately administered on regular basis.

A study reported in the United States[6-8] showed that although guidelines suggest specific approaches for the emergency management of insect sting allergy, concordance with these guidelines appears low in patients with a severe insect sting reaction.

Conclusion

Insect-stung patients who visited the emergency unit of Al-Ain hospital were, mostly, incompletely reported, often with severe symptoms (class III), and under treated. The severity of the allergic reactions was associated with older

Table 2: Cross tabulation of age and the severity of allergy symptoms according to Muller Classification

Age Group Years	Classification				Total Number
	I n (%)	II n (%)	III n (%)	IV N (%)	
0-10	34 (57.6)	12 (20.3)	13 (22.0)	0(0)	59
11-20	13 (25.5)	5 (9.8)	30 (58.8)	3 (5.9)	51
21-30	57 (25.7)	13 (5.8)	142 (63.9)	10 (4.5)	222
31-40	66 (31.3)	15 (7.1)	124 (58.8)	6 (2.8)	211
>40	28 (32.6)	5 (5.8)	49 (56.9)	4 (4.7)	86
Total	198 (31.5)	50 (7.9)	358 (56.9)	23 (3.7)	629

Grade I – urticaria, pruritus, malaise; Grade II – angioedema, chest tightness, nausea, vomiting, abdominal pain, dizziness; Grade III – dispnoea, wheeze, stridor, dysphagia, hoarseness; Grade IV – hypotension, collapse, loss of consciousness, incontinence, cyanosis. According to Mueller.[4]

Table 3: Medication and discharge outcome of insect stung patients from emergency department

Treatment	Number (%)
Systemic antihistamine	709 (90.5)
Antihistamines tablets	534 (68.2)
Systemic corticosteroids	417 (53.3)
Ventolin Inhaler	327 (41.8)
Oxygen	78 (10)
Antihistamine injections	74 (9.5)
Steroid tablets	16 (2)
Adrenaline injections	8 (1%)
Discharge outcomes	774 (98.9)

age and male gender. Emphasis on training of healthcare providers could potentially improve the current level of diagnosis and management.

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Correspondence:
Dr. Shirina Alsowaidi,
Department of Internal Medicine,
Faculty of Medicine, UAE University, UAE.
E-mail: Shirina.alsowaidi@uaeu.ac.ae

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Gene Expression Profile in Leucocytes of Type 2 Diabetic Subjects

S Kaviarasan, S Muniandy[1], R Qvist, IS Ismail

Department of Medicine, [1]Department of Molecular Medicine, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

Abstract

Communication between insulin target tissues and beta-cells initiate compensatory responses which increase insulin production. Correlated changes, in gene expression between tissues, can provide evidence for such intercellular communication. We profiled gene expression studies in Type 2 diabetic subjects to unveil the mechanistic factors involved. Our results showed that genes involved in carbohydrate, lipid and amino acid metabolism pathways, glycan of biosynthesis, metabolism of cofactors and vitamin pathways, ubiquitin-mediated proteolysis, signal transduction pathways, neuroactive ligand receptor interaction were upregulated in diabetes compared to healthy subjects. In contrast, genes involved in cell adhesion, cytokine-cytokine receptor interaction, insulin signaling, PPAR signaling pathways were downregulated in subjects with type 2 diabetes mellitus (T2DM). β_2-microglobulin, a MHC class I molecule was strongly downregulated in diabetic subjects. Further, genes involved in inflammatory pathway are differentially expressed in subjects with T2DM. Hence, it was evident that genes concerned with pathways of carbohydrate, lipid and amino acid metabolism, neuronal function and inflammation play a significant role in the pathobiology of T2DM.

Key words: Gene expression profile, leucocytes, PCR array, Type 2 diabetes mellitus

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Introduction

Type 2 diabetes mellitus (T2DM) is a disorder that involves an increased demand for insulin brought about by insulin resistance, together with a failure to compensate with sufficient insulin production. Although insulin resistance occurs in most obese individuals, diabetes is generally forestalled through compensation with increased insulin. This increase in insulin occurs through an expansion of beta-cell mass and/or increased insulin secretion by individual beta-cells. Failure to compensate for insulin resistance leads to T2DM.

T2DM is one of the most challenging health problems in many developing and industrialized countries [1]. The exact cause of T2DM, which affects millions of people all over the world, is not known. One of the foremost challenges we face is to account mechanistically for not only the definition of hyperglycemia, but also for the myriad of other biochemical and physiological abnormalities, which are characteristics of this disease. The abnormalities include central obesity, hypertension, accelerated atherosclerosis, hypertriglyceridemia and low serum concentrations of high density lipoproteins [2,3].

One way to understand the pathophysiology of diabetes is to examine the coordinated changes in gene expression in T2DM. In each case, there are groups of genes that undergo changes in expression in a highly correlated fashion. Genomic approaches to determine differential expression profiles utilizing serial analysis of gene expression (SAGE) [4] and DNA microarrays [5] are now providing global views of the potential genes and pathways that are associated with diabetes. Utilizing these approaches, tissue-specific gene expressions in human pancreas, muscle and fat demonstrated differential regulation of approximately 800 genes in diabetes [5].

Systemically circulating peripheral blood mononuclear cells (PBMCs) are considered unique tissue affected by

the host condition and may reflect oxidative stress caused by high levels of glucose, insulin, free fatty acids, and tissue-derived circulating bioactive mediators. To verify the hypothesis that the gene expression of PBMCs changes in response to diabetic circumstances, we comprehensively compared global gene expression profiles of PBMCs between patients with and without T2DM by using Reverse Transcription Quantitative Real Time PCR array technology. Even though there is extensive literature relating peripheral blood cells to diabetic complications, the association of gene expression changes in PBMCs in T2DM is largely unknown. In this study, we examined gene expression profiles of peripheral blood cells in T2DM to identify potential gene signatures.

Material and Methods

Study subjects

The study population was composed of six type 2 diabetics and three normal subjects from the mixed races of Malaysian population. Patients were in the age group 30-65 years. .The clinical characteristics of the study subjects were recorded.

Anthropometric measurements

Anthropometric measurements, like weight and height, were obtained using standardized techniques as detailed elsewhere [6]. Height was measured with a tape to the nearest cm. Weight was measured with traditional spring balance that was kept on a firm horizontal surface. The body mass index (BMI) was calculated using the formula

$$BMI = \frac{\text{Weight (kg)}}{\text{Height } (m^2)}$$

Laboratory studies

After an overnight fast, venous blood samples were withdrawn from each patient in sterile EDTA tubes. Serum samples were assayed for plasma glucose, HbA1C, total cholesterol, triglycerides and HDL cholesterol.

Processing of blood samples

Blood was collected in EDTA tubes. PBMCs were isolated using Ficoll gradients within 4h of each blood draw; if not processed immediately, cells were lysed in RLT buffer containing β-mercaptoethanol and stored at minus 80°C.

Isolation of RNA from PBMCs

Mononuclear cells were isolated by the Ficoll density-gradient method as previously described [7]. Total RNA was extracted using an RNA isolation kit as per the manufacturer's protocol (SuperArray, catalog number PA-001). RNA integrity was assessed using an Agilent 2100 bio-analyzer (Agilent, Palo Alto, Ca).

PCR arrays

From 0.5–1.0 µg of total RNA, double-stranded cDNA was generated using a cDNA synthesis kit (SA Bioscience, Catalog. Number C-03) that eliminated genomic DNA contamination. cDNA was used as a template for SYBR-green based RT^2-qPCR array using the diabetes array plate (SA Biosciences).

Statistical analysis

All data is expressed as means plus/minus SD. Statistical analysis of the results was performed by student't' test. Values were considered significant when *p was less than* 0.0001.

Results and Discussion

In this study, we demonstrate the possibility that gene expression profile in PBMCs reflects the pathophysiology of T2DM. As T2DM is a multifactorial disorder [8], a comprehensive approach identifying biological pathways or co-regulated gene sets associated with the diseases is required to understand the molecular signature of T2DM [9]. Thus, we screened known human pathways and extracted information on the metabolic pathways that were significantly altered in the PBMCs of the diabetic subjects.

Clinical characteristics of control subjects and patients with diabetes are shown in Table 1. Age, BMI, and levels of fasting plasma glucose, HbA1c, total cholesterol, triglyceride and LDL-cholesterol were significantly increased while HDL-cholesterol was found to be decreased in patients with T2DM.

Table 1: Clinical characteristics of patients with T2DM (n = 6) and normal subjects (n=3)

	Healthy controls	T2DM
M:F	1:2	3:3
Age, Yr	34.7 ± 9.07	53.5 ± 7.4*
BMI, Kg/m2	22.07 ± 1.78	26.7 ± 1.93*
Fasting plasma glucose (mmol/L)	4.5 ± 0.45	11.5 ± 1.36**
HbA1c (%)	5.49 ± 0.13	11.1 ± 0.68**
Total cholesterol (mmol/L)	4.73 ± 0.25	7.03 ± 0.34**
Triglyceride (mmol/L)	1.70 ± 0.26	4.83 ± 0.44**
HDL cholesterol (mmol/L)	2.14 ± 0.17	1.04 ± 0.04**
LDL cholesterol (mmol/L)	2.25 ± 0.15	4.87 ± 0.18**

*Data are expressed as Mean ± SD. *P < 0.01, **P < 0.0001 as compared to control.*

Gene expression profiling is a powerful alternative strategy to test for differences in expression of pre-defined clusters or networks of genes rather than individual genes, thus reducing the number of tested hypotheses. Gene expression analysis using RNA extracted from peripheral blood cells of subjects with T2DM showed significant alterations in the expression of 84 candidate genes (Table 2). For the purpose of analysis, genes were clustered functionally into

1. Receptors, transporters and channels
2. Nuclear receptors
3. Metabolic enzymes
4. Secreted factors
5. Signal Transduction molecules
6. Transcription factors.

It is possible to predict and understand pathological

Table 2: Fold change in gene expression in subjects with T2DM over normal subjects

Unigene	Genbank	Position	Symbol	Description	Fold change
				T2D vs C	
Hs.54470	NM_000352	A01	ABCC8	ATP-binding cassette, sub-family C (CFTR/MRP), member 8	-1.35
Hs.298469	NM_152831	A02	ACE	Angiotensin I converting enzyme (peptidyl-dipeptidase A) 1	2.10
Hs.387567	NM_001096	A03	ACLY	ATP citrate lyase	1.45
Hs.2549	NM_000025	A04	ADRB3	Adrenergic, beta-3-, receptor	-1.20
Hs.19383	NM_000029	A05	AGT	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	1.48
Hs.631535	NM_001626	A06	AKT2	V-akt murine thymoma viral oncogene homolog 2	-1.35
Hs.130730	NM_000486	A07	AQP2	Aquaporin 2 (collecting duct)	-1.36
Hs.514821	NM_002985	A08	CCL5	Chemokine (C-C motif) ligand 5	1.48
Hs.644637	NM_000648	A09	CCR2	Chemokine (C-C motif) receptor 2	1.66
Hs.591629	NM_006139	A10	CD28	CD28 molecule	-1.70
Hs.512682	NM_001712	A11	CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)	-1.37
Hs.76171	NM_004364	A12	CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha	1.86
Hs.247824	NM_005214	B01	CTLA4	Cytotoxic T-lymphocyte-associated protein 4	-1.28
Hs.417962	NM_057158	B02	DUSP4	Dual specificity phosphatase 4	1.68
Hs.527295	NM_006208	B03	ENPP1	Ectonucleotide pyrophosphatase/phosphodiesterase 1	2.11
Hs.494496	NM_000507	B04	FBP1	Fructose-1,6-bisphosphatase 1	1.33
Hs.436448	NM_005251	B05	FOXC2	Forkhead box C2 (MFH-1, mesenchyme forkhead 1)	-1.50
Hs.632336	NM_005249	B06	FOXG1B	Forkhead box G1B	-1.19
Hs.247700	NM_014009	B07	FOXP3	Forkhead box P3	-1.51
Hs.212293	NM_000151	B08	G6PC	Glucose-6-phosphatase, catalytic subunit	2.11
Hs.461047	NM_000402	B09	G6PD	Glucose-6-phosphate dehydrogenase	-1.34
Hs.516494	NM_002054	B10	GCG	Glucagon	1.66
Hs.208	NM_000160	B11	GCGR	Glucagon receptor	1.36
Hs.1270	NM_000162	B12	GCK	Glucokinase (hexokinase 4, maturity onset diabetes of the young 2)	-1.61
Hs.389103	NM_002062	C01	GLP1R	Glucagon-like peptide 1 receptor	-1.34
Hs.524418	NM_005276	C02	GPD1	Glycerol-3-phosphate dehydrogenase 1 (soluble)	-1.19
Hs.445733	NM_002093	C03	GSK3B	Glycogen synthase kinase 3 beta	1.80
Hs.517581	NM_002133	C04	HMOX1	Heme oxygenase (decycling) 1	1.49
Hs.116462	NM_178849	C05	HNF4A	Hepatocyte nuclear factor 4, alpha	-1.54
Hs.643447	NM_000201	C06	ICAM1	Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	1.49
Hs.500546	NM_004969	C07	IDE	Insulin-degrading enzyme	-1.07
Hs.856	NM_000619	C08	IFNG	Interferon, gamma	1.67
Hs.635441	NM_000599	C09	IGFBP5	Insulin-like growth factor binding protein 5	-1.42
Hs.413513	NM_001556	C10	IKBKB	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	-1.20
Hs.193717	NM_000572	C11	IL10	Interleukin 10	-1.53
Hs.674	NM_002187	C12	IL12B	Interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation	1.69

Hs.513457	NM_000418	D01	IL4R	Interleukin 4 receptor	1.17
Hs.512234	NM_000600	D02	IL6	Interleukin 6 (interferon, beta 2)	1.17
Hs.523875	NM_001567	D03	INPPL1	Inositol polyphosphate phosphatase-like 1	1.48
Hs.89832	NM_000207	D04	INS	Insulin	-1.56
Hs.465744	NM_000208	D05	INSR	Insulin receptor	-1.69
Hs.32938	NM_000209	D06	PDX1	Pancreatic and duodenal homeobox 1	-1.68
Hs.471508	NM_005544	D07	IRS1	Insulin receptor substrate 1	-1.53
Hs.442344	NM_003749	D08	IRS2	Insulin receptor substrate 2	-1.34
Hs.588289	NM_001315	D09	MAPK14	Mitogen-activated protein kinase 14	1.20
Hs.138211	NM_002750	D10	MAPK8	Mitogen-activated protein kinase 8	1.66
Hs.21160	NM_002395	D11	ME1	Malic enzyme 1, NADP(+)-dependent, cytosolic	-1.82
Hs.72981	NM_002500	D12	NEUROD1	Neurogenic differentiation 1	-1.60
Hs.431926	NM_003998	E01	NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	1.48
Hs.653170	NM_000603	E02	NOS3	Nitric oxide synthase 3 (endothelial cell)	-1.51
Hs.298069	NM_005011	E03	NRF1	Nuclear respiratory factor 1	-1.35
Hs.431279	NM_006178	E04	NSF	N-ethylmaleimide-sensitive factor	-1.34
Hs.177766	NM_001618	E05	PARP1	Poly (ADP-ribose) polymerase family, member 1	1.79
Hs.497487	NM_002646	E06	PIK3C2B	Phosphoinositide-3-kinase, class 2, beta polypeptide	-1.20
Hs.518451	NM_005026	E07	PIK3CD	Phosphoinositide-3-kinase, catalytic, delta polypeptide	-1.19
Hs.132225	NM_181504	E08	PIK3R1	Phosphoinositide-3-kinase, regulatory subunit 1 (p85 alpha)	-1.36
Hs.103110	NM_005036	E09	PPARA	Peroxisome proliferative activated receptor, alpha	1.67
Hs.162646	NM_015869	E10	PPARG	Peroxisome proliferator-activated receptor gamma	-1.20
Hs.527078	NM_013261	E11	PPARGC1A	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	-1.27
Hs.591261	NM_133263	E12	PPARGC1B	Peroxisome proliferator-activated receptor gamma, coactivator 1 beta	-1.06
Hs.43322	NM_006251	F01	PRKAA1	Protein kinase, AMP-activated, alpha 1 catalytic subunit	-1.27
Hs.549162	NM_016203	F02	PRKAG2	Protein kinase, AMP-activated, gamma 2 non-catalytic subunit	-1.20
Hs.460355	NM_002738	F03	PRKCB1	Protein kinase C, beta 1	1.47
Hs.417549	NM_002827	F04	PTPN1	Protein tyrosine phosphatase, non-receptor type 1	1.78
Hs.282417	NM_002863	F05	PYGL	Phosphorylase, glycogen; liver (Hers disease, glycogen storage disease type VI)	1.27
Hs.296169	NM_004578	F06	RAB4A	RAB4A, member RAS oncogene family	-1.51
Hs.283091	NM_020415	F07	RETN	Resistin	1.87
Hs.82848	NM_000655	F08	SELL	Selectin L (lymphocyte adhesion molecule 1)	-1.20
Hs.380691	NM_001042	F09	SLC2A4	Solute carrier family 2 (facilitated glucose transporter), member 4	-1.21
Hs.511149	NM_003825	F10	SNAP23	Synaptosomal-associated protein, 23kDa	-1.35
Hs.167317	NM_003081	F11	SNAP25	Synaptosomal-associated protein, 25kDa	-1.51
Hs.592123	NM_004176	F12	SREBF1	Sterol regulatory element binding transcription factor 1	-1.44
Hs.83734	NM_004604	G01	STX4	Syntaxin 4	-1.07
Hs.288229	NM_003165	G02	STXBP1	Syntaxin binding protein 1	-1.34
Hs.534352	NM_006949	G03	STXBP2	Syntaxin binding protein 2	-1.51
Hs.191144	NM_006481	G04	TCF2	Transcription factor 2, hepatic; LF-B3; variant hepatic nuclear factor	-1.21
Hs.645227	NM_000660	G05	TGFB1	Transforming growth factor, beta 1	1.48
Hs.94367	NM_003317	G06	TITF1	Thyroid transcription factor 1	-1.52
Hs.241570	NM_000594	G07	TNF	Tumor necrosis factor (TNF superfamily, member 2)	1.32
Hs.279594	NM_001065	G08	TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	1.66
Hs.516826	NM_021158	G09	TRIB3	Tribbles homolog 3 (Drosophila)	1.67
Hs.66708	NM_004781	G10	VAMP3	Vesicle-associated membrane protein 3 (cellubrevin)	-1.32
Hs.653207	NM_194434	G11	VAPA	(vesicle-associated membrane protein)-associated protein A, 33kDa	-1.35
Hs.73793	NM_003376	G12	VEGFA	Vascular endothelial growth factor A	1.39

conditions by studying gene expression in blood cells [10,11]. Hence, we used such an approach to delineate similarities and differences in gene expression between normal and type 2 diabetic patients.

Hierarchical clustering and heat map of the genes significantly regulated in diabetes showed distinct gene clusters that are unique to T2DM (Figure 1a and b). This demonstrates that gene expression signatures in PBMCs could potentially provide a mechanism to distinguish diabetic state from the normal state or to explain the pathophysiology of diabetes. Sixty per cent of significantly altered genes in the diabetic group were down regulated while 40% were up regulated when compared with the non-diabetic groups (Figure 2a and b). This relationship emphasizes how strikingly different the pattern of expression is between these two groups.

We found that distinct pathophysiology of patients with type 2 diabetes was reflected in coordinate alterations in the gene expression levels of pathways involving carbohydrate, lipid and amino acid metabolism, insulin biosynthesis, secretion and its signaling mechanisms in the liver, pancreas, skeletal muscle and adipocytes and its co-factors.

Metabolic homeostasis has long been considered a major component in the pathophysiology of diabetes. In PBMCs, the genes encoding enzymes regulating carbohydrate and fat metabolism showed significant differential expression in diabetes.

This shows that insulin regulation of energy homeostasis in PBMCs is distinctly different from other target tissue such as skeletal muscle and pancreas [12].

Genes encoding metabolic enzymes, receptors, substrates and signaling molecules (ENPP1, IDE, NEUROD1, INSR, ABCC8, IRS1, IRS2, INS, PXD-1, SNARE protein complex) that showed significant differential expression (Table 2) are known to play a role in insulin signaling and homeostasis. Protein kinases such as mitogen activated protein kinases (MAPK) were found to be up regulated and AMP-activated protein kinases (AMPK) were down regulated in T2DM but their association with the pathogenesis of diabetes is unclear.

The liver is regarded as one of the central metabolic organs in the body, regulating and maintaining homeostasis. It performs most of the reactions involved in the synthesis and utilization of glucose. Diabetes results in a decrease in glucose utilization, an increase in glucose production,

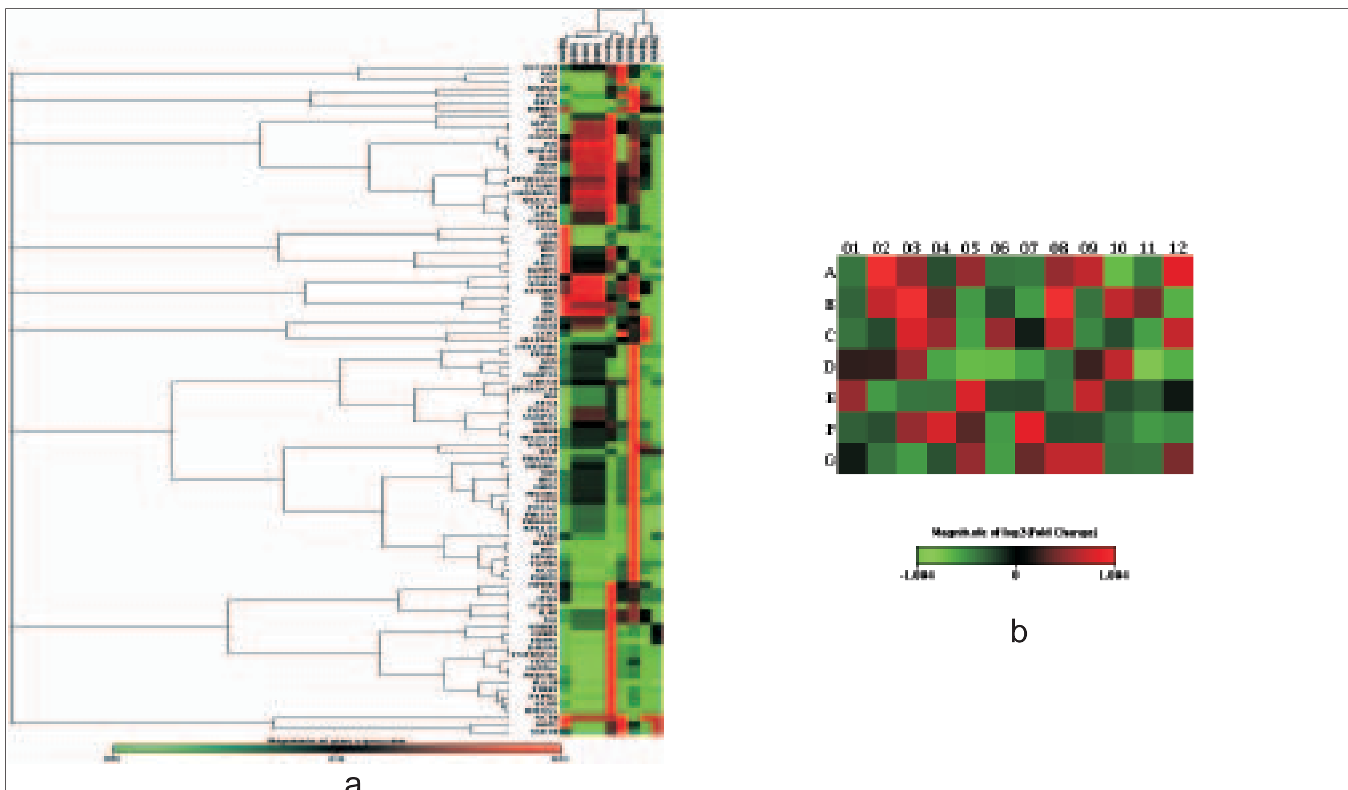

Figure 1: *Hierarchical cluster (a) and Heat map (b) illustration of gene-expression profiles of peripheral-blood mononuclear cell (PBMC) samples from type 2 diabetics (n=6) and healthy controls (n=3). "Heat map," which is the part of the figure containing colors (red, green, and black). The color represents the expression level of the gene. Red represents high expression, while green represents low expression. A01 to G12 refer to positions shown in Table 2*

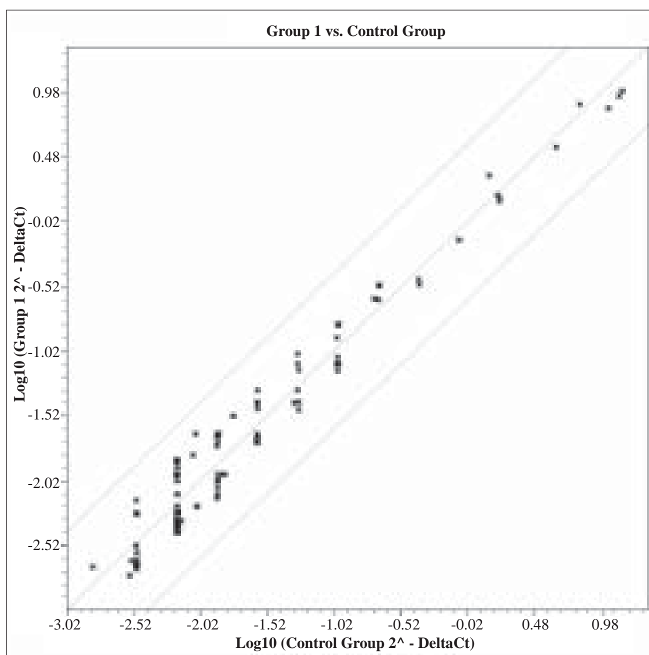

Figure 2 (a): *Scatter plot analysis of gene expression profiling on T2DM patients demonstrates differential gene expression. The y-axis represents log scores from T2DM patients (group 1) and the x-axis represents log scores from normal controls. Each symbol represents one gene within two-fold cutoff threshold*

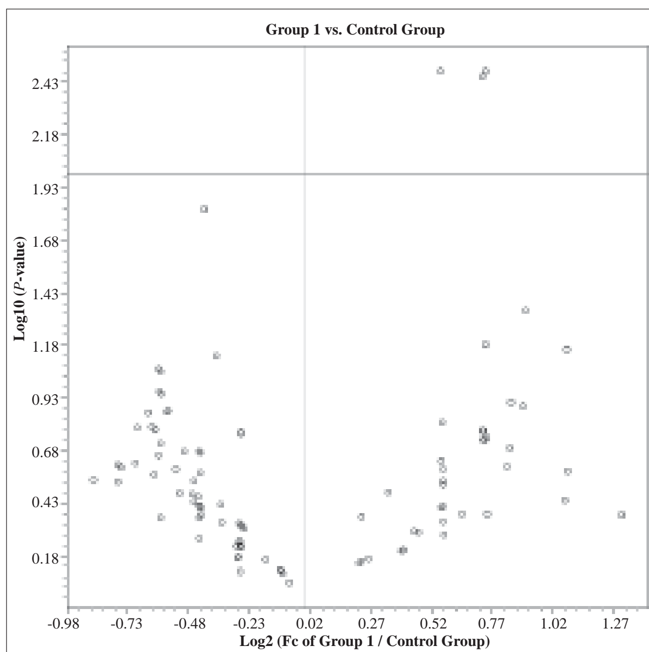

Figure 2 (b): *Volcano plot of log intensities for Control vs.T2DM. Each circle corresponds to one gene represents the average log-ratio (log fold-change) in a two group comparison. The two-fold change method selects as differentially expressed all genes above the line 0.02 (up regulation) and below the line minus 0.02 (down regulation)*

increased angiogenesis, a reduced stress-defence system, and altered mitochondrial oxidative phosphorylation (OXPHOS) in insulin-dependent tissues such as liver [13,9,14]. A marked increase in the hepatic lipid concentration has been observed during diabetes [15-17]. In contrast, the rate of hepatic lipogenesis and related enzymes is decreased. Decreased glycolysis, impeded glycogenesis and increased gluconeogenesis are some of the changes of glucose metabolism in the diabetic liver [18]. Patti *et al*. [19] in their study showed decreased expression of PGC1α coupled with reduced expression of NRF1 and PPARγ eventually result in decreased oxidative phosphorylation and lipid oxidation, accumulation of lipid in skeletal muscle and ultimately diabetes. In our studies on PBMCs, NRF1 and PPARγ were down regulated 1.35- and 1.20-fold, respectively.

Immunoregulation plays a significant role in diabetes and its pathogenesis. Enhanced expression and activation of transcription factor, nuclear factor κB and degradation of IκB triggers activation of genes involved in immune responses such as the pro-inflammaory cytokines (IL-4, TNF-α, IFN-γ). IL-4, TNF-α and IFN-γ expression was up regulated in our experiments, an indication of activation of the pathways of inflammation. An imbalance between Th1 and Th2 cells has been shown to results in decreased activity of CD28 and CTLA4-mediated immunosuppressant and activation of macrophage-mediated inflammation involved in the process of β-cell destruction [20]. In PBMC both CD28 and CTL4 are downregulated and may be indicative of similar processes.

In human eosinophils, intracellular signaling molecules ERK, p38 MAPK and c-Jun N-terminal protein kinase (JNK), together with proinflammatory cytokines IL-6, IL-10, chemokines MCP-1/C-C chemokine receptor-2 (CCR2), CXCL9/monokine induced by interferon-γ (MIG), CCL5/regulated upon activation normal T cell expressed and secreted (RANTES) and CXCL10/IFN-γ inducible protein-10 (IP-10) form a network in orchestrating inflammation in diabetes [21,22]. Our results show upregulation of CCL2, CCL5, IL-6 and MAPKs 8 and 14 in diabetes indicating triggering of proinflammatory pathways.

Regulation of cell adhesion molecules plays an important role in vascular complications in T2DM. Intracellular adhesion molecule-1 (ICAM1) is a member of the immunoglobulin super-family of adhesion molecules. This type I membrane protein mediates leukocyte-endothelial cell adhesion and signal transduction, may play a role in the development of atherosclerosis. In the retina, its increased expression contributes to the microcirculation dysfunction by increasing leucocyte adhesion, aggregation

and migration in retinal vasculature [23]. In PBMC, ICAM1 expression is markedly elevated and this would enhance leucocyte adhesion. Plasma concentrations of ICAM1, in patients with T2DM are more reflective of hyperglycemia than hyperinsulinemia or insulin resistance [24].

In summary, the current study demonstrates that 84 gene transcripts, involved in a variety of functions, were altered in PBMCs of people with T2DM. Results of this study suggest that there are significant differences in the expression of various genes concerned with carbohydrate, lipid, and protein metabolism, ubiquitin-mediated proteolysis, signal transduction pathways, neuroactive ligand-receptor interaction, cell adhesion molecules, cytokine-cytokine receptor interaction, insulin signaling and immune system pathways, oxidative phosphorylation, and PPAR signaling pathways in subjects with T2DM compared to normal.

These alterations in gene transcripts may represent the response to increased circulating insulin levels necessary to maintain normal glucose levels in these patients. Further, studies on important known and novel targets regulated in T2DM in peripheral blood cells identified in this study will provide new insights in the role of peripheral blood cells in insulin action, insulin resistance and interactions with key target tissues such as skeletal muscle and endocrine pancreas.

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Correspondence:
Sekaran Muniandy,
Department of Molecular Medicine, Faculty of Medicine,
University of Malaya, Kuala Lumpur-506 03, Malaysia.
E-mail: sekaran@um.edu.my

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Effect of *Centella asiatica* L. extract Against Ethinylestradiol-induced Genotoxic Damage on Cultured Human Lymphocytes

Yasir Hasan Siddique, Tanveer Beg, Mohammad Afzal

Human Genetics and Toxicology Laboratory, Section of Genetics, Department of Zoology, Faculty of Life Sciences, Aligarh Muslim University, Aligarh, India

Abstract

In this study the effect of *Centella asiatica* L. extract was studied against genotoxic doses of ethinylestradiol on human lymphocytes culture. The different doses of *C. asiatica* L. extract i.e. $1.075x10^{-4}$, $2.125x10^{-4}$ and $3.15x10^{-4}$ g/ml were treated separately with 5 μM of ethinylestradiol. Similar treatments were given with 10 μM of ethinylestradiol. The treatments result in a significant dose dependent decrease in chromosomal aberrations and sister chromatid exchanges on human lymphocyte cultures induced by 5 and 10 μM of ethinylestradiol. The selected doses of *C. asiatica* L. extract were not genotoxic itself. Hence it is concluded that *C. asiatica* L. extract reduced the genotoxic damage during the ethinylestradiol therapy in patients and thereby reducing the chances of cancer development in humans.

Key words: *Centella asiatica* L, chromosomal aberrations, human lymphocytes, natural products, sister chromatid exchanges

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Introduction

Centella asiatica L. belongs to the family Umbellifera. It is known as Mandukparni or Indian Penny Wort. It is found in swampy areas of India, commonly as a weed in crop fields and in other waste places throughout India up to an altitude of 600 m [1]. The crude extract of *C. asiatica* and the products derived from it have various pharmacological properties [2-6]. The crude extract of *C. asiatica* was shown to be non-toxic in normal human lymphocytes [7], and reduced the genotoxic effects of methyl methanesulphonate and cyclophosphamide in cultured human lymphocytes [8].

Conjugated estrogens, have been listed in the fourth annual report on carcinogenesis (ROC), as human carcinogens [9]. A number of individual steroidal estrogens including estradiol-17β and mestranol were listed in the ROC as reasonably anticipated human carcinogens [9]. Steroidal estrogens are placed in a Group 1 as carcinogenic to humans, on the basis of sufficient evidence of carcinogenicity in humans [10]. Ethinylestradiol is used with various progestogens, in combined oral contraceptive formulations [11]. There are sufficient evidences for the carcinogenicity of ethinylestradiol in experimental animals [12]. It has also been reported to cause aneuploidy in Chinese Hamster DON Cells *in vitro* [13], aneuploidy and polyploidy both, in V79 cells *in vitro* [14]. Estrogens are used in the treatment of sexual and metabolic disorders and also in oral contraceptives [15]. Prolonged use of estrogens has been reported to develop various types of malignancies in human and experimental animals [15]. The genotoxic effects of estrogens/ progestins can be reduced by the use of antioxidants [16-22] and natural plant products [23-28]. The genotoxicity testing provides human a risk assessment. An increase in frequency of chromosomal aberrations in peripheral blood lymphocytes is associated with an increased overall risk of cancer [29,30].

Any reduction in the genotoxicity gives an indication of the lesser possibility of carcinogenesis [31]. The majority of populations use traditionally natural preparations derived from the plant material for the treatment of various diseases, and for that reason it becomes necessary to assess the mutagenic potential or modulating action of plant extract in combination with other substances. In an earlier study, ethinylestradiol was found to be genotoxic at five and 10 μM [32]. Herbal preparations can be useful in taking care of the genotoxic effects of certain drugs. The aim of this work is to study the effect of *C. asiatica* L. extract against the genotoxic doses of ethinylestradiol.

Material and Methods

Chemicals

Ethinylestradiol (CAS No.: 57-63-6, Sigma); RPMI 1640, Fetal calf serum, Phytohaemagglutinin-M, Antibiotic-antimycotic mixture (Gibco); Dimethyl sulphoxide, 5-Bromo-2-deoxy uridine; Colchicine (SRL, India), Giemsa stain (Merk).

Extract preparation

C. asiatica L. leaves were collected from the nursery of Forest Research Institute (FRI), Dehradun (UA) and were air dried and ground to fine powder. Extraction was performed by soaking samples (30 g of dry weight) in 300 ml of acetone for 8-10h at 40-60°C in Soxhlet's apparatus. After filtration, the excess of solvent was removed by rotatory evaporator. The extract concentrations of $1.075x10^{-4}$, $2.127x10^{-4}$ and $3.15x10^{-4}$ g/ml of culture medium were established [8].

Human lymphocyte culture

Duplicate peripheral blood cultures were prepared according to Carballo *et al*. [33]. Briefly, heaprinized blood samples (0.5 ml), were obtained from healthy female donors and were placed in a sterile culture bottles containing seven ml of RPMI-1640 medium, supplementd with fetal calf serum (1.5 ml), antibiotic-antimycotic mixture (1.0 ml) and phytohaemagglutinin (0.1 ml). The culture bottles were kept in an incubator at 37°C for 24 hours.

Chromosomal aberration analysis

After 24 hours, 5 µM of ethinylestradiol treatment (dissolved in dimethylsulphoxide, 5 µl/ml) was given separately with $1.075x10^{-4}$, $2.125x10^{-4}$ and $3.15x10^{-4}$ g/ml of *Centella asiatica* L. extract. Similarly, 10 µM of ethinylestradiol was also given with the same three doses of *C. asiatica* extract. About, 0.5 ml of S9 mix (supplement with NADP) was also given with the treatments for six hours. The cultures were washed with fresh RPMI-1640 and kept for another 48 hours in an incubator at 37°C. After 47 hours, 0.2 ml of colchicine (0.2 µg/ml) was added to the culture bottles. Cells were centrifuged at 800 g for 10 min. The supernatant was removed and five ml of pre-warmed (37°C), KCl hypotonic solution (0.075 M) was added. Cells were resuspended and incubated at 37°C for 15 minutes. The supernatant was removed by centrifugation at 800 g for 10 minutes and five ml of chilled fixative (methanol: glacial acetic acid; 3:1) was added. The fixative was removed by centrifugation and the procedure was repeated twice. The slides were stained in three per cent Giemsa solution in phosphate buffer (pH 6.8) for 15 minutes. Three hundred metaphases were examined for the occurrence of different types of abnormally. Criteria to classify the different types of aberrations were in accordance with recommendation of EHC46 for environmental monitoring of human population [34].

Sister chromatid exchange analysis

For sister chromatid exchange analysis, bromodeoxyuridine (10 µg/ml) was added at the beginning of the culture. After 24 h, 5 µM of ethinylestradiol (dissolved in dimethylsulphoxide, 5 l), the treatment was given separately with $1.075x10^{-4}$, $2.125x10^{-4}$ and $3.15x10^{-4}$ g/ml of *C. asiatica* extract respectively. Similar treatments with the three dosages of *C. asiatica* extract were given separately with 10 µM of ethinylestradiol. About, 0.5 ml of S9 mix (supplemented with NADP) was also given along with the treatments for 6 h. The cultures were washed with fresh RPMI 1640 and kept for another 48 hours, in an incubator. Mitotic arrest was performed by adding 0.2 ml of colchicine (0.2 µg/ml). Hypotonic treatment and fixation were performed in the same way as described for the chromosomal aberration analysis. The sister chromatid exchange average was taken from an analysis of 50 metaphases during second cycle of division [35].

Statistical analysis

Student 't'-test was used for analysis of CAs and SCEs. Regression analysis was performed using Statistica Soft Inc.

Results

Ethinylestradiol induced a significant increase of abnormal metaphases as compared to the untreated at 5 and 10 µM. A significant dose dependent decrease in number of abnormal metaphase was observed when 5 and 10 µM of ethinylestradiol was treated separately, with the different doses of *C. asiatica* extract, i.e. $1.075x10^{-4}$, $2.125x10^{-4}$ and $3.15x10^{-4}$ g/ml (Table 1). For sister chromatid exchange analysis, a significant increase was observed at both of the studied doses of ethinylestradiol i.e. 5 and 10 µM (Table 2). A significant decrease in sister chromatid exchanges per cell was observed when 5 and 10 µM of ethinylestradiol was treated, separately, with the different doses of *C. asiatica* extract i.e. $1.075x10^{-4}$, $2.125x10^{-4}$ and $3.15x10^{-4}$ g/ml (Table 2). Regression analysis was also performed to determine the dose effect of *C. asiatica* extract on 5 and 10 µM of ethinylestradiol. For a number of abnormal metaphases and sister chromatid exchanges, a decrease in slope of linear regression lines with increase in dose of the extract was observed in each treatment. For abnormal metaphases the treatment of 5 µM ($F = 29.14$; P less than 0.04) and 10 µM (F is equal to 12.65; $P < 0.05$) of ethinylestradiol, with the increase in the doses of *C. asiatica* extract results in the decrease in the slope of the linear regression lines (Figures 1 and 2).

Table 1: Effect of Centella asiatica L.extract on chromosomal aberrations (CAs) induced by ethinylestradiol

Treatments	Cells scored	Abnormal metaphases without gaps		Total structural chromosomal aberrations		
		Number	Mean±SE	Gaps	CTB	CSB
Ethinylestradiol (μM)						
5	300	12	4.00±1.13[a]	9	8	4
10	300	18	6.00±1.37[a]	11	12	6
Ethinylestradiol (μM)+ CAE (g/ml)						
5+1.075x10^{-4}	300	8	2.67±0.93[b]	4	6	2
5+2.125x10^{-4}	300	6	2.00±0.80[b]	3	4	2
5+3.15x10^{-4}	300	5	1.67±0.73[b]	2	4	1
10+1.075x10^{-4}	300	13	4.33±1.17[b]	5	9	4
10+2.125x10^{-4}	300	10	3.33±1.03[b]	4	6	4
10+3.15x10^{-4}	300	9	3.00±0.98[b]	3	6	3
CAE (g/ml)						
1.075x10^{-4}	300	2	0.67±0.47	1	2	-
2.125x10^{-4}	300	3	1.00±0.57	2	2	1
3.15x10^{-4}	300	4	1.33±0.66	2	3	1
Untreated	300	2	0.67±0.47	3	2	-
Negative control (DMSO, 5 μl/ml)	300	2	0.67±0.47	1	2	-
Positive control (CP, 0.16 μg/ml)	300	29	9.67±1.70[a]	16	18	10

CAE: Centella asiatica extract; DMSO: Dimethylsulphoxide; CP: Cyclophosphamide; CTB: Chromatid break; CSB: Chromosome break; CTE: Chromatid exchange; DIC: Dicentric; S.E.: Standard error.

Table 2: Effect of Centella asiatica L. extract on sister chromatid exchanges (SCEs) induced by ethinylestradiol

Treatment	SCEs / Cell (Mean ± SE)	Range
Ethinylestradiol (μM)		
5	6.92 ± 0.72[a]	2-8
10	7.62 ± 0.84[a]	2-9
Ethinylestradiol (μM) + CAE (g/ml)		
5+1.075x10^{-4}	3.24 ± 0.34[b]	0-5
5+2.125x10^{-4}	2.94 ± 0.29[b]	0-5
5+3.15x10^{-4}	2.78 ± 0.26[b]	0-5
10+1.075x10^{-4}	4.88 ± 0.54[b]	1-5
10+2.125x10^{-4}	3.94 ± 0.42[b]	0-5
10+3.15x10^{-4}	3.54 ± 0.38[b]	0-5
CAE (g/ml)		
1.075x10^{-4}	2.18 ± 0.18	0-5
2.125x10^{-4}	2.24 ± 0.21	0-5
3.15x10^{-4}	2.72 ± 0.23	0-5
Untreated	1.12 ± 0.12	0-5
Negative control (DMSO, 5 μl/ml)	1.88 ± 0.16	0-5
Positive control (CP, 0.16 μg/ml)	12.24± 0.96[a]	3-13

CAE: Centella asiatica extract; DMSO: Dimethylsulphoxide; CP: Cyclophosphamide; S.E.: Standard error.

For sister chromatid exchange analysis, the treatment of 5 μM ($F = 35.19$; $P < 0.01$) and 10 μM ($F = 19.69$; $P < 0.03$) of ethinylestradiol, with the increase in the dosages of C. asiatica extract, the decrease in slope of linear regression lines was observed (Figure 3 and 4).

Discussion

The results of the study reveal that the selected dosages of the plant extract were not genotoxic *per se,* but reduced the genotoxic damage of ethinylestradiol on human lymphocytes *in vitro*. In our earlier study, three doses of ethinylestradiol (1, 5 and 10 μM) were studied [32]. Ethinylestradiol was found to be genotoxic at five and 10 μM only in the presence of S9 mix supplemented with NADP. International Agency on Cancer (IARC), mainly on the basis of epidemiological studies classifies steroidal estrogens and estrogen progestins combinations among agents carcinogenic to humans (Group 1), progestins as possibly carcinogenic (Group 2) and androgenic anabolic steroids, as probably carcinogenic (Group 2A) [36]. Cancer is characterized by genetic instability, i.e. gross chromosomal abnormalities, which consist of translocation and gene amplifications. Molecular and cytogenetic evidence indicates that the induction of structural and numerical chromosome aberrations in cell play an important role in the neoplastic development of certain tumors [37]. An increase in frequency of chromosomal

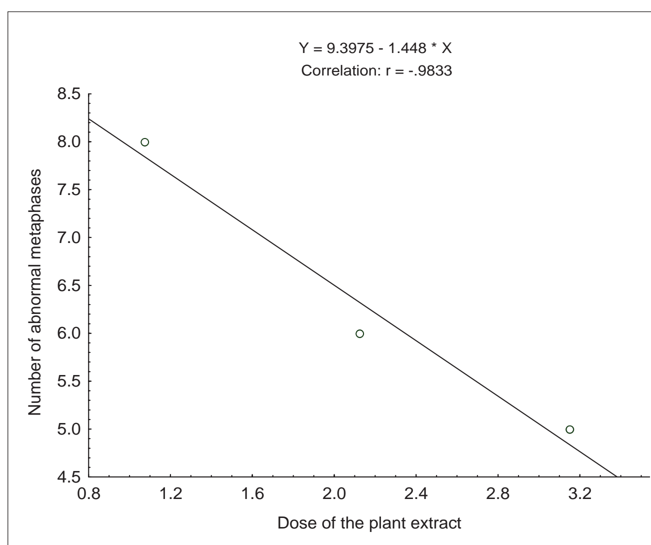

Figure 1: *Regression analysis for the dose effect of Centella asiatica extract on number of abnormal metaphases treated with 5µM of ethinylestradiol*

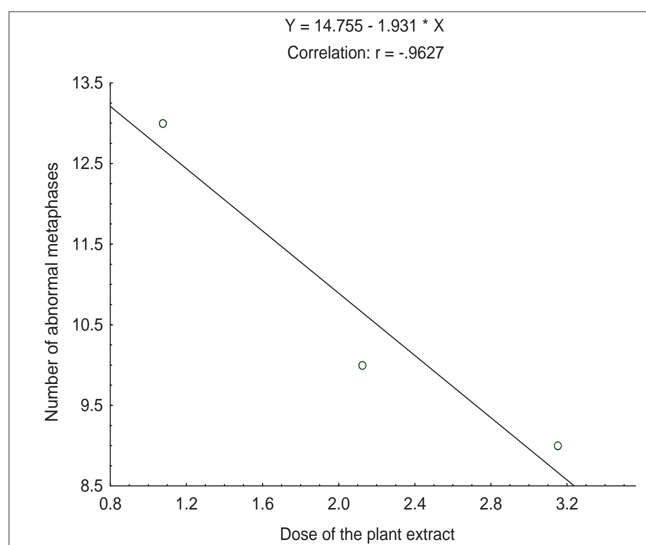

Figure 2: *Regression analysis for the dose effect of Centella asiatica extract on number of abnormal metaphases treated with 10 µM of ethinylestradiol*

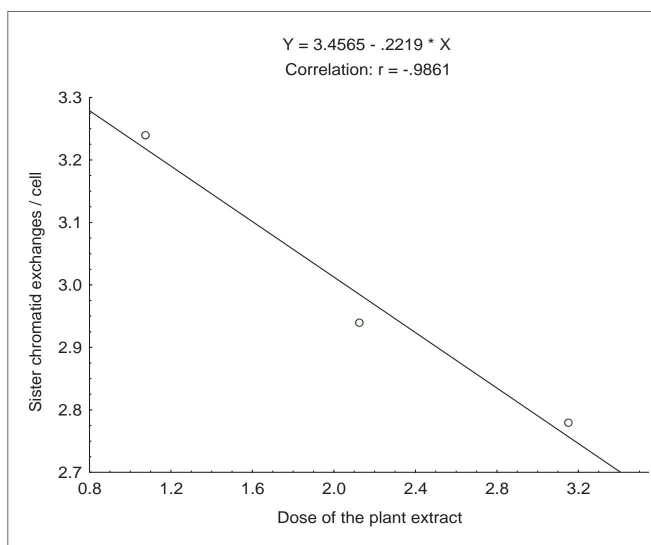

Figure 3*: Regression analysis for the dose effect of Centella asiatica extract on sister chromatid exchanges / cell treated with 5 µM of ethinylestradiol*

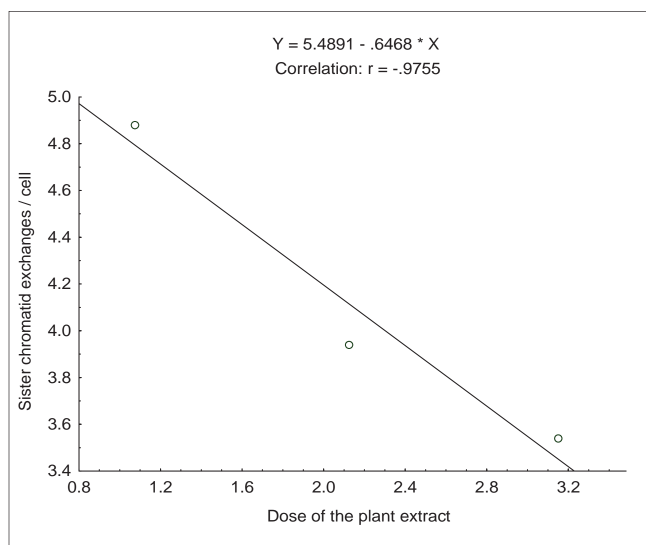

Figure 4*: Regression analysis for the dose effect of Centella asiatica extract on sister chromatid exchanges / cell treated with 10 µM of ethinylestradiol*

aberrations in peripheral blood lymphocytes is associated with an increased overall risk of cancer [29,30]. Genetic toxicology tests are *in vitro* and *in* vivo assays designed to detect compounds that induce genetic damage directly or indirectly.

Any kind of chromosomal damage may play an important role in many malignancies. The ready quantifiable nature of sister chromatid exchanges with high sensitivity for revealing toxicant-DNA interaction and the demonstrated ability of genotoxic chemicals to induce significant increase in sister chromatid exchanges in cultured cells has resulted this endpoint being used as indicator of DNA damage in blood lymphocytes of individual exposed to genotoxic carcinogens [31]. The above genotoxic endpoints are well known markers of genotoxicity and any reduction in the frequency of these genotoxic endpoints gives us an indication of the anti-genotoxicity of a particular compound [31].

At present much attention of preventive medicine research is focused on natural antioxidants, leading to isolation and identification of new biologically active molecules by the pharmaceutical industry. The crude extract of plants may

contribute to total intake of plant antioxidants and be even better source of dietary antioxidants [38].

The compounds present in the extract may inhibit the promutagen bioactivation by blocking the oxidation processes or reacts with electrophilic metabolites of promutagens [39]. The verification of the possible mutagenic and/or anti-mutagenic effects of medicinal plants infusion/ extracts is another important factor in studies. Such effects have been elucidated in some plant species by using various test systems [40]. Medicinal herbs contain complex mixtures of thousand of compounds that can exert their antioxidant and free radical scavenging effect either separately or in synergistic ways [41]. Many plant products protect against xenobiotics either by inducing defoxifying enzymes or by inhibiting oxidative enzymes [42]. Detoxification systems play a major role in preventing carcinogenesis, but also an important role in preventing xenobiotic as well as indogenous toxicity. Phytochemicals plays an important role in inhibition of carcinogenesis [43]. Some plant extract may possess substances that can modulate the genotoxicity of other compounds.

The data obtained in this study suggests that the compounds present in the extract of *C. asiatica* are not mutagenic on their own or when associated with ethinylestradiol in the presence of NADP.

The protective effect observed in the present study i.e. significant reduction in the frequency of cells with chromosomal damage and sister chromatid exchanges may be due to the direct action of the compounds present in the extract on ethinylestradiol by inactivating it enzymatically or chemically. The genotoxic effects of ethinylestradiol have been attributed to the bio-activation of ethinylestradiol in the presence of S9 mix supplemented with NADP [32]. The compounds present in the extract may also enhance the DNA repair system or DNA synthesis or even may prevent the bioactivation of certain chemicals [44]. Our earlier reports on synthetic progestins showed that they are genotoxic in *in vitro* [45-48] and *in vivo* [49,50]. A reduction in the frequency of SCE and chromosomal aberrations in the test system thereby indicates the possibility of reducing the chances of carcinogenesis during the ethinylestradiol therapy in patients.

Conclusion

The extract of *Centella asiatica L.* is potent enough to reduce the genotoxic damage of ethinylestradiol. The results of the present study suggest that the plant extract can form the basis of herbal medicine as it has antioxidants that are responsible for the reduction of the genotoxic damage of the drugs. The prolonged use of ethinyl estradiol has been reported to induce cancer. Any reduction in the frequency of CAs and SCEs thereby gives an indication of little chances of developing cancer. The extract of *Centella asiatica* reduces the frequency CAs and SCEs induced by ethinyl estradiol.

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Correspondence:
Yasir Hasan Siddique,
Human Genetics and Toxicology Laboratory,
Section of Genetics, Department of Zoology,
Faculty of Life Sciences, Aligarh Muslim University,
Aligarh-202 002 (U.P.), India.
E-mail: yasir_hasansiddique@rediffmail.com

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Holoprosencephaly: A Rare Complication of Maternal Hypothyroidism

Shailja Puri, Ashish Gupta, Natu SM, Shailendra Singh[1]

Departments of Pathology, [1]TB and Chest, Chhatrapati Shahuji Maharaj Medical University, Lucknow, Uttar Pradesh, India

Abstract

Maternal hypothyroidism is known to cause an array of complications, maternal and fetal. Some of them include infertility, abortion, still birth, pre-term labor, fetal distress, birth defects, low birth weight and low IQ. We present a case of 32-year-old multiparous woman with hypothyroidism whose pregnancy was terminated in mid-gestation because of development of holoprosencephaly. Holoprosencephaly is failure of the forebrain to divide into distinct lateral cerebral hemispheres. To the best of our knowledge this is the first case of holoprosencephaly associated with maternal hypothyroidism.

Key words: Maternal hypothyroidism holoprosencephaly, pregnancy

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Introduction

Maternal hypothyroidism causes various maternal and fetal complications like infertility, abortion, still birth, preterm labor, fetal distress, birth defects, low birth weight and low IQ. The fetus is able to produce thyroid hormones by 8-10 week gestation but prior to that time is totally dependent on maternal thyroid hormones [1]. The fetal gland becomes operational near mid-gestation [2]. We report here a singleton pregnancy with Alobar Holoprosencephaly in a 32-year-old multiparous hypothyroid woman during early pregnancy. Holoprosencephaly denotes an incomplete or absent division of embryonic forebrain into distinct lateral hemispheres. The prevalence of holoprosencephaly during embryogenesis is 1:250 and 1:16,000 in new born infants [3,4]. Holoprosencephaly has been classified into three grades of severity: Alobar Holoprosencephaly - complete absence of midline forebrain division resulting in a monoventricle and fused cerebral hemispheres. Semilobar Holoprosencephaly - incomplete forebrain division resulting in partial separation of cerebral hemispheres, typically, posteriorly and Lobar Holoprosencephaly - complete ventricular separation with focal areas of incomplete cortical division or anterior falcine hypoplasia [5].

Case Report

A 32-year-old multiparous woman P_{2+3} blood group O Rh negative presented to the Chemical Laboratory, Department of Pathology, Chhatrapati Shahuji Maharaj Medical University, Lucknow, Uttar Pradesh, India. Married for 17 years, non-consanguineous marriage, she tested negative for VDRL, HIV 1 and 2, Toxoplasma, Rubella, Cytomegalovirus and herpes simplex virus. Fasting blood glucose was normal (95 mg/dl). No history of smoking or alcohol intake. Her first pregnancy was after eight years of marriage. Result: spontaneous abortion at 15-16 week gestation. Second pregnancy in 2000: Uneventful pregnancy delivered a healthy male child. Third pregnancy 2006: Conceived six years after second pregnancy. During early pregnancy she presented with excessive weight gain (90 kg - before pregnancy 52 kg), myxoedema, hypercholesterolemia (270 mg/dl) and hypertension (144/90 mm Hg). Thyroid function test showed high Thyroid stimulating hormone (TSH), low tri-iodothyronine (T3) and low tetra- iodothyronine (T4) (Table 1). At 25-week gestation Alobar Holoprosencephaly was detected by ultrasonography. Parameters noted on ultrasound: Female fetus, head circumference 21 cm, total length 28 cm, foot length 5 cm corresponding to gestational age of 24 weeks. This pregnancy was terminated at 25-week gestation. Weight of fetus was 625 gm. Internal

Table 1: Thyroid hormone profile during pregnancy

Date	TSH normal range (0.5-4.7 mIU/L)	T3 normal range (0.92-2.78 nmol/L)	T4 normal range (58-140 nmol/L)	Remark
16/5/05	2.73 mIU/L	1.75 nmol/L	137 nmol/L	
7/1/06	9.67 mIU/L	0.90 nmol/L	55 nmol/L	Patient had Hypothyroidism During early pegnancy
10/02/06	8.00 mIU/L	0.85 nmol/L	51 nmol/L	
25/02/06	8.53 mIU/L	0.87 nmol/L	54 nmol/L	Thyroxin started
18/5/06	1.99 mIU/L	1.80 nmol/L	100 nmol/L	
12/07/07	0.98 mIU/L	2.0 nmol/L	135 nmol/L	Holoprosencephaly Detected on Ultrasonography At 25 week. Gestation
19/09/07	0.02 mIU/L	2.6 nmol/L	152 nmol/L	
29/4/08	2.41 mIU/L	13.7nmol/L	150 nmol/L	
30/5/08	1.66 mIU/L	5.4 nmol/L	142 nmol/L	
11/7/08	2.81 mIU/L	3.73 nmol/L	138 nmol/L	
30/8/08	0.75 mIU/L	2.67 nmol/L	150 nmol/L	
12/9/08	1.17 mIU/L	5.13 nmol/L	151 nmol/L	
25/11/08	2.18 mIU/L	1.55 nmol/L	132 nmol/L	

examination showed malformation in brain, thalami fused, no separation of cerebral hemispheres, mono-ventricle covered by thin cortex, olfactory tract not seen. These findings were suggestive of Alobar Holoprosencephaly. No malformation of any other organ was seen. Parents refused to get an autopsy done and karyotype was not done. Fourth pregnancy 2007: A year later, spontaneous abortion at six to eight-week gestation. Fifth pregnancy 2008: Uneventful, delivered a full term male child with club feet and patchy pneumonities at birth.

Discussion

Thyroid hormone is critical for normal fetal brain development, neuronal multiplication, migration and structural organization [2]. A lack of adequate maternal thyroid hormones can lead to disruption of normal brain growth and development in the fetus manifesting itself in a variety of ways such as poor cognitive development, mental retardation and cerebral palsy further in life [6,7]. In this case there are clinical features of hypothyroidism namely excessive weight gain, myxoedema, hypercholesterolemia and hypertension in early third pregnancy. Table 1 illustrates that the thyroid function tests done during this pregnancy. The results showed high TSH value and low level of thyroid hormones in early pregnancy when maternal thyroid hormones are critical for brain growth and development. Lack of thyroid hormones probably leads to development of alobar holoprosencephaly which is detected at 25-week gestation ultrasonographically. After starting thyroxin, thyroid hormone profile came to normal and remained in normal range in follow up. The causative factors of Holoprosencephaly are manifold of which chromosomal disorders account for not more than 40-50% mutations-mis-sense, non sense, deletion, insertion and frame shift [8]. Suggested non-genetic risk factors for HPE are maternal diabetes, infections during pregnancy-syphilis, toxoplasmosis, rubella, herpes, cytomegalovirus and various drugs taken during pregnancy-alcohol, aspirin, lithium, thorazine, anti-convulsants, hormones and retinoic acid [9]. These varied causes attest to the extreme heterogeneity of the disease. Since disturbance of brain development resulting in holoprosencephaly occurs before day 28 of embryonic life, basically, any factor that causes perturbation to forebrain development at the critical phase of mid to late gastrulation period of embryogenesis can result in holoprosencephaly [10]. All known causes of holoprosencephaly are ruled out in this case. The most probable factor instrumental in early pregnancy is low level of maternal thyroid hormones. Based on these findings we present an association between maternal hypothyroidism and holoprosencephaly.

To the best of our knowledge this is the first case of maternal hypothyroidism causing holoprosencephaly. Thyroid diseases are the commonest endocrinal disorders affecting woman of reproductive age group so expectant mothers should be screened for thyroid hormone status.

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Correspondence:
S. M. Natu,
Department of Pathology,
Chhatrapati Shahuji Maharaj Medical University,
(King George's Medical University)
Lucknow-226 003, Uttar Pradesh, India.
E-mail: smnatu.natu@gmail.com

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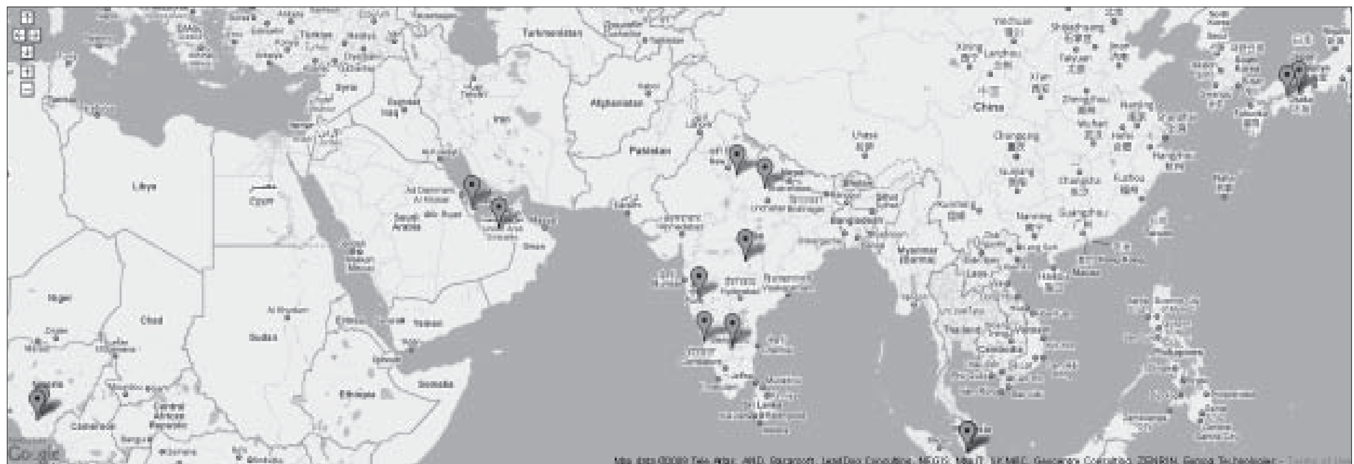

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Prescription Pattern of Fixed Dose Drug Combination in Tertiary Care Hospital: An Evaluation Study

Eesha BR, Tara Shanbhag, Adiga MNS, Smitha Shenoy

Department of Pharmacology, Kasturba Medical College, Manipal, India

Abstract

The fixed dose combination (FDC) therapy varies among countries. The available combination drugs in the market include both rational and irrational ones. The use of irrational fixed dose combination has a great impact on consumers, society and the nation. Since a few studies on prescribing patterns of fixed dose combination are available in literature, there is need for such a study. In this prospective study, carried out in a tertiary care teaching private hospital, one thousand prescriptions from the hospital pharmacy were collected, classified according to anatomic therapeutic and chemical (ATC) classification. The fixed drug combination used was compared with the essential drug list of World Health Organization (WHO) and other countries. In one thousand prescriptions, 3151 drugs were prescribed, of which, 960 were fixed dose combination drugs. Among those, the anti-infective agents for systemic use (17.5%) and drugs affecting the central nervous system (17.08%), cardiovascular system (15.41%) and alimentary system (11.14%) mainly contributed to the fixed dose combinations. The highest percentage of FDCs was seen among the nervous system (36.6%), anti-infective (33.67), anti-neoplastic (31%), respiratory system (31%) and various drugs (35.90%) category which were more than the average percentage of fixed dose combination drug use (30.47%). Majority of the FDCs prescribed (73.25%) were not approved in the WHO essential drug list. There is a significant burden of these combinations on consumers, physicians and policy makers which needs to be handled with care.

Key words: Essential drug list, fixed dose combination, prescription, rationality

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Introduction

The goal of drug therapy is to achieve the desired therapeutic response without producing toxicity, i.e., maximizing efficacy yet minimizing untoward effects. Traditional approach with monotherapy is successful in only 50% of instances, because multiple mechanisms are involved in the pathogenesis of most diseases, and a single drug may not interdict all offending pathways [1]. Low adherence to the prescribed medication for chronic conditions is well documented in literature [2,3]. The reason could be specific condition being treated, health system, the social and economic conditions and more importantly the therapy itself [4]. Concomitant use of two or more drugs (polypharmacy) adds to the complexity of individualization of drug therapy. The dose of each drug should be adjusted to achieve the optimal benefit. To obviate these problems, fixed dose combination of drugs (FDC) were developed [5].

The FDC of drugs is defined as product of two or more active ingredients in a defined composition. They are accepted when the dosage of each ingredient meets the requirement of a defined population and if the advantages outweigh the added risks of using two or more drugs [6]. Even though the WHO included only 25 FDCs in its essential drug list [7], the major chunk of prescription includes these combinations. This is because there has been little scientific study of these preparations but much emotion and rhetoric has been expended both for and against them [8]. Hence, there is a need to study the pattern of prescription from time to time to evaluate their rationality. In this context we undertook this study to know the prescription pattern of FDC in our setting.

Material and Methods

In this prospective observational study, we collected prescriptions from the pharmacy of Kasturba Hospital, a

1650 bedded teaching, tertiary care hospital catering to the healthcare needs of South-West districts of Karnataka, Goa and Northern districts of Kerala. A total of 1000 prescriptions were collected in the pharmacy after obtaining approval from the Kasturba Hospital Ethics Committee. The prescriptions of patients consulting both in-patients as well as out patient departments of internal medicine, surgery, obstetric and gynecology, pediatrics, dermatology, orthopedics, otorhinolaryngology, ophthalmology and subspecialities like cardiology, neurology, gastroenterology, urology, pulmonary and chest medicine were collected from the hospital pharmacy over a period of one month. The case sheets of patients were referred to if information about the drug was not clearly mentioned in the prescription. The prescribed fixed dose combination drugs and other drugs were categorized according to ATC classification. Descriptive statistics was used to analyze data. The percentage of FDCs used in each class and their contribution to overall FDCs were calculated. Data was compared with FDCs approved in WHO's essential drug list and other countries.

Results

In 1000 prescriptions, a total of 3151 drugs were prescribed. Of this, 960 were FDCs and 2191 were others. The highest percentage of FDCs were seen in nervous system (36.6%), anti-infective (33.67%), anti-neoplastic (31%), respiratory system (31%) and various drugs class (35.90%). The group-wise distribution of drugs, according to ATC classification and FDCs in that category, is given in the Table 1. Of the total 960 FDC prescriptions, majority were anti-infective agents for systemic use (17.5%), central nervous system (17.08%), cardiovascular system (15.41%) and alimentary system (11.14%) respectively as shown in the Table 2. Among drugs affecting the central nervous system, the most common FDC was a combination of ibuprofen and paracetamol (56.7%) followed by nimesulide and paracetamol (20.12%) as shown in the Figure 1. Levodopa and carbidopa combination constituted for 9.74% which was the only FDC approved in WHO's essential drug list. In the anti-infective class of drugs, the most common FDCs prescribed were combinations of amoxicillin and clavulanic acid (53.57%), sulfamethoxazole and trimethoprim (17.85%) and isoniazid and rifampin (5.95%) which are approved by WHO. The rest of the FDCs used were approved by either DCGI (Drug Controller General of India) or the state drug statutory board shown in the Figure 2.

Many anti-hypertensive FDCs were prescribed in our hospital. Among them were combinations of ramipril and hydrochlorothiazide (29.07%), losartan and hydrochloro-thiazide (20.61%), amlodipine and atenolol (20.61%) and

Table 1: ATC class of drugs used in study

ATC Class of drug	Non FDC (%)	FDC (%)
Alimentary tract & metabolism	274 (71)	107 (29)
Blood and Blood forming organs	103(76)	48 (31.78)
Cardiovascular system	367 (71.27)	131 (32.91)
Dermatologic	98(82)	21 (18)
Genitourinary and sex hormones	82(78)	23 (22)
Systemic hormone preparation	132(72)	51(28)
Anti infective for systemic use	331 (66.33)	168 (33.67)
Antineoplastics & Immunomodulators	38 (69)	17 (31)
Musculoskeletal system	52(71)	21 (29)
Nervous system	284 (63.4)	164(36.6)
Anti parasitic & insecticides	10 (66.6)	5(33.4)
Respiratory system	189 (69)	87(31)
Sensory Organs	42 (79)	11(21)
Various drugs	189 (64.1)	106(35.9)

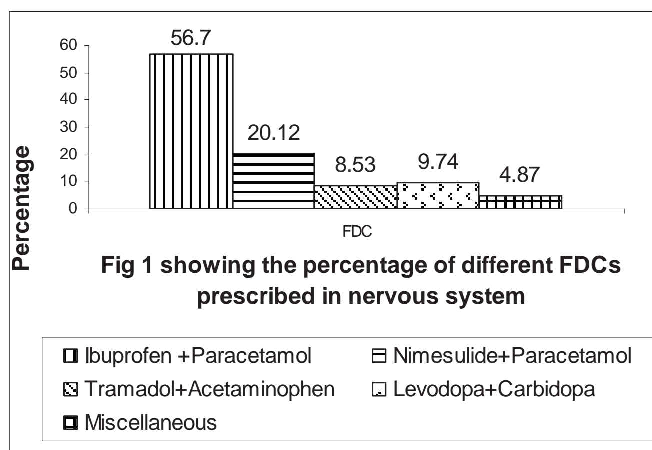

Figure 1: *Percentage of different FDCs prescribed in nervous system*

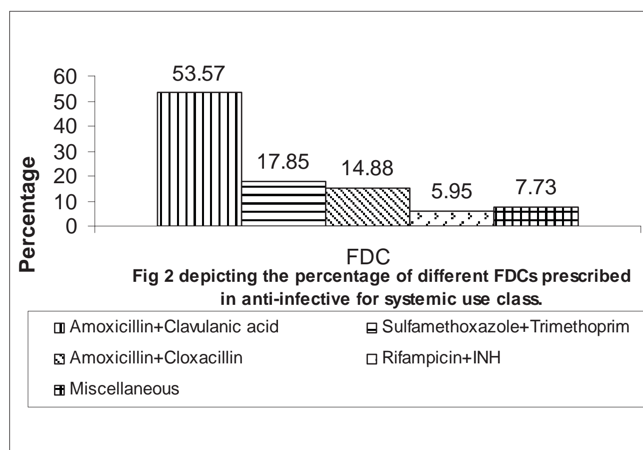

Figure 2: *Percentage of different FDCs prescribed in anti-infective for systemic use class*

Table 2: Percentage of different FDCs prescribed in our set up (n=960)

ATC class of drug	FDC	Percentage
Alimentary tract & metabolism	107	11.14
Blood and Blood forming organs	31	3.22
Cardiovascular system	148	15.41
Dermatologic	21	2.1
Genitourinary and sex hormones	23	2.4
Systemic hormone preparation	51	5.31
Anti infective for systemic use	168	17.5
Antineoplastics & Immunomodulators	17	1.78
Musculoskeletal system	21	2.1
Nervous system	164	17.08
Anti parasitic & insecticides	5	0.52
Respiratory system	87	9.06
Sensory organs	11	1.14
Various	106	11.04
Total	960	100

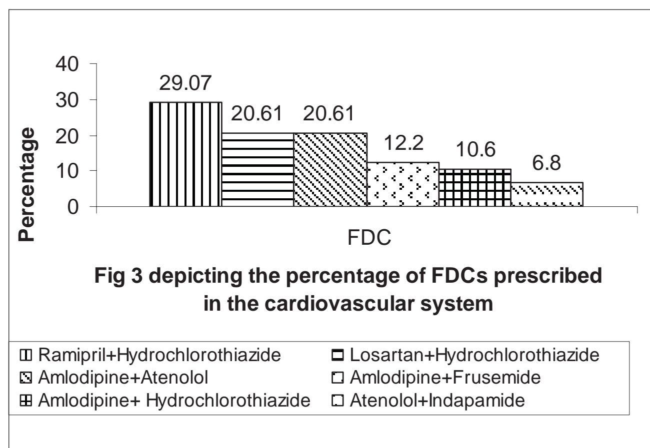

Figure 3: *Percentage of FDCs prescribed in cardiovascular system*

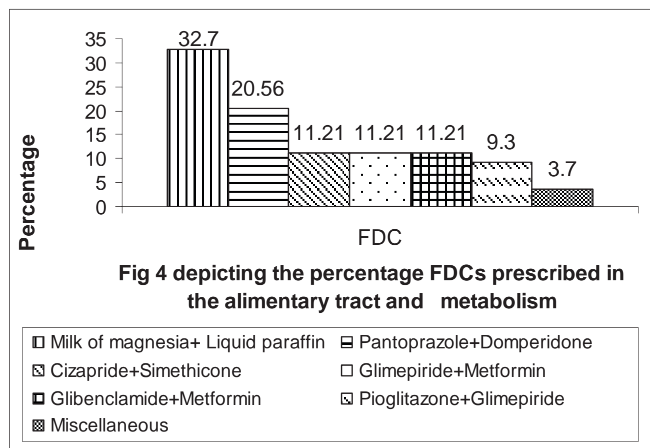

Figure 4: *Percentage of FDCs prescribed in alimentary tract and metabolism*

amlodipine and frusemide (12.2%) as depicted in Figure 3. The commonly prescribed FDCs for diseases of alimentary tract and metabolic disorders were milk of magnesia, liquid paraffin,pantoprazole and domperidone, glibenclamide and metformin (Figure 4). Majority of the FDCs prescribed (73.25%) in our study were not approved in the WHO essential drug list (Figure 5).

Discussion

In this era, poly-pharmacy is very common in clinical practice adding to the complexity of individualization of drug therapy. There is an increasing tendency to combine drugs, more often without a sound rational basis for doing so.

The 15th list of essential medicines by WHO has only 25 FDCs, whereas there are currently 156 fixed dose combinations available in the Indian market after the withdrawal of 138 combinations recently [9]. There is confusion among physicians prescribing the drugs, since these are available in the market but not included in standard textbooks. There are only few published studies, available in literature, which addressed this scenario in India. Hence, the study was undertaken to focus on the growing concern in this field. The prescription pattern of FDCs in our study ranged from 18-36.6%, in different classes of drugs, according to ATC classification. Among them, drugs affecting the nervous system and dermatologic system are the highest and lowest respectively, with an average prescription of 30.47% in our study (Table 1). Major FDCs belonged to the nervous system, anti-infective agents used for systemic use, cardiovascular system and alimentary system respectively (Table 2). The prescribing pattern of FDC in general is high (30.47%) when compared to western literature in which the average prescription of FDCs was 17, 25 and 20% in USA, Britain and Israel

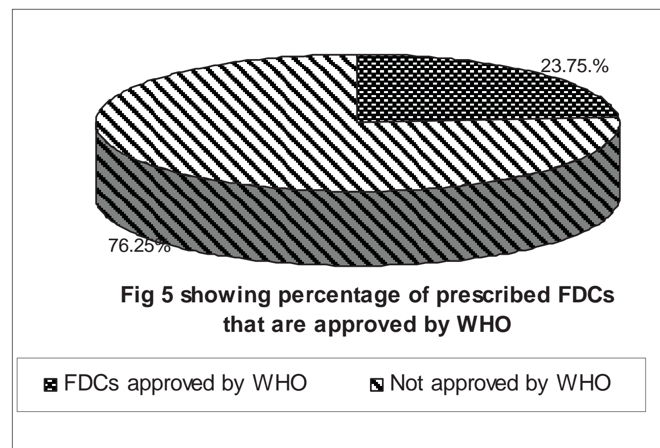

Figure 5: *Percentage of prescribed FDCs approved by WHO*

respectively (data has been taken from PDR 1997 in USA, BNF, 1997 and MEDIC, 1997, respectively). A similar trend is seen in individual groups [10]. Being a tertiary care service provider, there is a possibility of over prescription of such combinations as the last option. The high percentage of use of FDCs in our study could be due to the fact that Indian pharmaceutical industry revolves around marketing the new formulations rather than investing on research and development. This is partly due to the business strategy (less investment, more returns) and partly due to legal loopholes permitting the companies to thrive on this (lack of co-ordination between state regulatory agency and the DCGI) resulting in innumerable FDCs available in the market without any streamline approach. The monthly index of medical specialities published in June 2007 has mentioned 136 FDCs whose rationality is questionable [11]. The combinations prescribed in the nervous system, alimentary tract and metabolism, amoxicillin and cloxacillin combinations have been excluded from the WHO essential list.

Are we not responsible for this unscientific irrational combination? (If not all; at least quite significant proportions).The mudslugging game between pharmaceutical companies, health care professionals and regulatory authorities is going on, finally affecting the consumers. There should be a sound scientific basis to have FDCs which can be tailor-made according to local needs, thereby reducing adverse drug reactions, unnecessary hospitalization and financial burden substantially. By strictly minimizing and mentioning the approved FDCs in standard textbooks, physicians can clearly minimize the prescription of such drugs. Periodic studies highlighting FDC use at all levels should be encouraged and results should be publicized so that we can overcome this problem in the near future.

In conclusion, there is a clear over-prescription of FDCs in our set-up. This can be minimized by proper co-ordination and support from regulatory bodies, academicians and policy makers

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Correspondence:
Sachidananda MN Adiga,
Department of Pharmacology,
Kasturba Medical College,
Manipal-576 104, India.
E-mail: adigaiscool@yahoo.com

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Palm oil γ-tocotrienol and α-tocopherol increased apoptosis of hepatoma Alexander cells

Narimah AH Hasani[1], A Gapor MT[2], Khalid BAK[3], Wan Ngah WZ[3]

[1]Faculty of Medicine, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia, [2]Chemistry Division, Malaysian Palm Oil Board, Jalan Persiaran Ilmu, 43600 Bangi, Selangor, Malaysia, [3]Faculty of Medicine, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia

Abstract

Gamma-tocotrienol (GTT) has been reported to exhibit anti-proliferation effect on breast cancer cell lines such as MDA-MB-435 and MCF7 possibly through enhancement of apoptosis. The effect of GTT and α-tocopherol (ATF) was determined in a hepatoma cell line (Alexander) and a normal liver cell line (Chang). Cell proliferation was determined using 5-Bromo-2'-deoxy-uridine (BrdU) detection method. Alexander cells was sensitive to -tocotrienol-anti proliferation effect with 59.7% to 69.1% ($P < 0.001$) inhibition, starting at a concentration of 100µM. Treatment with ATF showed a lesser inhibition in the proliferation activity of Alexander cells by 16.9% to 19.6% ($P < 0.001$) starting at a concentration of 200µM. However, both the compounds had no effect on Chang cells. Gamma-tocotrienol showed an IC_{50} inhibition of Alexander cells growth at a dose of 66µM ($P < 0.05$). Growth inhibition of Alexander cells by ATF was lower with IC_{20} value of a concentration of 300µM ($P < 0.05$). GTT induced maximum apoptotic activity of Alexander cells at a concentration 150µM of treatment as compared to ATF at a concentration 500µM. Apoptosis was also shown by the presence of the cellular DNA laddering fragments by both the compounds. GTT and ATF also induced a 50% ($P < 0.05$) and 11% ($P < 0.05$) of nuclear apoptotic morphological changes in Alexander cells, respectively as detected using propidium iodide staining. The results suggested that palm oil GTT exhibited a higher anti-proliferation effect compared to ATF on Alexander cells probably through induction of apoptosis.

Key words: Apoptosis, γ-tocotrienol, α-tocopherol, Alexander cells

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Introduction

Palm oil vitamin E consists of a mixture of 78% to 82 % of α-, γ-, δ-tocotrienols and 18% to 22% of α-, γ-, δ-tocopherols, respectively [1]. Each of these molecules was similar in their molecular structures consisting of a chromanol ring and an isoprenoid tail. The tocopherol and tocotrienol isomers (α-, β-, γ-, δ-) differ in the number and position of the methyl groups on the chromanol ring. Tocopherols have saturated tails, whereas tocotrienols have three double bonds at 3', 7' and 11' position in their isoprenoid tails [2]. It is believed that these unique differences enable tocotrienols to display anti-proliferative activities [3] and as excellent antioxidants [2], however tocotrienols are 40 to 50 times more effective in their antioxidant capabilities [4]. The reason may be due to more effective uptake of tocotrienols by cells and better distribution throughout cell membranes as compared to tocopherols [5].

The protective effect of vitamin E in cancer has been suggested to be through the quenching of free radicals and control of tumor growth via the induction of differentiation, cell cycle inhibition at G_1-S transition phase and increased apoptosis [6].

In vitro studies have demonstrated that α-, γ-, δ-tocotrienols effectively inhibit the growth of human cervical carcinoma (HeLa) and breast cancer cells (non-estrogen-responsive, MDA-MB-435 and estrogen-responsive, MCF-7) by inducing apoptosis [7]. Another study reported that γ-tocopherol inhibits the growth of several human cancer cell lines, including prostate (androgen-resistant, PC-3

and androgen-sensitive, LNCaP) and lung (A549) cells. However, it has no effect on normal prostate epithelial (PrEC) cells [8]. However, whether the anti-proliferative effect of these isomers of vitamin E extends to other type of human cancer cells is of interest. This is to determine the anticancer effect of vitamin E isomers and its potential use as anticancer agents. The antiproliferative effect of γ-tocotrienol on a cervical carcinoma cell line, CaSki cells was reported to occur via increase in apoptosis [9].

Vitamin E succinate acts as a potent apoptotic inducer for an extensive variety of both epithelial and lymphoid human cancer cells that comprise of more than 90% of all human malignancies [10]. Studies indicated that α-tocopheryl succinate induces apoptosis in human breast cancer cell via the translocation of Bax from the cytosol to the mitochondria and the releases cytochrome c from the mitochondria to the cytosol [11]. Another study has demonstrated that α-tocopheryl succinate also induces apoptosis in prostate cancer cell through the inhibition of Bcl-xL/Bcl-2 function [6]. Apoptosis is natural programmed cell death that complements proliferation [12] to maintain the homeostasis of cells [13]. The ability of tumor cells to detect cellular damage and activate the apoptotic response may determine the ultimate success of cancer chemotherapy treatments [14].

Our study intends to demonstrate that palm oil γ-tocotrienol and α-tocopherol could inhibit cell proliferation of a human hepatoma cell (Alexander) and a normal liver cell (Chang) via the selective induction of apoptosis.

Materials and Methods

Cell culture

Cells were cultured in EMEM with Earle's balanced salts and supplemented with 10% fetal bovine serum, 20mM HEPES, 20mM sodium bicarbonate, 2mM L-glutamine and 1% penicillin and streptomycin. Cells (American Type Cell Collection, USA) were grown in culture flask (Falcon, Becton Dickinson, NJ, USA) as a monolayer to approximately 80% confluence in 5% CO_2, at 37°C. Culture media and the above chemicals were purchased from FLOWLAB, Sydney, Australia.

Vitamin E treatment

Palm oil γ-tocotrienol and α-tocopherol were obtained as 80% concentration (single peak by HPLC) from the Palm Oil Research Institute of Malaysia, Kuala Lumpur. Stock solutions of both the vitamins were dissolved in absolute alcohol at 500μM and then diluted so that the final concentration of alcohol in the culture media was <0.1% and stored in a dark bottle at -20°C before use.

Cell proliferation assay

The 2 x 10^4 cells were treated separately with different concentrations of γ-tocotrienol and α-tocopherol (0, 10 M, 50μM, 100μM, 150μM, 200μM and 300μM) and incubated in 5% CO_2 at 37°C for 48 hours. The effect of both compounds on the cell proliferation was determined using a 5-Bromo-2'-deoxy-uridme (BrdU) labelling and detection method (Bohringer Mannheim, Germany).

Cell growth assay

The 2 x 10^4 cells were treated separately with γ-tocotrienol at IC_{50} value of 66μM and α-tocopherol at IC_{20} value of 300μM on different days (0, 2, 4, 6, 8, 10, 12, 14). Cells were stained with 10% trypan blue (Flow General Company, USA) and counted on different days of treatment as above. Cells without test compound were also cultured as controls.

Apoptosis assay

(i) Analysis of DNA fragmentation activity

The 2 x 10^4 cells were treated separately with γ-tocotrienol and α-tocopherol at different concentrations (0, 10μM, 50 M, 100μM, 150μM, 200μM, 300μM and 500μM) and incubated in 5% CO_2 at 37°C for 24 hours. The cellular DNA fragmentation induced by both compounds was measured using a Cellular DNA Fragmentation-ELISA method (Bohringer Mannheim, Germany).

(ii) Detection of DNA laddering via electrophoresis

The 1 x 10^6 cells were treated separately with γ-tocotrienol and α-tocopherol at a concentration of 150μM and 500μM, respectively (values obtained from the maximum apoptotic activity assay) for 24 hours. Cells were treated with 10μL of 0.5mg/mL Proteinase K containing 10mM EDTA (pH 8.0), 0.5% SDS and 50mM Tris-HCl (pH 8.0) at 50°C for 1 hour. After 1 hour of incubation in 10μL of RNase A [0.5mg/mL RNase A, 10mM EDTA (pH 8.0), 50mM Tris-HCl (pH 8.0)] at 50°C for 1 hour, DNA samples and loading buffer [10μL 10mM EDTA (pH 8.0), 1% (w/v) low-gelling-temperature agarose, 0.25 % (w/v) bromophenol blue, 40% (w/v) sucrose] were loaded into 2% agarose gel stained with 0.5μg/mL ethidium bromide and electrophoresis in 10 x Tris-phosphate buffer [0.9M Tris-phosphate, EDTA 0.2M pH (8.0)] at 75 volts for 3 hours. All the above chemicals were obtained from Sigma Chemical Co, St. Louis, MO, USA.

(iii) Morphological evaluation of propidium iodide-stained cells

The 1 x 10^6 cells were treated separately with γ-tocotrienol and α-tocopherol at maximum apoptotic doses obtained from the apoptotic activity assay above for 48 hours. Cells without test compound were cultured as controls. After washing, the cells was fixed with 1% folmaldehyde and stained with 10μg/mL of propidium iodide for 30 minutes.

Finally, the cells were viewed using a Leitz Dialux 20 EB fluorescent microscope at 630 of magnification, counted and photographed. All the above chemicals were purchased from Sigma Chemical Co, St. Louis, MO, USA.

Determination of cellular uptake of γ-tocotrienol and α-tocopherol by HPLC

The 10 x 10^6 cells were incubated separately with γ-tocotrienol and α-tocopherol at different concentrations (0, 50μM, 100μM, 200μM and 300μM) in 5% CO_2 at 37°C for 24 hours. At the end of incubation, the cells were washed thrice with ice-cold phosphate buffer saline (10mM NaF, 0.9% NaCl, pH 7.2) and 50μL of 10mg/mL β-hydroxytoluene was added to prevent oxidation of vitamin E isomers. After the final washing with phosphate buffer saline, 100μL ethanol and β-hydroxytoluene were added and the cells were homogenized for 40 seconds. The samples were dissolved into 100μL HPLC grade-hexane containing β-hydroxytoluene, vortex and centrifuged at 300g for 3 minutes to separate the phases. Aliquots of the top phase were air dried using a vacuum concentrator and again dissolved in 100μL of hexane. The samples were filtered with 0.4μm nitrocellulose membrane and injected into a 150 x 4.6mm normal silica column with a mobile phase of hexane:methanol (99.75:0.25) at a rate of 1.50mL/min. Vitamin E compounds were quantified with a fluorescence detector at 294nm and emission detection at 330nm. All the above chemicals were obtained from Sigma Chemical Co, St. Louis, MO, USA.

Statistical analysis

ANOVA was used to compare between control and different levels of each treatment on the measured parameters. Significance was set at *P* less than 0.05.

Results

Effect of γ-tocotrienol and α-tocopherol on cell proliferation

The effects of both the vitamin E compounds on cell proliferation were examined by determining the incorporation of BrdU into freshly synthesized cellular DNA. Gamma-tocotrienol, at concentrations of 100μM and above, effectively suppressed the proliferation of Alexander cells by 59.7% to 69.1% ($P < 0.001$) with IC_{50} value of 66μM (Figure 1A). Alpha-tocopherol reduced the proliferation of Alexander cells by 16.9% to 19.6% ($P < 0.001$) beginning at a concentration of 200μM with IC_{20} value of 300μM (Figure 1B). However, both compounds had no effect on the cell proliferation of Chang cells at all concentrations used.

Effects of γ-tocotrienol and α-tocopherol on cell growth

Gamma-tocotrienol and α-tocopherol at IC_{50} and IC_{20} values of 66μM and 300μM reduced the growth of Alexander cells by 50% ($P < 0.05$) and 20% (*P* less than 0.05, n is equal to 4), respectively as compared to untreated culture.

Induction of apoptosis by γ-tocotrienol and α-tocopherol by DNA fragmentation

To further analyze the possible anti-proliferation mechanism induced by Vitamin E on Alexander cells, the apoptotic properties of both compounds was investigated for the presence of cellular DNA fragmentation by electrophoresis (Figure 2a) and by using BrdU-labeled DNA fragments released into the cytoplasm during apoptosis using Bohringer Mannheim Cellular DNA Fragmentation-ELISA kit (Figure 2b). Morphological changes were determined using propidium iodide staining (Figure 2c).

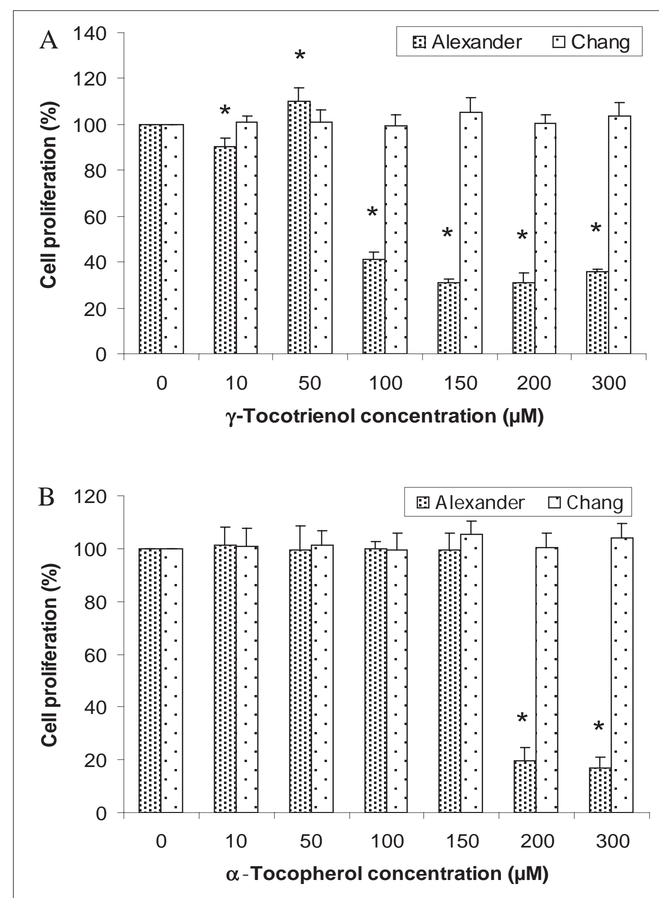

Figure 1: *The effect of γ-tocotrienol (GTT; 1A) and α-tocopherol (ATF; 1B) on cell proliferation of Alexander and Chang cells. Gamma-tocotrienol inhibits the proliferation activity of Alexander cells by 59.7% to 69.1% (P < 0.001)) beginning at a concentration of 100μM with* IC_{50} *of 66μM (1A), while α-tocopherol (ATF) at 200μM and above reduced 16.9% to 19.6% (P< 0.001) with* IC_{20} *of 300μM (1B). However, both compounds have no effect on Chang cells at all concentrations used. * significant as compared to control p<0.001.*

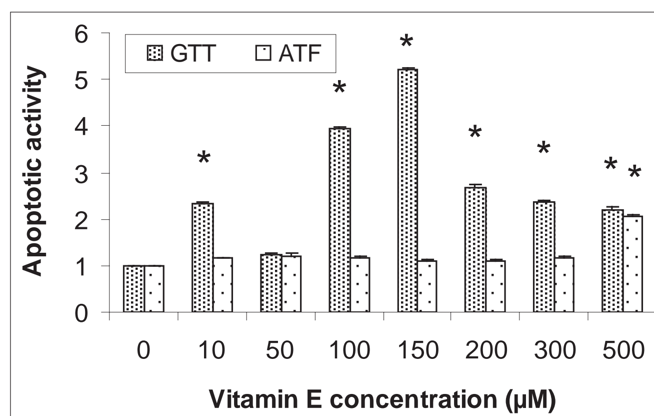

Figure 2a: *The effect of γ-tocotrienol (GTT) α-tocopherol (ATF) on the apoptotic DNA fragmentation activity of Alexander cells. Gamma-tocotrienol and α-tocopherol enhanced the maximum apoptotic activity of Alexander cells by 5.2-fold ($P < 0.01$) and 2.1-fold ($P < 0.01$) at 150μM and 500μM, respectively. * significant as compared to control $p<0.01$.*

Treatment with γ-tocotrienol at a concentration of 10μM enhanced the apoptotic activity of Alexander cells by 2.3-fold ($P < 0.01$) but had no effect at 50μM. Interestingly, at a concentration of 100μM, γ-tocotrienol enhanced the apoptotic activity by 3.9-fold (*P* less than 0.01, n is equal to 4) with a maximum apoptotic activity of 5.2-fold ($P < 0.01$) at a concentration of 150μM. At higher concentrations of 200μM to 500μM, the apoptotic activity induced by γ-tocotrienol were slightly reduced to 2.7-fold, but the effect was still significant at *P* less than 0.01 as compared to untreated cultures. Alpha-tocopherol enhanced the apoptotic activity of Alexander cells by 2.7-fold ($P < 0.01$) and 2.1-fold ($P < 0.01$) beginning at a much higher concentration of 500μM (Figure 2a).

The apoptotic effect of γ-tocotrienol and α-tocopherol was confirmed by the DNA fragmentation patterns extracted from Alexander cells.

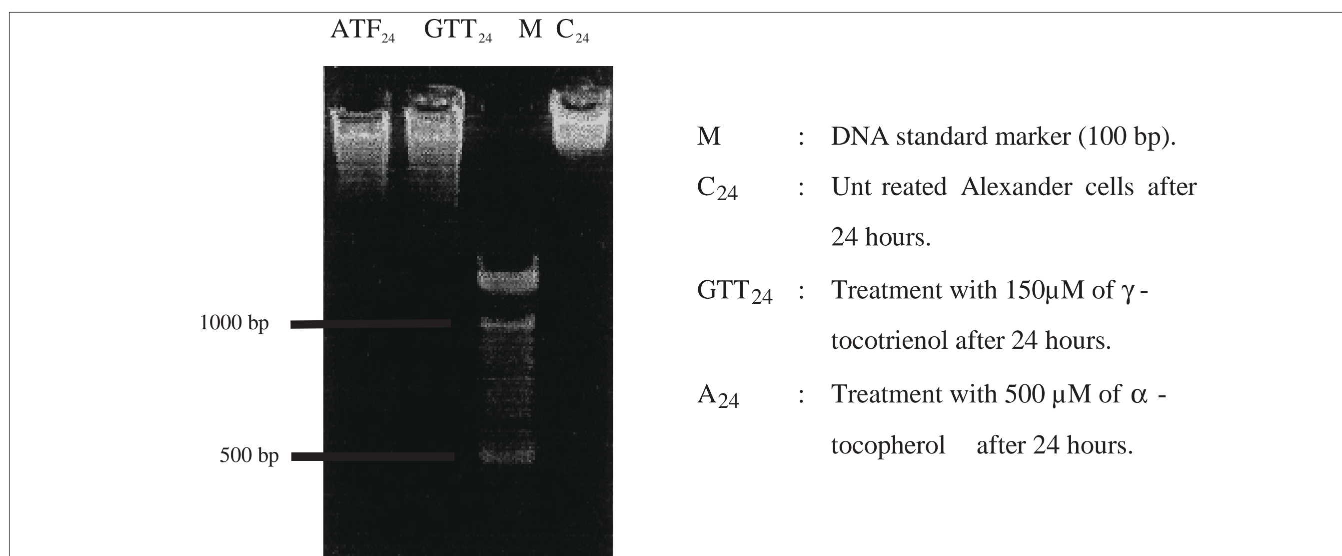

Figure 2b: *DNA laddering fragmentation induced by γ-tocotrienol (GTT) and α-tocopherol (ATF) in Alexander cells.*

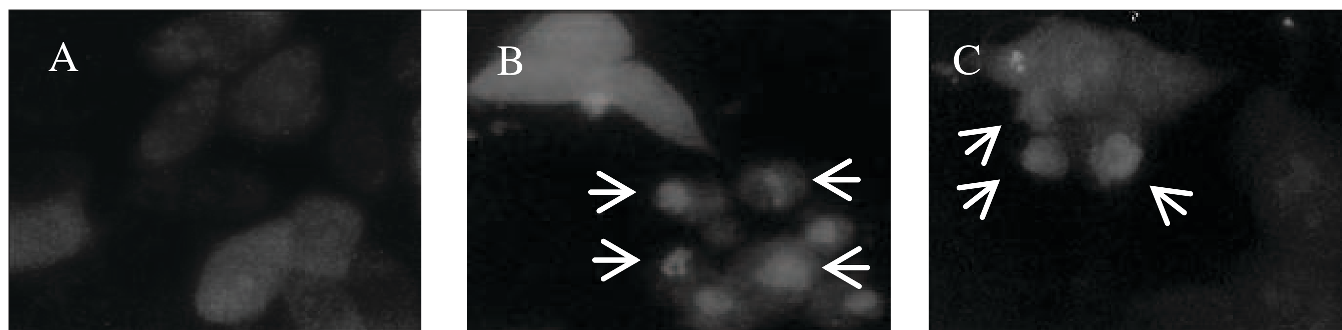

Figure 3c: *Morphological changes induced γ-tocotrienol and α-tocopherol in Alexander cells. Gamma-tocotrienol and α-tocopherol at 150μM (B) and 500μM (C), respectively induced a 50% ($P < 0.05$) and 11% ($P < 0.05$) of the nuclear chromatin and cytoplasmic condensation in Alexander cells as compared to untreated cultures (A).*

Vitamin E uptake by cells

Gamma-tocotrienol content in the Alexander cells increased with increasing concentration of γ-tocotrienol used (Figure 3A). In contrast, smaller amount of γ-tocotrienol ($P < 0.01$) was detected at concentrations from 150μM to 300μM of treatments with Chang cells (Figure2A). On the other hand, α-tocopherol (Figure 3B) were taken-up by both cells at all concentrations used ($P < 0.01$). However, α-tocopherol was most efficiently absorbed by Alexander compared to Chang cells ($P < 0.01$).

Discussion

Treatment of cancer cells with high doses of 13-*cis*-retinoic acid, α-tocopheryl succinate, or β-carotene alters the expression of specific genes, levels of proteins, and translocation of certain proteins from one cellular compartment to another, causing differentiation, proliferation inhibition, and apoptosis, depending on the type and form of antioxidant, treatment schedule, and type of tumor cell [15]. The alterations in gene expressions and protein levels are directly related to inhibition of proliferation and apoptosis [16].

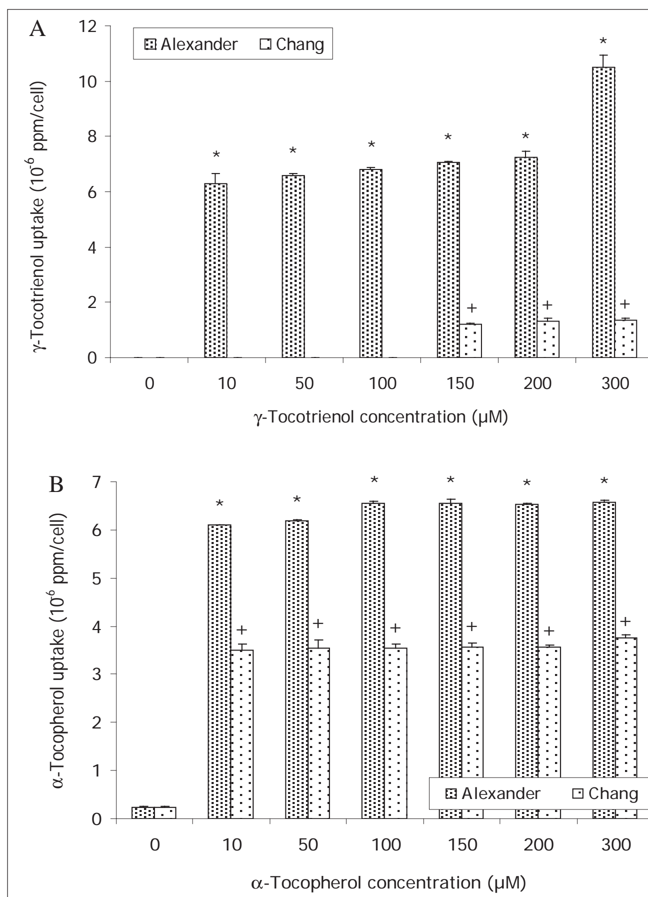

Figure 3: *Gamma-tocotrienol (A) and α-tocopherol (B) uptake by Alexander and Chang cells. Gamma-tocotrienol (A) and α-tocopherol (B) was taken-up most efficiently by Alexander cells as compared to Chang cells (P < 0.01) at all concentrations used. * significant as compared to untreated Alexander cells extract p<0.01.+ significant as compared to untreated Chang cells extract p<0.01.*

Our findings demonstrated that treatment with concentrations from 100μM to 300μM of γ-tocotrienol efficiently inhibited the proliferation of Alexander cells by more than 60%, whereas α-tocopherol treatment caused proliferation inhibition of about 20% with IC_{50} and IC_{20} values of 66μM and 300μM, respectively. This was probably due to the more efficient uptake of γ-tocotrienol in Alexander cells compared to α-tocopherol. Gamma-tocotrienol had little or no effect on Chang cells probably due to reduced uptake of γ-tocotrienol in Chang. On the other hand α-tocopherol had no antiproliferative effect although absorbed by the cells.

Other *in vitro* studies have reported that α-, γ-, δ-tocotrienol and α-tocopherol efficiently function as potent inhibitors of DNA synthesis in human breast cancer cells (non-estrogen-responsive, MDA-MB-435 and estrogen-responsive, MCF-7). The effectiveness of growth inhibition by tocotrienols is independent of estrogen sensitivity [17]. Treatment with a concentration of 180μg/mL and 2μg/mL of palm oil γ-tocotrienol reduced the proliferation of MDA-MB-435 and MCF-7 by 50%, respectively as compared to palm oil α-tocopherol at a concentration of 125μg/mL in the later cells [16]. Another study reported that treatment with a concentration of γ-tocopherol at concentrations of 25μM and 50μM, inhibited the growth of several human cancer cell lines, including prostate (androgen-resistant, PC-3 and androgen-sensitive, LNCaP) and lung (A549) cells. In contrast, at similar concentrations α-tocopherol had no effect on normal prostate epithelial (PrEC) cells [8]. In our previous study [9], we also observed that γ-tocotrienol at 100 μM and α-tocopherol at 50μM inhibited the proliferation of cervical carcinoma, CaSki cells through the apoptotic induction mechanism.

Increased apoptosis as indicated by DNA fragmentation and morphology evaluation was observed. Gamma-tocotrienol was a more potent as apoptotic inducer as compared to α-tocopherol. Our findings is in accordance with other observations which reported that δ-tocotrienol, RRR-α-tocopheryl succinate possessed the ability to induce cancer cells to undergo apoptosis without affecting normal cells. However, RRR-α-tocopherol did not induce cancer cells to undergo apoptosis [17]. Increased cellular DNA laddering was observed with γ-tocopherol or combination with δ-tocopherol treatment induced apoptosis in androgen-sensitive (LNCaP) but not the androgen-resistant (PC-3) human prostate cancer cells through the induction of

cytochrome c release, activation of caspase-9 and -3, cleavage of poly-ADP-ribose polymerase and involvement of caspase-independent pathways [8]. Another study reported that α-tocopheryl succinate induced apoptosis of human breast cancer cells via translocation of Bax from the cytosol to the mitochondria and releasing cytochrome c from the mitochondria to the cytosol [10].

The greater apoptotic effect on the Alexander cells by γ-tocotrienol compared to α-tocopherol may be due to its unsaturated isoprenoid tail that allowed easier mobility, more uniform distribution in cell membranes and greater recycling activity as compared to α-tocopherol [4]. This is in agreement with our findings of cellular uptake of the compounds by Alexander cells.

In conclusion, our results showed that palm oil γ-tocotrienol effectively induced apoptosis of hepatoma cells, Alexander. The apoptosis inducing ability of γ-tocotrienol makes this compound a promising candidate for further characterization of its antitumor profile *in vivo*.

Acknowledgments

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Correspondence:
Wan Zurinah Wan Ngah,
Department of Biochemistry, Faculty of Medicine,
UKM Medical Molecular Biology Institute (UMBI),
Universiti Kebangsaan, Kuala Lumpur-503 00, Malaysia.
E-mail: zurina@medic.ukm.my

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Interventional Role of Piperazine Citrate in Barium Chloride Induced Ventricular Arrhythmias in Anaesthetized Rats

Ghasi S, Mbah AU, Nze PU[1], Nwobodo E[2], Ogbonna AO, Onuaguluchi G

Department of Pharmacology and Therapeutics, [1]Department of Anaesthesiology, University of Nigeria College of Medicine, Enugu, Nigeria, [2]Department of Physiology, Nnamdi Azikiwe University College of Health Sciences, Nnewi, Nigeria

Abstract

Interventional potential of piperazine in Barium Chloride (BC) -induced ventricular arrhythmias was investigated in the rats. Various forms of arrhythmias were induced in 10 rats and piperazine (30mg/kg) was given in each case to reverse arrhythmia to sinus rhythm. Five out of six cases of induced ventricular tachycardia (83.3%) were reverted to sinus rhythm by piperazine. Again, 33% success was seen when ventricular fibrillation was induced. One of the three cases was reverted to the sinus rhythm as was also the only case of pulsus bigeminus observed. Piperazine, therefore, has the potential of a good anti-arrhythmic agent. Piperazine was shown to be a more effective antiarrhythmic agent than propranolol against BC-induced ventricular fibrillation. Propranolol not only failed to revert any of the ventricular fibrillations to sinus rhythm, but in two of four cases was not able to reverse the induced ventricular tachycardia. Although piperazine failed to control ventricular fibrillation with the same degree of effectiveness, piperazine has a remarkable therapeutic value in the management of ventricular tachycardia.

Key words: Piperazine citrate, arrhythmias, electrocardiogram, rat

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Introduction

Arrhythmia refers to disruption of normal sequence of electrical impulses causing abnormal heart rhythms. It is a potentially lethal cardiovascular condition. The incidence of ventricular arrhythmias is not well documented. Atrial fibrillation is the most commonly sustained of various kinds of cardiac arrhythmias [1]. It has a prevalence of six per cent in the population over 65 years of age [1]. Atrial flutter has an incidence of 88/100,000 persons per year and increases with age [2]. Patients over 80 years have been documented to have an incidence of 587/100,000 [2].

Anti-arrhythmic drugs act by either slowing conduction or lengthening the refractory period of cardiac tissue [3]. Arrhythmias have been induced with drugs or whole heart ischaemia [4-7] and the ability of a test drug to reverse the induced arrhythmia to sinus rhythm is taken as evidence of anti-arrhythmic effect.

Prompt management of arrhythmic conditions, especially ventricular arrhythmia, is imperative as a proportion of the population presenting with ventricular arrhythmias will be at high risk of sudden cardiac death [8]. Although a giant stride has been taken in understanding basic cardiac electrophysiology, many facts about arrhythmogenesis remain largely unknown. This has made treatment of various tachyarrhthimas an empirical trial often inadequate in preventing life threatening arrhythmias. This has necessitated the introduction of many anti-arrhythmia devices which include traditional pacing systems such as inhibited single and dual chamber pacemakers (AA1, VVl, DVl, and DDD) for the control of bradyarrhythmia and for overdrive suppression of certain tachyarrhythmia [9], radio-frequency ablation, and burst pacing systems and implantable cardioverters for control of both su-praventicular tachycardia and fibrillation [10,11], and synthesis of wide spectrum of drugs with varying electro-physiologic properties [12]. Many of these agents had been used for other ailments before serendipity, coupled with some good sense, necessitated their use as antiarrhythmic drugs. Quinidine for instance was first used as an antimalarial agent before Jean Baptiste de Senac [13] noted its anti-arrhythmic effect and lignocaine, a local anaesthetic, was discovered fortuitously during a cardiac surgery to have anti-arrhythmic

properties [14]. The search for anti-arrhythmic drugs still continues, as each drug is associated with adverse effects some of which may be quite serious.

Recently, Onuaguluchi and Ghasi [15] showed that piperazine citrate treatment in human volunteers might be of some value in the management of dysrhythmic conditions. Furthermore, cardioprotective effect of piperazine in the rat has also been demonstrated [16]. Consequently, it was decided to study the effects of piperazine on BC-induced arrhythmias in the anaesthetized albino rat connected to an electrocardiographic machine. For comparative purposes, effects of propranolol, a standard anti-arrhythmic drug, were also evaluated in the same animal model.

The rat has been chosen as the animal model for this study as it withstands the rigors of cannulation more than most other animals, and has been used by many other investigators to determine the ECG changes due to various factors even though the rat has short QT intervals and no ST segment [17-23].

Material and Methods

Albino Wistar rats of either sex weighing between 200 and 250g were used. They were anaesthetized with thiopentone sodium (50mg/kg) intra-peritoneally and placed in a supine position with the four limbs tied to a dissecting board. A longitudinal incision about 1.5cm in length was made in the middle of the neck and the skin reflected laterally to expose one of the external jugular veins. The vein was dissected of fat and other tissues. Two cotton threads for ligature were then passed under the vein. A small incision was made on the vein between these ligatures. A polythene cannula filled with heparinized saline (10 i.u. of heparin per ml of normal saline) was inserted into the vein and secured in position with the inferior ligature. The superior ligature was used to occlude the vessel about the point of cannulation.

The animal was then connected to an electrocardiographic (ECG) machine (Bioscience 400 series Washington Oscillograph) by means of pin electrodes inserted subcutaneously into the right forelimb and left hind limb. ECG records were obtained on Lead II channel of the ECG machine. ECG recordings were obtained at a paper speed of 10mm per second.

Singh *et al*. [24] had shown that BaCl2 of 3mg/kg i.v was adequate for the production of ventricular arrhythmia in dogs. However, in the present study it was found that the dosage between 12.5mg/kg and 15mg/kg of BaCl2 was required to induce ventricular tachycardia in the rat, which was sometimes found to induce ventricular fibrillation within 15 seconds. Therefore, cardiotoxicity response to BaCl2 would appear to show species variations. Because a consistent lethal dose was required for this study and BaCl2 at this dose range did not regularly produce ventricular fibrillation, a larger dose of BaCl2 was therefore employed. Ventricular fibrillation was induced by intravenous (jugular vein) administration of barium chloride (20 mg/kg). In all cases, the animals died within, if untreated, one minute of ventricular fibrillation.

To evaluate the anti-arrhythmic action of piperazine, ventricular dysrhythmia was established with BaCl2 (20mg/kg) in 16 of the animals. The effect of piperazine (30 mg/kg) on the BaCl2–induced arrhythmia was studied in 10 of the rats. Ability of the drug to revert the arrhythmia to sinus rhythm was taken as an indication of its anti-arrhythmic activity on the particular preparation. For comparative purposes, the anti-arrhythmic effect of propranolol (50mcg/kg) on the BaCl2-induced arrhythmia in the rat was similarly undertaken in the remaining 6 rats.

Results

BaCl2 of 20mg/kg was used to induce arrhythmias in 21 Wistar rats of either sex. Five of the rats that were not treated with piperazine following BaCl administration died within one minute from ventricular fibrillation. Among the 10 rats with BaCl2-induced dysrhythmia, treated with piperazine, five of the six cases of ventricular tachycardia were successfully reverted to sinus rhythm. Figure 1 shows the electrocardiograms in which the BaCl2-induced ventricular tachycardia was reverted to sinus rhythm. Ventricular fibrillation was induced in another three rats.

In one of the three animals, induced fibrillation was reverted to tachycardia (Figure 2). Interestingly, in one rat, piperazine reversed the BaCl2-induced fibrillation to sinus rhythm (Figure 3). However, piperazine at a dose of 30mg/kg failed to reverse the ventricular fibrillation induced in one of the rats to the sinus rhythm (Figure 4). The only case of pulsus bigeminus seen was reverted to sinus rhythm by piperazine (Figure 5).

In another group of six rats with ventricular arrhythmias, propranolol at a dose of 50mcg/kg reversed two of the four cases of ventricular tachycardia to sinus rhythm. Figures 6 shows the electrocardiogram of one of the two rats where ventricular tachycardia was reverted to sinus rhythm while Figure 7 is an electrocardiogram showing inability of propranolol to revert barium chloride-induced ventricular tachycardia to sinus rhythm.

Discussion

The results of this study have shown that piperazine is a

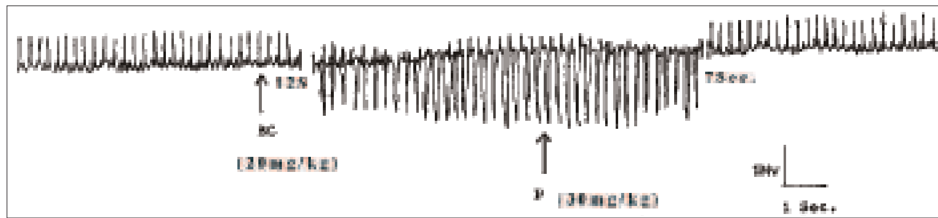

Figure 1: *BC-induced ventricular tachy-cardia in the rat was reverted to sinus rhythm by piperazine (P)*

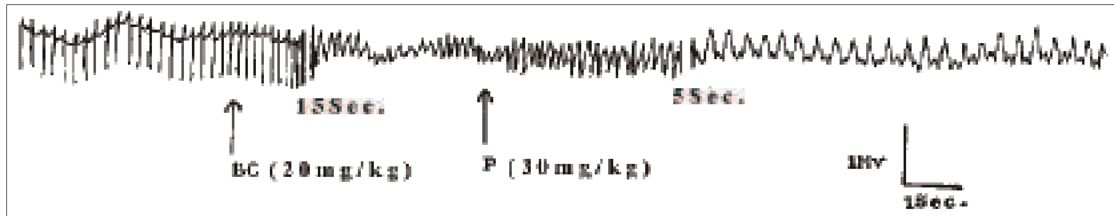

Figure 2: *BC-induced ventricular fibrillation in the rat was reverted to ventricular tachycardia by piperazine (P)*

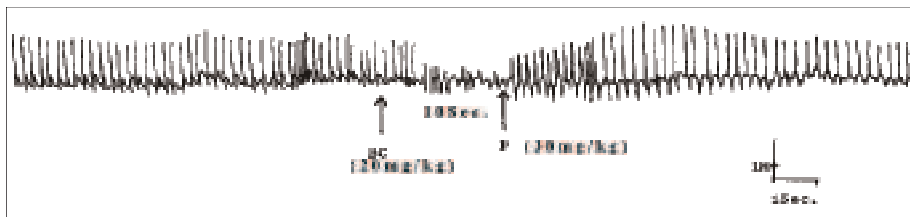

Figure 3: *BC-induced ventricular fibrillation in the rat was reverted to sinus rhythm by piperazine (P) 30mg/kg*

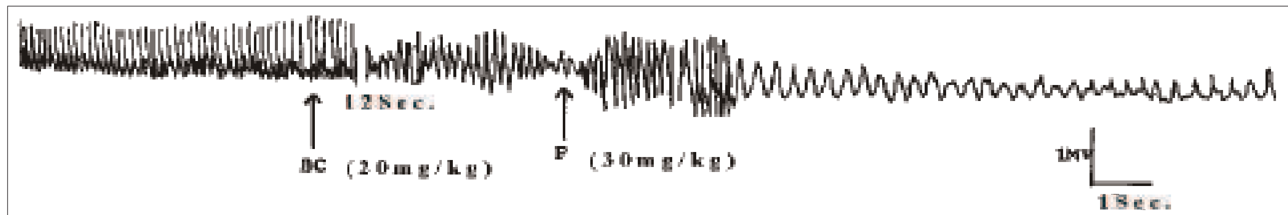

Figure 4: *Piperazine (P) failed to re-vert ventricular fibrillation induced by BaCl2 (BC) in the rat to sinus rhythm*

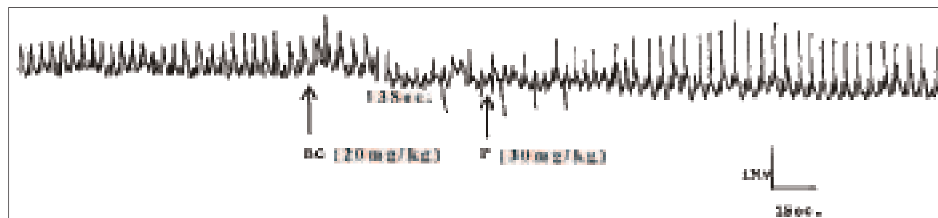

Figure 5: *BC-induced pulsus bigeminus in the rat was reverted to sinus rhythm by piperazine (P)*

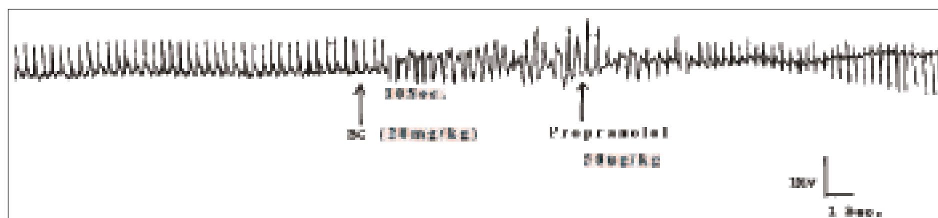

Figure 6: *BC-induced ventricular tachycardia in the rat was reverted to sinus rhythm by propranolol*

definite and potent antiarrhythmic agent. The drug has been shown to affect various forms of BC-induced ventricular arrhythmia.

Five out of 6 cases of induced ventricular tachycardia (83.3%) were reverted to sinus rhythm by piperazine. Again, 33% success was seen when ventricular fibrillation was

Figure 7: *Propranolol failed to revert ventricular tachycardia induced by BaCl2 (BC) in the rat to sinus rhythm. The animal died within one minute of barium chloride administration*

induced. One of the three cases was reverted to the sinus rhythm. Piperazine, therefore, has the potentials of a good anti-arrhythmic agent.

Barium is known to stimulate all muscles in the mammalia causing strong vasoconstriction, violent peristalsis, convulsive tremors, and increased excitability and force of contraction of the heart [25]. In terms of ionic fluxes, the effects of barium on the heart and other excitable membranes are largely attributable to the decline in the outward diffusion of K+ from the cell (that is, inhibition of the transient outward and delayed rectifier currents) without any decrease in the actively transported influx, that is, the inward rectifier protein [26]. The result is accumulation of K+ ions within the cells at the expense of extracellular K+. Actually, hypokalaemia is the main electrolyte disturbance in barium toxicity [25,27,28]. Indeed cardiac toxicity induced by Ba2+ has been successfully treated with potassium salt solution given intravenously [25,28,29]. This is because the most important concern regarding K+ depletion is its influence on ventricular fibrillation, which is the leading cause of sudden cardiac death and a major contributor to cardiovascular mortality [30]. Studies in experimental animals have demonstrated that K+ depletion lowers the threshold for electrically induced ventricular fibrillation in the ischaemic myocardium and also increases spontaneous ischaemic ventricular fibrillation [31,32]. The fact that Ba2+ is able to reactivate myocardial Na+-K+ATPase after depression by inhibitors of the enzyme such as ouabain *in vitro* [33], suggests that Ba2+ may in fact enhance active transport of K+ into the cell *in vivo*.

Piperazine is a direct non-specific, non-vascular smooth muscle relaxant. It has been shown to inhibit barium chloride, histamine, 5HT and acetylcholine-induced contractions in the guinea-pig ileum and rabbit duodenum [34-36]. It also antagonizes adrenaline-induced contraction of the guineapig vas deferens and oxytocin-induced contractions in the rat uterus [34,35].

The normal cardiac cell at rest maintains a trans-membrane potential approximately 80 to 90mV negative to the exterior. This gradient is established by pumps, especially Na+-K+ATPase, and fixed anionic charges within cells [30]. There is both an electrical and a concentration gradient that would move Na+ ions into resting cells. However, Na+ channels, which allow Na+ to move along this gradient, are closed at negative transmembrane potentials so Na+ does not enter normal resting cardiac cells until it is depolarised above a threshold potential. In contrast, a specific type of K+ channel protein (the inward rectifier protein) is in an open conformation at negative potentials [30].

Since BaCl2 does not have any negative effect on the potassium inward current and may indeed increase K+ influx at negative transmembrane potentials (increased phase 4 slope), it is understandable why BaCl2 elicits automaticity of the cardiac muscle.

From the foregoing discussion, piperazine has been shown to inhibit the effects of BaCl2. It is therefore reasonable to conclude that its predominant ionic effect is blocking the potassium channels.

Piperazine may, therefore bring about its anti-arrhythmic action by decreasing Ca2+ current, and inhibiting transient outward, delayed rectifier, and especially inward rectifier K+ currents since action potential duration may be influenced by several ion currents simultaneously [37]. It has also been established that transient outward and delayed rectifier currents actually result from multiple ion channel sub-types [38,39], and that acetylcholine-evoked hyperpolarization results from activation of a K+ by hetero-oligomerization of multiple, distinct channel proteins [40]. Piperazine may, therefore, be affecting any of these potassium ion channel sub-types. Incidentally, some piperazine derivatives have been shown to inhibit the potassium channels [41,42].

Potassium channel block would be expected to produce a series of desirable effects, such as, decreased automaticity, reduced defibrillation energy requirement, and inhibition of ventricular fibrillation due to acute ischaemia [43,44]. Furthermore, the increase in action potential duration because of the prolongation of the Q-T interval would increase refractoriness which should be an effective way of treating re-entry rhythm [45,46].

Beta-adrenoceptor agonists, like Ba2+, decrease the plasma concentration of K+ by promoting the uptake of the ion. Beta-blocking agent such as propranolol negates this buffering effect [47], and contributes to its anti-arrhythmic action. Similarly, piperazine may negate the arrhythmogenic effect of BaCl2 by blocking the K+ channels. Since most K+ channel blocking drugs also interact with beta-adrenergic receptors, such as sotolol that prolongs cardiac action potential by inhibiting K+ currents [48], and other channels (example, amiodarone), a multiple mechanism of action may equally be possible in the case of piperazine. Sotolol is more effective for many arrhythmias than other beta-blocking agents, probably because of its additional K+ channel-blocking actions [30].

It is, therefore, understandable why piperazine in this study proved to be a more effective antiarrhythmic agent than propranolol, as propranolol does not inhibit K+ currents. Propranolol not only failed to revert any of the ventricular fibrillations to sinus rhythm but in two instances it was not able to reverse the induced ventricular tachycardia to the sinus rhythm. Piperazine, on the other hand, was successful in reverting five of the six cases of BaCl2–induced ventricular tachycardia to sinus rhythm.

The therapeutic value of piperazine in the management of ventricular tachycardia was very excellent. It, however failed to manage ventricular fibrillation with the same measure of success. It is, however, interesting to witness any success at all as ventricular fibrillation is a rare phenomenon usually observed by a very few physicians who have by chance recorded the incident at the time of death. The patients were best treated not with drugs but with DC-cardioversion, the application of a large electric current across the chest [30]. Therefore, piperazine should have its proper place as an antiarrhyhmic drug, since it is affordable and is well tolerated with minimal adverse effects.

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Correspondence:
S. Ghasi,
Department of Pharmacology and Therapeutics,
University of Nigeria College of Medicine, Enugu, Nigeria.
E-mail: samuelghasi@yahoo.com

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Biochemical Changes in Alcoholic Hepatitis with Phyllanthus Amarus Therapy: A Study

Padmaja Nikam, Shashikant Nikam, Ajit Sontakke[1], Chitra Khanwelkar[2]

Department of Biochemistry, Belgaum Institute of Medical Sciences, Belgaum, Karnataka, Departments of [1]Biochemistry and [2]Pharmacology, KIMS, Karad, India

Abstract

Alcoholic hepatitis is a leading cause of morbidity and mortality. This study focuses on Phyllanthus Amarus therapy and its effect on liver in alcoholic hepatitis. The therapy tries to protect the liver by investigating liver profile enzymes, antioxidant enzymes, antioxidant vitamins and lipid peroxidation. The study consists of 50 well diagnosed alcoholic hepatitis males aged between 33 to 55 years. The control group includes 50 age-matched normal healthy persons. Oxidative stress was assessed by estimating lipid peroxidation [LPO]. Parameters like serum bilirubin, total proteins and activity of liver profile enzymes were chosen. The activity of enzymatic antioxidants, superoxide dismutase [SOD], glutathione peroxidase [GPx], catalase, and levels of non- enzymatic antioxidant vitamin E and vitamin C were measured in plasma or erythrocytes. Methods used in the study are mainly enzyme kinetics by auto-analyzer and turbidimetry. Plasma LPO levels were significantly high but activity of SOD, GPx, catalase, and levels of vitamin E and vitamin C were significantly lowered in alcoholic hepatitis when compared with controls. After phyllanthus amarus therapy, for four and eight weeks, plasma LPO levels significantly decreased and activity of SOD, GPx, catalase and levels of vitamin E and C significantly increased in alcoholic hepatitis. This study concludes that the imbalance between oxidative stress and anti-oxidants may play an important role in alcoholic hepatitis. Elevated free radicals may cause hepatic cell loss and play a role in pathogenesis of alcoholic hepatitis. This study strongly suggests that the therapy of phyllanthus amarus increases activity antioxidants and reduces lipid peroxidation; protects liver from damage due to free radicals in alcoholic hepatitis.

Key words: Alcoholic hepatitis, antioxidants, phyllanthus amarus, oxidative stress

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Introduction

Hepatitis is a global public health problem, which is responsible for major chunk of morbidity and mortality [1]. Alcoholic hepatitis is a leading cause of morbidity and mortality throughout the world [2]. It is a major health care problem, accounting for 40% of deaths from cirrhosis and more than 30% cases of hepatocellular carcinoma [3]. Consistent heavy drinking or binge drinking is a primary risk factor for alcoholic hepatitis.

There is no specific treatment for alcoholic hepatitis. Complete abstinence from alcohol is the single most important treatment for alcoholic hepatitis. Patients with severe alcoholic hepatitis may benefit from treatment with corticosteroids, pentoxifylline, methionine, vitamin C, vitamin E and vitamin B- complex.

Alcohol-induced liver injury is linked to an oxidative stress resulting from raised free radical generation and lowered antioxidant defense and play a vital role in pathogenesis of alcoholic hepatitis [4]. The plant Phyllanthus amarus is bitter, astringent, stomachine, diuretic, febrifuge and antiseptic. Every part of the plant is used in dropsy, gonorrhoea, menorrhagia and other genital infections [5,6]. It is found effective in treatment of hepatitis without adverse effects. It has most promising application in

hepatitis-B [7]. Considering these facts we planned to study the effect of Phyllanthus amarus therapy in protection of liver in alcoholic hepatitis with the help of investigating liver profile enzymes [serum glutamate oxaloacetate transminase (SGOT), serum pyruvate oxaloacetate transaminase (SGPT), alkaline phosphatase (ALP), γ glutamyl transferrase (GGT), 5' nucleotide phosphatase (5' NTP) and lactate dehydrogenase (LDH)], antioxidant enzymes (SOD, Gpx, catalase), antioxidant vitamins (vitamin-E and vitamin-C) and lipid peroxidation.

Materials and Methods

The research design included three study groups:

Group I: 50 healthy control male subjects.
Group II: 25 patients suffering from alcoholic hepatitis for past six months.
Group III: 25 patients suffering from alcoholic hepatitis for over six months.

All the 50 male patients of well diagnosed alcoholic hepatitis, aged between 33 to 55 years, were selected from the Out Patient Department (OPD) of Corporation hospital Sangli, Govt. Medical College hospital Miraj and Civil hospital Sangli. All the patients in this study group were diagnosed with the help of complete medical history, personal history including drinking habits, physical examination, specific laboratory tests, liver function tests, cellular blood counts, bleeding time, electrolyte tests and ultrasonographic examination.

There were symptoms and signs like abdominal tenderness, spider like blood vessels in the skin, ascites, poor appetite, jaundice, low grade fever, fatigue, portal hypertension, mental confusion etc. All these patients were kept totally free from alcohol in the time of therapy. Age-matched 50 normal healthy males as per International Federation of Clinical Chemistry (IFCC) were included as control group in this study. Patients and controls gave consent to participate in this study when informed of the details, purpose of study. For this study, the institution's ethical committee approval was taken. Patients with associated renal diseases, non-alcoholic liver diseases, lung diseases, thyroid diseases, gastro-intestinal diseases, tobacco chewers and smokers that could alter the required parameters were excluded from the study.

Sample Collection

About 10 ml of fasting venous blood samples were collected under sterile condition from alcoholic hepatitis patients before starting any therapy and from normal healthy subjects. After four weeks and eight weeks therapy 10 ml of fasting venous blood samples were collected from alcoholic hepatitis patients under sterile condition. Five ml blood was taken in sterile dry and acid washed ethylenediaminotetra acetic acid (EDTA) bulbs and five ml blood in plain bulbs. Plasma was separated by centrifuging the blood at 3000rpm for 20 minutes at 4°c [8,9]. This plasma was used for estimation of malondialdehyde (MDA) and vitamin E. The packed cells were used for the analysis of vitamin C, SOD, catalase and GPx.

The sera separated were used for the investigation of parameters like total bilirubin, total proteins and activity of enzymes SGPT [10], SGOT [11], ALP [12], GGT [13], 5' NTP [14] and LDH [14]. All these parameters were estimated in healthy subjects and all cases before and after the therapy with phyllanthus amarus. At the end of four, eight weeks, changes in these parameters from base line values were taken. MDA was determined as the measure of thiobarbituric acid reactive substances (TBARS) [15].

Erythrocytes ascorbic acid levels were estimated by the method of Tietz [16]. Plasma separated was used for estimation of vitamin E by the method of Baker H *et al*. [17] SOD was determined in the hemolysate by the method of Mishra and Fridovich based on the inhibition of autoxidation of epinephrine to adenochrome at pH 10.2 [18]. Catalase activity was measured by the method of Beer and Seazer [19] and GPx activity by paglia and valentine in erythrocytes [20].

Statistical Analysis

All values are presented as mean plus/minus SD. Statistical significance was analyzed by Student's 't' test.

Results

The levels of lipid peroxidation, vitamin E, vitamin C and activity of SOD, catalase and GPx are presented in Tables 1 and 2. The levels of lipid peroxidation were significantly higher in group II and group III in comparison with controls.

The levels of vitamin E, vitamin C and activity of antioxidant enzymes SOD, GPx and catalase were significantly lower in group II and group III patients.

Table 1: Lipid peroxidation and antioxidants in alcoholic hepatitis before and after therapy in group II patients

Sl. No.	Parameters	Controls (n=50)	Alcoholic hepatitis before therapy. (n=25)	Alcoholic hepatitis after 4 weeks therapy. (n=25)	Alcoholic hepatitis after 8 weeks therapy. (n=25)
1.	LPO n moles/ml.	2.22±0.64	4.18 ±0.02	3.12± 0.38*	2.20±0.72**
2.	SOD U/gm of Hb.	3.65±0.82	2.50±0.76	2.84±0.47*	3.05±0.22**
3.	GPx U/gm of Hb.	58.25±5.58	18.18±0.18	21 ±3.50*	34.95±3.06**
4.	Catalase n mole/ H_2O_2 decomposed /min	640±110.05	550 ±95.50	575±64.0*	603±0.92**
5.	Vitamin E mg/dl.	7.86±2.52	5.90±1.43	6.15 ±1.58*	6.65±2.50**
6.	Vitamin C mg/dl.	1.66±0.46	0.85±0.10	0.96±0.17*	1.23±0.26**

*Values are expressed in mean ± SD, n= Number of observations. *Indicates P< 0.01 when compared with before therapy. **Indicates P< 0.001 when compared with 4 weeks therapy.*

Table 2: Lipid peroxidation and antioxidants in alcoholic hepatitis before and after therapy in group III patients

Sl. No.	Parameters	Controls (n=50)	Alcoholic hepatitis before therapy. (n=25)	Alcoholic hepatitis after 4 weeks therapy. (n=25)	Alcoholic hepatitis after 8 weeks therapy. (n=25)
1.	LPO n moles/ml.	2.22 ± 0.64	5.22 ± 0.18	4.30 ± 0.50*	3.07 ± 0.29**
2.	SOD U/gm of Hb.	3.65 ± 0.82	2.08 ± 0.58	2.80 ± 0.46*	3.25 ± 0.25**
3.	GPx U/gm of Hb.	58.25 ± 5.58	12.24±0.18	22 ± 3.55*	36.78 ± 4.26**
4.	Catalase n mole/ H_2O_2 decomposed /min	640 ± 110.05	502 ± 115	568 ± 72.0*	597 ± 0.91**
5.	Vitamin E mg/dl.	7.86 ± 2.52	5.18 ± 2.67	5.86 ± 1.42*	6.10 ± 2.15**
6.	Vitamin C mg/dl.	1.66 ± 0.46	0.73 ± 0.13	0.87 ± 0.14*	1.18 ± 0.21**

*Values are expressed in mean ± SD, n= Number of observations. *Indicates P< 0.01 when compared with before therapy. **Indicates P< 0.001 when compared with 4 weeks therapy.*

Table 3: Changes in activity of liver profile enzymes after and before therapy in alcoholic hepatitis in group II patients

Sl. No.	Parameters	Controls (n=50)	Alcoholic hepatitis before therapy (n=25)	Alcoholic hepatitis after 4 weeks therapy (n=25)	Alcoholic hepatitis after 8 weeks therapy (n=25)
1.	Serum total bilirubin mg %	0.62 ± 0.019	4.80 ± 2.95	3.20 ± 1.25**	1.42 ± 0.9**
2.	Serum total protein gm%	7.28 ± 0.5	6.68 ± 0.62	6.98 ± 0.02*	7.35 ± 0.18*
3.	SGPT IU/L	28.90 ± 7.66	310 ± 80	220 ± 58**	120 ± 72.2**
4.	SGOT IU/L	18.20 ± 0.40	298 ± 102	145 ± 58**	65 ± 32.0**
5.	ALP IU/L	112 ± 4.84	198 ± 1.9	155 ± 4.0**	121 ± 4.20**
6.	GGT IU/L	27.90 ± 0.68	120 ± 3.90	88 ± 2.98**	50.51 ± 3.30**
7.	5' NTP IU/L	15.55 ± 3.68	20.17 ± 1.50	17 ± 1.32*	15.50 ± 2.35*
8.	LDH IU/L	242 ± 28.28	260 ± 25	252 ± 20•	245 ± 22.27•

*Values are expressed in mean ± SD, n= Number of observations. Indicates *P < 0.01, **Indicates P< 0.001. •indicates P > 0.05.*

The levels of total brilirubin (Tables 3 and 4) and the activity of liver profile enzymes SGPT, SGOT, ALP, GGT and 5' NTP were significantly increased in group II and group III in comparison with control group. However LDH did not show any statisticaly significant changes in both group II and group III.

Discussion

MDA estimation is one of the most commonly used

Table 4: Changes in activity of liver profile enzymes after and before therapy in alcoholic hepatitis in group III patients

Sl. No.	Parameters	Controls (n=50)	Alcoholic hepatitis before therapy (n=25)	Alcoholic hepatitis after 4 weeks therapy (n=25)	Alcoholic hepatitis after 8 weeks therapy (n=25)
1.	Serum total bilirubin mg %	0.62 ± 0.019	6.45 ± 3.15	3.10 ± 1.15**	1.48 ± 0.92**
2.	Serum total protein gm%	7.28 ± 0.5	6.50 ± 0.60	6.97 ± 0.09*	7.28 ± 0.20*
3.	SGPT IU/L	28.90 ± 7.66	425± 121	250 ± 82**	115 ± 73.33**
4.	SGOT IU/L	18.20 ± 0.40	358 ± 155	125 ± 62**	70 ± 40.0**
5.	ALP IU/L	112 ± 4.84	238 ± 2.9	180 ± 4.9**	140 ± 5.33**
6.	GGT IU/L	27.90 ± 0.68	190 ± 5.33	100 ± 3.30**	60 ± 5.15**
7.	5' NTP IU/L	15.55 ± 3.68	25.18 ± 2.18	20.20 ± 1.93*	16 ± 1.60*
8.	LDH IU/L	242 ± 28.28	290 ± 20.32	250 ± 18.52•	228± 22.27•

*Values are expressed in mean ± SD, n= Number of observations. Indicates * P < 0.01, ** Indicates P< 0.001.•indicates P > 0.05*

methods to monitor lipid peroxidation in biochemical samples. Lipid peroxidation is used as an indicator of oxidative stress in cell and tissues. Thiobarbituric acid reactive substances, the indicator of lipid peroxidation were significantly elevated in alcoholic hepatitis. There is association between increased levels of MDA and progression of alcoholic hepatitis. Alcohol may be reducing the activity of acetaldehyde dehydrogenase thus there is accumulation of acetaldehyde [21,22]. This may result in the formation of free radicals; such radicals may be attacking unsaturated fatty acids in membrane and organelles to produce lipid peroxides. This may cause decrease in membrane permeability. Thus, change in membrane fluidity causes cellular damage and necrosis.

The activity of liver profile enzymes were significantly increased in group II and group III. These enzymes are localized in the cell cytoplasm and cell mitochondria and found in bile as well. Elevated serum enzymes like SGPT, SGOT, ALP, 5' NTP, GGT and LDH are indicative to cellular damage and loss of functional integrity of cell membrane in liver. Damage of liver cells causes leakage of cellular enzymes into serum [23]. In group II and group III after four- and eight-week therapy of Phyllanthus amarus there was significantly decreased activity of liver profile enzymes including the reduction in liver cell damage.

The increased concentration of bilirubin and significant rise activity of liver profile enzymes could be taken as an index of liver damage. After four-week therapy of Phyllanthus amarus there was significant decrease in LPO and it resumes to normal after eight weeks in group II and group III. Effect of alcoholic hepatitis showed increased oxidative stress by decreasing non enzymatic antioxidants vitamin E and vitamin C and increasing LPO. An alcoholic hepatitis increased oxidative stress may increase consumption of vitamin E and C. Vitamin E traps free radicals and interrupts the chain reaction that damage the cell [25]. Thus in group II and group III there is increased utilization of vitamin E and vitamin C due to oxidative stress.

SOD is an important antioxidant enzyme having scavenging effect against superoxide anion and catalase is responsible for detoxification of H_2O_2 produced by action of superoxide dismutase and inhibits formation of superoxide radicals [24]. Due to increased oxidative stress there may be increased utilization of enzymes in group II and group III to balance the decreased activity of antioxidant enzymes by oxidation through reactive free radicals. Thus, the activity of SOD, GPx, and catalase may decrease in group II and group III of alcoholic hepatitis. Decreased antioxidant enzymes and vitamin C and vitamin E might be causing oxiradical mediated injury and thus may contribute to liver damage. After eight weeks of therapy with Phyllanthus amarus in group II and in group III decreased levels of vitamin E and vitamin C has been comes to near normal. This indicates that the utilization of vitamin E and C is decreased and this may be responsible for raised levels of Vitamin E and vitamin C. Increased activity of antioxidant enzymes and vitamins in group II and group III of alcoholic hepatitis indicate that there might be regeneration of liver cells after therapy, which helps in curing hepatitis.

Phyllanthus amarus can detoxify the alcohol by

scavenging free radicals formed from acetaldehyde and has an antioxidant activity [26]. It has been shown to increase protein biosynthesis [27] and increase the rate of regeneration of necrosed cells [28].

This study concludes that oxidative stress and antioxidants may be playing an important role in alcoholic hepatitis. Elevated free radicals may cause hepatic cell damage and play a role in pathogenesis of alcoholic hepatitis. The Phyllanthus amarus therapy is found to be equally effective in group II and group III patients of alcoholic hepatitis. The present study strongly suggests that the therapy with Phyllanthus amarus increases various antioxidants and reduces lipid peroxidation of hepatic cellular and intracellular membranes. Hence it ultimately protects liver damage due to free radicals in alcoholic hepatitis.

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Correspondence:
Padmaja Nikam,
Department of Biochemistry,
Belgaum Institute of Medical Sciences,
District Hospital Campus,
Belgaum-590 001, Karnataka, India.
E-mail: nikam31@gmail.com

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A Comparative Study of Serum Myeloperoxidase Activity in Type 2 Diabetes and Diabetic Nephropathy

Kusuma KS, Vasudha KC, Vanitha Gowda MN

Department of Biochemistry, M. S. Ramaiah Medical College, MSR Nagar, Gokula, Bangalore, India

Abstract

Inflammatory burden is high in End Stage Renal Disease (ESRD) patients. In Diabetic ESRD patients, the balance between pro- and anti-oxidant activities is shifted towards an oxidative stress. Myeloperoxidase (MPO), a bactericidal enzyme plays an active role in induction and evolution of the endothelial dysfunction associated with Type 2 Diabetes Mellitus (DM). However, whether MPO can serve as a marker in diabetic ESRD patients is doubtful. Hence, the present study was undertaken. The Fasting Blood Glucose (FBG), Serum Creatinine, Blood Urea Nitrogen (BUN) and Serum Myeloperoxidase levels were estimated in three different groups with 30 subjects each. The Control group includes healthy individuals, group I constitutes Type 2 diabetes with no nephropathy and group II includes Type 2 diabetes with nephropathy patients. Glomerular Filtration Rate (GFR) was calculated using Cockcroft Gault formula corrected for the Body Surface Area. Statistical software, namely, SPSS 15.0, Stata 8.0, MedCalc 9.0.1 and Systat 11.0 were used for the analysis of the data and Microsoft word and Excel have been used to generate graphs, tables etc. Reference Ranges for MPO were established in our study. An increase in MPO levels was noticed in Group I, whereas a differential behaviour of MPO levels was noted in group II. Uremic diabetic nephropathy patients with a low MPO level may be at a lesser risk for any cardiac event compared to those uremic patients with high MPO levels. Hence, MPO may be taken as a biomarker to predict coronary events in diabetic ESRD.

Key words: End stage renal disease, glomerular filtration rate, myeloperoxidase, oxidative stress, reactive oxygen species, type 2 diabetes mellitus

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Introduction

"The quality of life is dependent upon the quality of the life of your cells. If the blood stream is filled with waste products, the resulting environment does not promote a strong, vibrant healthy cell life nor a biochemistry capable of creating a balanced emotional life for an individual"
- *Anthony Robbins* [1].

Diabetes mellitus is characterized by elevated blood glucose levels, a continuous low–grade inflammation and endothelial activation state [2,3]. Type 2 DM is prevalent in our country to an extent of 5.9% [4]. MPO (E.C.No. 1.11.1.7), a hemoprotein traditionally viewed as bactericidal enzyme secreted by neutrophils plays an active role in the induction and evolution of endothelial dysfunction associated with Type 2 DM [3].

The inflammatory burden is high in ESRD patients. In diabetic ESRD patients, the balance between pro- and anti-oxidant capacities is shifted towards a state of oxidative stress [5]. As increased oxidative stress and inflammation are both common features of ESRD, it has been speculated that there may be an association between them and endothelial dysfunction, contributing to increased risk for cardiovascular disease [5]. As MPO predicts endothelial function in humans, probably by regulating nitric oxide bioavailability, increased MPO activity serves as a mechanistic link among the inflammation (activated leukocytes), oxidative stress and endothelial dysfunction in ESRD [6].

There is limited data available regarding the study

of serum MPO activity in patients with type 2 DM with ESRD in India. Considering this, the study was undertaken to see if MPO can serve as a Biomarker in type 2 DM with ESRD.

Materials and Methods

A cross sectional study was conducted for a period of one year, starting from Jan 2007, at the M.S.Ramaiah Medical College Teaching Hospital (MSRMTH), Bangalore. Informed Consent was taken from all the participants. The Ethical Clearance for this study was obtained from the Ethical Review Board of M.S.Ramaiah Medical Teaching Hospital. The subjects were selected by Simple Random Sampling.

Study groups

The subjects were divided into three groups of 30 subjects each. All the subjects were above 45 years of age and of either sex.

1. Control group: healthy subjects, attending the Out Patient Department (OPD) of MSRMTH.
2. Group I: Type 2 diabetes patients with no nephropathy, attending the OPD or admitted in the wards of MSRMTH.
3. Group II: Type 2 diabetes patients with nephropathy, attending the OPD or admitted in the MSRMTH.

Relevant clinical history was taken in OPD or in wards and the clinical examination was performed. The height and weight of the patient was recorded at the same time.

Patients with type 2 diabetes mellitus with uncontrolled hyperglycemia, acute coronary syndromes, cerebrovascular accidents or stroke and severe peripheral vascular artery diseases were excluded from group II whereas type 2 DM with nephropathy were excluded from the group I.

About 5 ml of blood sample was collected in the vacutainer bulb without any anti-coagulant. The blood sample was collected after 10 hours of fasting. It was centrifuged and the following investigations were performed immediately- fasting blood glucose, serum creatinine & blood urea nitrogen. They were measured on an auto-analyzer – Dade Behring Dimension series R_XL Max. Blood glucose was estimated by an adaptation of the hexokinase – glucose 6-phosphate dehydrogenase method [7]. The remaining serum sample was stored between 0 – 4˚C, to measure the MPO levels [8]. The creatinine and BUN were estimated by modification of kinetic Jaffe's reaction reported by Larsen [9] and urease glutamate dehydrogenase coupled enzymatic technique according to Talke H and Schubert [10] respectively.

Serum MPO levels were estimated by a spectrophotometric method using O–Dianisidine Dihydrochloride as a substrate [11]. Myeloperoxidase was assayed as follows: 0.1 ml of the serum was mixed with 2.9 ml of 50 mM phosphate buffer, pH 6.0, and containing 0.167 mg/ml O-Dianisidine Dihydrochloride (HI-MEDIA Co.) and 1% Hydrogen peroxide (E–Merck) [11]. Some modifications were made to the original procedure to enhance sensitivity of the assay. The change in absorbance was measured at 470 nm using a spectrophotometer (ELICO SL 134 UV – VIS Double Beam Spectrophotometer) for a period of three minutes (kinetic assay) and MPO was measured using the formula [12]. One unit of MPO activity was defined as that degrading one µmole of peroxidase per minute at 25˚C [13]. The Statistical software namely SPSS 15.0, Stata 8.0, MedCalc 9.0.1 and Systat 11.0 were used for the analysis of the data and Microsoft word and Excel have been used to generate graphs, tables etc [14-16].

Calculations [12]:

$$\text{MPO (U/L)} = \frac{\Delta A/\text{min} \times 3200\ \mu l \times 10^6\ \mu\ \text{mol/mol}}{11300\ \text{L.mol}^{-1}.\text{cm}^{-1} \times 1.0\ \text{cm} \times 100\ \mu l}$$

$$= \Delta A/\text{min} \times 2832\ \mu\ \text{mol/min}$$

$$= \Delta A/\text{min} \times 2832\ \text{U/L}$$

where, $\Delta A/\text{min} = A_3 - A_2 / 2$, A_3 = absorbance at three min at 470 nm & A_2 = absorbance at one min at 470 nm.

11300 L mol^{-1}. cm^{-1} = molar absorptivity co-efficient [17].

Glomerular Filtration Rate (GFR) was calculated using Cockcroft Gault formula corrected for the Body Surface Area [18,19].

Results

The Reference Ranges for MPO was established in our study (Table 1). MPO levels were increased notably in Group I (F is equal to1406.22; P less than 0.001), whereas a differential behaviour was noted in Group II. The blood glucose levels were increased in both Group I and Group II as compared to the controls (Table 1). The levels of BUN and Serum Creatinine were increased in group II as compared to the controls (Table 1). GFR levels were decreased in Group II as compared to the controls and Group I (Table 1).

Group II was divided into two subgroups, Group IIa and Group IIb based on the GFR values (Table 2), to see if MPO can serve as a biomarker in End Stage Renal

Table 1: Comparison of study parameters in three groups

Study parameters	Controls	Group I	Group II	*P* value
FBS (mg/dl)	96.77±11.99	216.20±65.65	213.87±43.14	<0.001**
BUN (mg/dl)	11.03±2.88	11.70±4.53	60.89±30.68	<0.001**
S. Creatinine (mg/dl)	0.89±0.19	0.95±0.17	6.56±3.07	<0.001**
GFR (ml/min/1.73m^2)	112.54±29.22 (63.73-168.71)	101.87±24.81 (60.36-158.95)	19.66±13.56 (6.88-57.86)	F=1406.22; *P*<0.001**
MPO (U/L)	165.44±74.17 (83.54-332.76)	494.59±218.48 (354-1268.7)	379.82±311.27 (84.96-1274.4)	F=140.662; *P*<0.001**

Table 2: Comparison of study parameters in three groups

Study parameters	Controls	Group I	Group II_a GFR < 15 ml/min/1.73m^2	Group II_b GFR > 15 ml/min/1.73m^2	*P* value
GFR (ml/min/1.73m^2)	112.54±29.22 (63.73-168.71)	101.87±24.81 (60.36-158.95)	11.06±2.45 (6.88-14.92)	30.89±13.95 (15.12-57.86)	F=100.472; *P*<0.001**
MPO (U/L)	165.44±74.17 (83.54-332.76)	494.59±218.48 (354-1268.7)	297.06±230.27 (84.96-882.17)	488.08±375.57 (135.94-1274.0)	F=13640; *P*<0.001**

Depending on the Glomerular filteration rate, Group II has further been divided into two groups, Group IIa and Group IIb. Group IIa : GFR ≤ 15ml/min/1.73m^2 –Stage V Diabetic nephropathy/ End Stage Renal Disease (ESRD). Group IIb : GFR ≥ 15ml/min/1.73m^2 – Stage III/ Stage IV Diabetic nephropathy.

Table 3: Pair-wise significance

Study parameters	Controls-Group I	Controls-Group IIa	Controls-Group IIb
GFR (ml/min/1.73m^2)	0.278	<0.001**	<0.001**
MPO (U/L)	<0.001**	0.200	<0.001**

Disease. GFR was measured using the Cockroft Gault formula. The F value for GFR in various study groups is 100.472 with P less than 0.001 and hence is strongly significant (Table 2). The MPO levels are significantly higher in Group I when compared to the control group. In Group IIb, the raise in MPO is again significant with a Mean plus/minus S.D is equal to 488.08±375.57 when compared to the control group (Table 2). In the Group IIa, there is a raise in MPO with a Mean plus/minus S.D is equal to 297.06 plus/minus 230.27 compared to the control group, but the pair wise significance (Table 3) is 0.200 (not significant). The pair wise significance between the Control group and Group I and Control group and Group IIb is less than 0.001 (strongly significant) (Table 3). The F value between the various groups for MPO is 13640 with a P value of less than 0.001 suggesting that it is strongly significant.

The sensitivity, specificity and the accuracy of the test is 100% when the MPO values are ≥ 354 U/L in type 2 diabetes, whereas in diabetic nephropathy no such value of MPO exists (Tables 4 and 5). The Area under the curve for MPO in type 2 diabetes is around 1.0000, which indicates that it is an excellent test (Figure 1),

Table 4: Sensitivity, specificity of MPO levels in diabetics with respect to controls

MPO levels U/L	Sensitivity %	Specificity %	Accuracy %	LR+	LR-
≥ 254.88	100.00	80.00	90.00	5.00	0
≥ 260.54	100.00	90.00	95.00	10.00	0
≥ 312.94	100.00	93.33	96.67	15.00	0
≥ 332.76	100.00	96.67	98.33	30.00	0
≥ 354	100.00	100.00	100.00	-	**1**
≥ 356.83	0.00	100.00	50.00	-	-

The test is 100% sensitive and specific when the value of MPO is greater than or equal to 354 U/L, though it is significant even when the MPO value is just greater than or equal to 254.88 U/L in Diabetics. LR+ - Likelihood ratio of the occurrence of the disease,. LR- Likelihood ratio of the absence of the disease.

Table 5: Comparison of study parameters in the two groups in diabetic nephropathy

MPO levels U/L	Sensitivity %	Specificity %	Accuracy %	LR+	LR-
≥ 254.88	50.00	80.00	65.00	2.50	0.63
≥ 260.54	46.67	90.00	68.33	4.67	0.59
≥ 283.2	46.67	93.33	70.00	7.00	0.57
≥ 303.02	43.33	93.33	68.33	6.50	0.61
≥ 312.94	40.00	93.33	66.67	6.00	0.64
≥ 332.76	40.00	96.67	68.33	12.00	0.62

There is no such significance obtained in the Diabetic nephropathy.

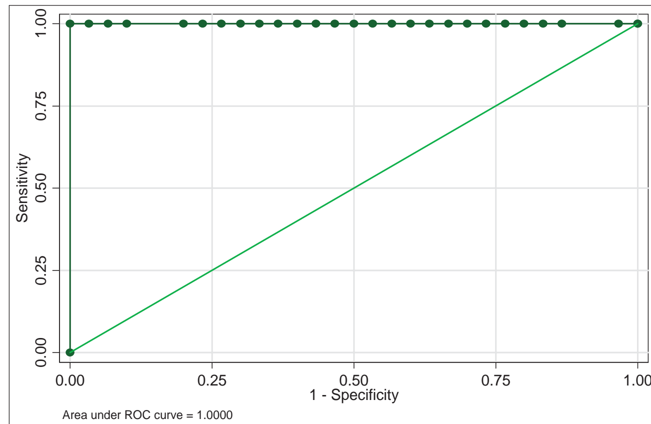

***Figure 1:** ROC Curve for MPO in type 2 diabetes*

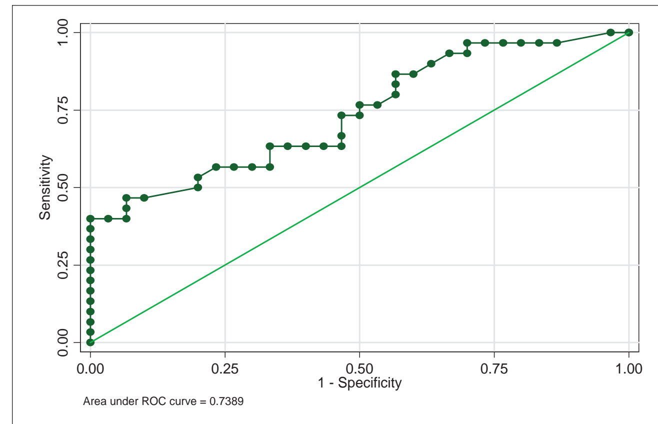

***Figure 2:** ROC Curve for MPO in Diabetic nephropathy*

whereas in diabetic nephropathy patients, the area under the curve for MPO is 0.7389 which indicates that it is a fair test (Figure 2).

Group II cases are further divided into two groups based on the MPO levels:

1. Group X: Diabetic Nephropathy with an increase in Myeloperoxidase levels. (greater than or equal to 354 U/L)
2. Group Y: Diabetic Nephropathy with a decrease in Myeloperoxidase levels. (less than or equal to 354 U/L).

The Group II was again divided into two other subgroups based on MPO levels (Table 6) in order to explain the behaviour of MPO enzyme in diabetic nephropathy individuals. The subgroups are: Group X: Diabetic nephropathy with increased MPO levels (greater than or equal to 354 U/L), Group **Y**: Diabetic nephropathy with decreased MPO levels (less than or equal to 354 U/L).

Table 6: Comparison of study parameters in the two groups in diabetic nephropathy

Study parameters	Group X	Group Y	P value
GFR (ml/ min/1.73m^2)	25.35±14.97 (8.45-44.96)	16.36±11.84 (6.88-57.86)	t=1.820; P= 0.079+
MPO (U/L)	714.95±276.64 (424.80-1274.40)	185.79±72.22 (84.96-339.84)	t=7.973; P<0.001**

Discussion

Myeloperoxidase is an abundant heme protein that is released by activated neutrophils, monocytes and tissue – associated macrophages after inflammatory stimuli [6]. End Stage Renal Disease patients are subjected to enhanced oxidative stress, as a result of reduced anti – oxidant systems (Vitamin C and Selenium deficiency, reduced intracellular levels of Vitamin E, reduced activity of glutathione systems) and increased pro – oxidant activity (advanced age, high frequency of diabetes, chronic inflammatory state, uremic syndrome, bio-incompatibility of dialysis membranes and solutions) [5].

Many patients in Chronic Renal Failure exist in a chronic inflammatory state. Patients affected by ESRD experience an excess morbidity and mortality due to cardiovascular disease [20]. Haemodialysis also serves as a potent stimulator of circulating phagocytes [21]. Hyperglycemia of diabetes mellitus and its attendant metabolic syndromes – insulin resistance, hypertension,

obesity, dyslipidemia and some social characters of these patients e.g. smoking and the use of xenobiotics predispose to diabetic nephropathy. These accelerate free radical generation and attenuate the anti-oxidant defence system creating oxidative stress [22]. Hence diabetic nephropathy is a state of disease where in there is increased oxidative stress and an increase in MPO is expected which explains the elevation of MPO levels in group I and group II_b.

Table 6 shows the details of GFR and MPO levels in group **X** and group **Y**. In group **Y** (19 patients), the MPO levels are reduced (less than or equal to 354 U/L). These are diabetic nephropathy patients and hence classically a rise in serum MPO would be expected. Instead a decrease in MPO is seen which is due to a rise in the urea levels. The uremic plasma seriously interferes with a variety of normal functions. When urea is present in high concentrations in the body fluids, it may decompose spontaneously into ammonia and cyanate. Cyanate is a potential toxin which combines irreversibly with normal protein in cells and affects their function [23]. Cyanate inhibits both peroxidative and halogenating activities of MPO and inhibits the enzyme within intact neutrophils. The cyanate also inhibits Cl^-/H_2O_2/MPO mediated bacterial killing [24]. In Group $II_{b,}$ the fall in MPO noted with increased BUN levels was for the same reason.

In Group **X** (11 patients), the MPO was raised in all of them (greater than or equal to 354 U/L). In this group a few of them were on haemodialysis (four patients) whereas the rest of them were on conservative medical line of treatment (seven patients). Uremia represents a unique clinical condition in which immune dysregulation appears to be closely associated with phagocyte derived oxidative stress. The latter is dramatically exacerbated by haemodialysis (HD) treatment. This aggravation is commonly attributed to the recurrent activation of neutrophils during blood passage through the dialysis circuits and subsequent generation of activated complement components, following the contact with bio-incompatible membranes and/ or the retrodiffusion of endotoxins from the dialysate [25]. Some studies show that during hemodialysis treatment, even with use of bio-compatible membrane and ultra pure dialysate, it may contribute to an increase in leucocyte activation and enhanced oxidative stress [5,26,27].

In group X some patients who were on conservative line of treatment had an increase in BUN levels. We have already seen that MPO values decrease with an increase in BUN level. But in this group of patients, there was an increase in MPO with an increased BUN level. The raise in MPO in this group of patients may be due to the following reasons. In a study [25], which emphasized the respective role of uremic toxins and myeloperoxidase in uremic state, the workers have measured MPO by two different methods. One is an ELISA method and the other is the measurement of a colored product quinoneimine, which is formed due to the co-oxidation of phenol and aminoantipyrine (AAP). A discrepancy was noted between the MPO values measured by these two different methods. To understand the discrepancy in CKD plasma between low immunoreactive MPO levels detected by ELISA and high MPO enzymatic activity levels measured by quinoneimine formation, they investigated whether the oxidative coupling of phenol and AAP resulted mainly from MPO activity in the presence of hydrogen peroxide or whether other oxidative plasma components were directly involved in this oxidation. Hence comparative assays were performed. The plasma of predialysis patients is devoid of peroxidase and the oxidation level as measured by quinoneimine formation is constant irrespective of both H_2O_2 and MPO presence suggesting mediation by other oxidative plasma components [25]. The other oxidative plasma components are the free plasma aminothiols (homocysteine, cysteine, cysteinylglycine, glutathione). These plasma aminothiols have been found to be excessively oxidized in uremia. The plasma of the ESRD patients on hemodialysis has increased levels of these free plasma aminothiols before dialysis. Hemodialysis procedure serves to remove these and restore the redox status [28]. Hence the increase in MPO levels in patients on hemodialysis appears to be a true reflection of the MPO activity. In pre-dialysis patients, exogenous compounds, such as uremic toxins or active redox compounds, could mediate the oxidative coupling of phenol and aminoantipyrine, leading to quinoneimine formation, independently of MPO enzymatic activity [25]. They form the good candidates to perform redox mediation of aminopyrine and phenol and thus stimulate the spectrophotometric detection of quinoneimine [25] suggesting an apparent increase of MPO activity rather than a true MPO activity.

In the present study, a differential behavior of the MPO has been noticed in the uremic patients and the causes for the same have been discussed above. Diabetics are prone for coronary events and cardiovascular disease is a major cause of morbidity and mortality in patients with type 2 diabetes mellitus [29]. Hence, we may consider that the precipitation of a cardiac event leading on to death is higher in those uremic patients with a high MPO than in those with low MPO levels. Therefore Myeloperoxidase

enzyme may be considered as a biomarker for predicting coronary events in End Stage Renal Disease.

However, further studies with follow-up and large trial groups are required to validate the results of our study.

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Correspondence:
Kusuma K.S,
No.9, Sri Satya Sai Nilaya,
5th ‘C’ cross, Sharadambanagar,
Jalahalli, Bangalore-560 013, Karnataka, India.
E-mail: sai10kusupabbi@yahoo.com

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What does initiate parturition?

Amar Chatterjee

Faculty of Medicine, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia

Abstract

Several hormonal and other factors have been proposed to be involved in the initiation of parturition. However, definitive sequential events leading to initiation of parturition are still lacking. Conceptual evolution on the mechanism of initiation of parturition since 1891 has been briefed.

Key words: ERα, PR-A, cytokines, CRH, parturition

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Introduction

More than a century back, Spiegelberg [1] reported that parturition results from substances produced by the fetus. Later, it is suggested that some portion of the central nervous system and fetal adrenal are important [2]. Experiments of Newton [3] and van Wagenen and Newton [4] have shown that following fetectomy, pregnancy continues and placental delivery occurs at or near to normal term in mice and monkeys. Liggins [5] however, reported that ACTH or cortisol infusion into pregnant lambs induces parturition on the 6th day of infusion. Conversely, Chatterjee *et al.* [6] have documented that the administration of glucocorticoid to pregnant rats close to term delays parturition. Liggins [7] later recorded that human fetuses with adrenal hypoplasia are born at or close to term. Administration of large doses of glucocorticoid to pregnant women [8] or sheep [9] before term also failed to induce premature labor.

Molokwu and Wagner [10] have proposed that an increased level of glucocorticoid at the time of parturition is due to the stress of labor but not a cause of parturition. On the other hand, Talbert *et al.* [11] could not detect any significant difference in cord plasma levels of cortisol in infants born after elective Caesarian section compared to those born after spontaneous onset of labor.

Based on 89 publications, the International Planned Parenthood Federation suggested that at term, fetal ACTH by stimulating fetal adrenal glucocorticoid induces a concomitant decrease in progesterone with a concurrent increase in estrogen. As a result, a spontaneous elevation of fetal and maternal prostaglandins and oxytocin leads to softening of the cervix and a simultaneous uterine myometrial contraction which consequently results in parturition [12]. Cortisol, a progesterone agonist, has however been shown to exert a direct inhibitory effect on estrogen [13] and prostaglandin [14] synthesis.

Parturition is moreover claimed to be driven by a pulsatile pattern of oxytocin secretion [15], but neither oxytocin antiserum [16] nor its antagonist [17,18] is found to prolong gestation or delay parturition. Circulating oxytocin therefore, does not seem to be essential for the initiation of parturition [19].

Progesterone is known to support pregnancy and prevent parturition by promoting myometrial quiescence [20]. In contrast, estrogen stimulates parturition by augmenting myometrial excitability and contractility [21] with a corresponding stimulation of prostaglandins and ripening of the cervix [22]. Therefore to initiate parturition, transformation of myometrium from a quiescent to a contractile state requires a coordination of progesterone withdrawal and estrogen activation. However, in humans [23] and higher primates [24], maternal progesterone and estrogens levels are found to remain elevated during parturition.

RU 486, a progesterone antagonist and a potent abortifacient agent [18,25], not only increases myometrial estrogen receptor (ER) expression, it does also reduce progesterone concentration in several species including rats [26], monkeys [27] and humans [28]. RU 486 also exerts a profound softening action on the cervix [29].

It is now hypothesized that functional progesterone withdrawal occurs by increased expression of progesterone receptor-A (PR-A) type which suppresses myometrial

progesterone responsiveness [30].

Similarly, functional ERα activation is found to be linked with functional progesterone withdrawal [31]. Progesterone withdrawal consequently stimulates prostaglandin synthesis by human endometrium [32], myometrium and cervix [33] and also by the rat myometrium [34]. Released prostaglandin then induces gap junction formation between myometrial cells [35] which facilitates a synchronized propagated uterine contraction with a corresponding ripening of the cervix [36] and finally results in labor [37]. Sugimoto et al [38] have shown that female mice lacking receptors for prostaglandin F2α do not deliver fetuses at term.

The expression of prostaglandin in the uterine tissue is increased by cytokines [39]. IL-6 and IL-8 are the cytokines produced by human endometrium, myometrium, chorio-decidua and cervices [40]. Preterm labor due to uterine or intra-amniotic infection is being found to be associated with an increased synthesis and release of IL-6, IL-8 [41] and prostaglandins [42]. Progesterone and glucocorticoids, the well-established anti-inflammatory agents [43] cause a significant inhibition of IL-6 and IL-8 [44] as well as prostaglandin synthesis [45]. However, intraperitoneal infusion of proinflammatory cytokines does not cause activation of the pregnant rat uterus [46]. The integrated role between the cytokines and prostaglandins in the initiation of labor therefore remains enigmatic.

Corticotropic-releasing hormone (CRH), a peptide highly expressed in human placenta at the end of gestation has also been implicated in the process of labor [47]. An elevation of maternal serum CRH concentration has been documented as early as 18-week of gestation in patients who have subsequently aborted [48]. Estrogen stimulates CRH through its action on prostaglandin synthesis. On the other hand, CRH is found to increase the synthesis and release of prostaglandins from the cells of amnion, chorion and deciduas [49, 50].

In conclusion, the functional progesterone withdrawal with subsequent estrogen receptor activation possibly mediates the formation of prostaglandin which by interacting with cytokines and CRH may result in the initiation of parturition.

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Correspondence:
Professor Amar Chatterjee,
Faculty of Medicine, Universiti Teknologi MARA,
40450 Shah Alam, Selangor, Malaysia.
E-mail: amar.chatterjee@hotmail.com

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Menopausal Syndrome: Effect on Serum Lipid and Lipoprotein Profiles

Ebele J. Ikekpeazu, Emeka E. Neboh[1], Ignatius C. Maduka[2], Fidelis E. Ejezie, Silas Ufelle[3]

Department of Medical Biochemistry, [3]Department of Medical Laboratory Sciences, College of Medicine, University of Nigeria Enugu Campus (UNEC), Enugu State, Nigeria, [1]Department of Chemical Pathology, College of Medicine, Enugu State University Teaching Hospital (ESUTH), Enugu State Nigeria, [2]Department of Chemical Pathology, University of Nigeria Teaching Hospital (UNTH), PMB. 01129, Ituku-Ozalla, Enugu State, Nigeria

Abstract

Plasma lipids have been known to be altered by the within-month variations in the female hormonal system. The female hormonal system also controls the menstrual cycle in the pre-menopausal women. This study is, therefore, aimed at finding the possible effects of menopause on plasma lipids and lipoproteins, as shown by the lipid profile. The lipid profiles of 200 post-menopausal women and 100 pre-menopausal control women were estimated. The results obtained show a statistically significant increase (*P* less than 0.05) in the total cholesterol level of early menopausal subjects (6.05 plus/minus 1.03mmol/L) and slightly higher values in late menopausal subjects (6.80 plus/minus 0.81mmol/L), when compared with the control subjects (4.6 plus/minus 0.62mmol/L). Triglycerides (TG) showed slight but significant increase (*P* less than 0.05) in the early menopausal subjects (1.40 plus/minus 0.80 mmol/L) and higher levels in late menopausal subjects(1.96 plus/minus 0.45 mmol/L) in comparison with the control subjects (1.02 plus/minus 0.44 mmol/L). High density lipoprotein (HDL) levels were however reduced significantly (*P* less than 0.05) in early menopausal subjects (1.20 plus/minus 0.27 mmol/L) and even lower in late menopausal subjects (1.17 plus/minus 0.28 mmol/L) when compared to the controls; while Low density lipoprotein (LDL) and very low density lipoprotein (VLDL) levels were significantly increased (*P* less than 0.05) in early menopausal subjects (4.21 plus/minus 0.81 and 0.63 plus/minus 0.37 mmol/L), and higher in late menopausal subjects (4.70 plus/minus 0.55 and 0.81 plus/minus 0.41 mmol/L) when compared with the control subjects. The findings suggest the probability that menopausal syndrome can lead to hyperlipidaemia, dyslipidaemia and possibly predispose women to coronary artery disease.

Key words: Effect, menopausal syndrome, serum, lipid, lipoprotein, profile

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Introduction

A lipid profile is a battery of tests used in diagnosing, treating and predicting atherosclerosis. It consists of four tests and some calculated values. The measured tests are total cholesterol (TC), triglycerides (TG) and high density lipoprotein (HDL), while the calculated values are low density lipoprotein (LDL), very low density lipoprotein (VLDL) and a cardiac risk factor [1].

Coronary artery disease (CAD) results from the thickening of the inner layer of arterial walls and accounts for more than 50% of all deaths. It is the number one cause of death of women. Part of the body's cholesterol is derived from dietary intake, but majority is synthesized by the liver and other tissues [2]. Lipids have important roles in virtually all aspects of biological life – serving as hormone precursors, aiding digestion, providing energy storage and metabolic fuels, acting as functional and structural components in bio-membranes and forming insulation to allow nerve conduction or to prevent heat loss [3]. Menopause results from the loss of ovarian function, either naturally or surgically.

The female hormonal cycle is an exquisitely controlled system that includes the hypothalamus, pituitary, adrenal, thyroid and gonadal tissues; involving both positive and negative feed back loops [4]. At birth, every woman is endowed with one

to two million primodial follicles which decrease to about 300,000 by the time of menarche (puberty) [5]. By age 51, the median age for the final menstrual period, the ovary reserve is about 1000 and this corresponds with a significant drop in estrogen production, typical when symptoms of menopause occur [4]. It is significant to note that while a woman may stop menstruating at this time, endogenous cycling and ovulation may still occur for months, even years [5]. About 20% of estrogenemic effects on arteries are mediated through changes in lipids and lipoproteins [6]. The association between hypercholesterolemia and atherosclerotic heart disease is an established fact and thrombosis produce occlusive vascular lesions in humans, which may lead to such adverse clinical conditions as stroke, myocardial infarction or peripheral vascular disease [7]. While similar mechanisms operate to induce cardiovascular disease in women and men, gender-related differences exist in the anatomy and physiology of the myocardium, and sex hormones modify the course of the disease in women [8].

The hypo-estrogenic status that results from menopause leads to ovarian failure, resulting in a significant increase in total cholesterol and LDL-cholesterol, predisposing such women to the risk of coronary artery disease [9].

Material and Method

Subjects

The subjects in this study were 200 apparently healthy volunteers of post-menopausal age status (aged between 50 and 69 years), drawn from Enugu metropolis. A total of 100 apparently healthy pre-menopausal women (aged between 35 and 49 years) served as control subjects for the study. Informed consent was duly obtained from each subject that participated in the study.

Exclusion criteria

Subjects with a history of, or who were suffering from conditions which predisposes one to abnormal lipid profile, such as diabetes mellitus, hypercholesterolaemia and hyperlipidaemia, were excluded from the study.

Sample collection and preparation

Fasting whole blood samples {3.0 ml each} were collected from subjects while in the sitting position, by clean venepuncture from the antecubital vein. The samples were collected under asceptic conditions while avoiding haemolysis and dispensed into sterile plain tubes and allowed to clot. The clotted samples were centrifuged at 3000 rpm for five minutes and the separated clear serum supernatants were transferred into sterile tubes. These freshly drawn serum supernatants were used for total cholesterol, triglycerides and HDL-cholesterol assays. The sera were stored frozen and the analysis was carried out within one week.

Analytical method

Total cholesterol (TC) assay was done by enzymatic-spectrophotometric method [10]; while HDL-cholesterol (HDL-C) estimation was carried out using precipitation/ enzymatic-spectrophotometric method [11]. Enzymatic spectrophotometric method [12] was used for triglyceride (TG) estimation; while LDL and VLDL were calculated using Friedewald's formula [13].

Statistical method

The statistical analysis (students T-test) were done using graph pad prism computer software package. Results are reported as mean plus/minus standard deviation (mean plus/minus SD).

Result

The results are presented as mean plus/minus standard deviation (mean plus/minus SD). Table 1 shows a statistically significant difference (P less than 0.05) in the levels of the lipid profile parameters in the test and control subjects. There was a marked increase in the mean levels of TC, TG, LDL, VLDL and lower than normal HDL concentration for postmenopausal women 6.43 plus/minus 0.92; 1.68 plus/ minus 0.63; 4.46 plus/minus 0.68; 0.76 ± 0.39 and 1.19 plus/minus 0.28 mmol/L when compared to the controls (4.60 plus/minus 0.62; 1.02 plus/minus 0.44; 2.71 plus/ minus 1.13; 0.46 plus/minus 0.20 and 1.52 plus/minus 0.36 mmol/L, respectively). When late post-menopausal subjects (60-69 years) were compared with early post-menopausal subjects (50-59 years), there was a statistically significant difference (P less than 0.05) in the mean levels of the various parameters [Table 2].

TC, TG, LDL,VLDL and HDL for the late postmenopausal subjects were 6.80 plus/minus 0.81; 1.96 plus/minus 0.45;4.70 plus/minus 0.55; 0.89 plus/minus 0.41 and 1.17

Table 1: Mean ± SD (mmol/L) of lipid profile parameters in post-menopausal women and pre-menopausal (control) subjects

No. of Subjects	Test Group n = 200	Control Group n= 100	*P* value
T.C	6.43 ± 0.92	4.6 ± 0.62	<0.05*
T.G	1.68 ± 0.63	1.02 ± 0.44	<0.05*
HDL	1.19 ± 0.28	1.52 ± 0.36	<0.05*
LDL	4.46 ± 0.68	2.71 ± 1.13	<0.05*
VLDL	0.76 ± 0.39	0.46 ± 0.20	<0.05*

**= Statistically Significant*

Table 2: Mean ± SD (mmol/L) of lipid profile parameters in early menopausal, late menopausal and pre-menopausal (control) subjects

No. of Subjects	Early Menopausal {50-59 years} n = 100	Late Menopausal {60-69 years} n = 100	Control {35-49 years} n = 100	*P* value
T.C	6.05 ± 1.03	6.80 ± 0.81	4.60 ± 0.62	<0.05*
T.G	1.40 ± 0.80	1.96 ± 0.45	1.02 ± 0.44	<0.05*
HDL	1.20 ± 0.27	1.17 ± 0.28	1.52 ± 0.36	<0.05*
LDL	4.21 ± 0.81	4.70 ± 0.55	2.71 ±1.13	<0.05*
VLDL	0.63 ± 0.37	0.89 ± 0.41	0.46 ± 0.20	<0.05*

**= Statistically Significant*

plus/minus 0.28 mmol/L respectively, while that of the early menopausal subjects were 6.05 plus/minus 1.03; 1.40 plus/minus 0.80; 4.21 plus/minus 0.81; 0.63 plus/minus 0.37 and 1.20 plus/minus 0.27 mmol/L in the same order.

Discussion

The study is focused on the effect of age, early and late menopause on the lipid profile of postmenopausal women. The results obtained in the two age groups pf postmenopausal women show elevated TC, TG, LDL, VLDL and reduced HDL level (P less than 0.05), which could be attributed to gradual cessation in the synthesis of reproductive hormones due to the transition into menopause. This consequently leads to the accumulation of cholesterol in the blood. This finding agrees with an earlier study by [1].

Although the two groups of post-menopausal women showed elevated levels of lipids and lipoproteins, there was significantly higher levels among the late menopausal women (P less than 0.05), which might be linked to a total loss of estrogen production. This finding supports the work of [5], which stated that while a woman may stop menstruating at the onset of menopause, endogenous cycling may still occur for months or even years (as seen in the early menopausal age). The control subjects however showed a normal pattern of lipid profile which can be attributed to the continual metabolism of cholesterol, via the synthesis of hormones such as estrogens, which regulate the female menstrual cycle.

The study shows an association between early and late menopause and lipid profile of post-menopausal women; and also provides an explanation for the higher prevalence of hyperlipidemia and increased risk of atherosclerosis as observed in the late menopausal women.

Conclusion

The study shows that there is a probability that concentration of the lipid profile parameters in post-menopausal subjects might depend on menopausal age.

It also reveals possible predisposition to unfavorable lipid profile and risk of coronary heart disease with progress from early to late menopausal age. It is therefore recommended that post-menopausal women should always be screened for hyperlipidaemia to reduce the prevalence of coronary artery disease (CAD).

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Correspondence:
Ebele J. Ikekpeazu,
Department of Medical Biochemistry,
College of Medicine, University of Nigeria,
Enugu Campus, UNEC, Enugu State, Nigeria.
E-mail: iebelejoy@yahoo.com

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Prevalence and Clinical Aspects of Drug-induced Gingival Enlargement

Syed S. Ahmed, Afshan Bey[1], Sarwat H. Hashmi, Shishir Yadav[2]

Department of Oral and Maxillofacial Surgery, [1]Department of Periodontics and Community Dentistry, [2]Department of Oral Pathology, Oral Medicine and Dental Radiology, Dr. Ziauddin Ahmed Dental College, Aligarh Muslim University, Aligarh, India

Abstract

Drug-induced gingival enlargement is an iatrogenic condition which is usually not preventable. Certain factors like thorough tooth brushing, proper oral hygiene measures, regular dental flossing and professional care of oral cavity are helpful. Surgical excision of the gingival overgrowth is a treatment of choice but non-surgical methods should be adopted first. More research is needed to investigate measures that can prevent or reduce gingival overgrowth or prevent recurrence. A thorough review of literature reveals that inadequate data exist pertaining to prevalence and treatment options available for iatrogenic gingival hyperplasia. Studies with large number of samples, with statistical analysis, are required to make the data more scientific. Preventive measures have limited value and require regular assessment of patients. Hyperplasia does occur after surgical intervention but percentage of recurrence is not available. Hyperplasia can be managed to a certain extent by withdrawing or changing the medication. However, surgical intervention remains the mainstay of treatment. This paper reviews various aspects of prevalence, pathogenesis and treatment options available to manage drug-induced gingival hyperplasia.

Key words: Diltiazem, drug induced, gingival enlargement, nifedipine induced

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Introduction

Gingival enlargement is a clinical condition which has been widely studied and is directly associated with specific local or systemic conditions. There are several medications now available that cause gingival overgrowth. (Gingival enlargement or gingival overgrowth is the preferred term for all medication-related gingival lesions which were previously termed as “gingival hyperplasia”. The term gingival hyperplasia, in fact, did not reflect the histopatholgical composition of drug-induced gingival effects). These medications can be broadly divided into three categories: anti-convulsants, calcium channel blockers and immuno-suppressants [1]. Gingival overgrowth caused by these medications is not only aesthetically unacceptable but also impairs oral hygiene, resulting to periodontal disease, caries and nutrition [2].

Although the pharmacological effect of each of these drugs is different they produce a similar effect on gingival connective tissue causing common histopathological findings. This indicates common pathway of induction [3]. The exact mechanism of this effect is unclear, particularly of immunosuppressive agents [4]. However, the pathognomic mechanisms involve different factors such as dental plaque, presence of genetically predetermined gingival fibroblasts (named responders) and effect of drug itself, with all compounds affecting the trans-membrane flow of calcium [5]. This changes the ongoing metabolism of connective tissue fibroblasts, causing an increase in component of extra-cellular matrix [6-8]. Over all, there are at least two mechanisms which have been proposed. One is related to an interaction between the drug and gingival inflammation secondary to bacterial irritation. The other suggests that the drug might alter the complex cascade of biochemical events surrounding the inflammatory response, resulting in an increased gingival connective tissue production [9].

Prevalence

The prevalence of gingival overgrowth varies with drugs. Miranda, Brunet, Roset, Berini, Farré, Mendieta [10] performed a cross sectional study and compared the nifedipine-induced gingival growth to controls who had never received the drug. They found the prevalence of gingival enlargement was significantly higher in nifedipine-treated cases than in controls (33.8% versus 4.1%). The incidence occurred in 31% of the patients taking diltiazem and 21% in the verapamil-treated group. The prevalence of gingival enlargement was higher in the diltiazem (31%) than verapamil-treated patients (21%), however, in the diltiazem-treated group it was statistically significant [11]. The prevalence for cyclosporine therapy was 21-25% [12]. Hooda and Narula [13] in their study conversely indicated the prevalence with cyclosporine therapy as 9.5%. Ellis, Seymour RA, Steele *et al*. [14] performed a case control, community-based study to assess the prevalence of gingival overgrowth by calcium channel blockers and concluded that there was a remarkable variation in effect on the gingiva caused by different drugs and the subjects taking nifedipine appeared to be more at risk for developing clinically significant overgrowth than those taking amlodipine or diltiazem. The prevalence for nifedipine-induced gingival overgrowth was found to be 6.3% and males are three times sensitive as compared to females to develop clinically significant overgrowth.

Pathogenesis

Gingival epithelium plays an important role in protecting the periodontium from bacterial infections and mechanical trauma. It possesses the capacity of fast self renewal which contributes to gingival defense, since continuous desquamation of superficial epithelial cells prevents bacterial colonization. Therefore, it is suggested that changes in turnover rate of gingival epithelium may affect progression of periodontal disease [15].

Histologically, drug-induced gingival overgrowth is associated with thickening of epithelium with elongated rete pegs and fibrosis in the lamina propria, with increased number of fibroblasts [16,17]. Histo-chemically there is an increase in number of fibroblasts containing large amount of mucopolysaccharide sulphate in nifedipine and phenytoin induced gingival hyperplastic tissue [18].

Ramon *et al*. [19], from their studies, showed that the thickness of the oral epithelium in nifedipine-induced therapy was about five to 10 layers greater than in controls [19].

The volume density of oral epithelium significantly increased in cyclosporine A-induced gingival overgrowth as compared to non-medicated controls [20]. Epithelial thickening, induced by nifedipine [21] and cyclosporine A, is related to thickening of spinous layer [20]. Brown, Beawer, Bottomlay [22] suggested a functional heterogenecity between subgroups of fibroblasts in normal tissues. Fuji, Matsumoto, Nakao, Teshigawara, Akimoto [23] have shown that fibroblasts obtained from hyperplastic tissues synthesise protein approximately two times more than the cells of control group and accelerate the DNA and collagen synthesis. Satio, Mor, Iwakura, Sakamoto [24] have demonstrated that nifedipine and phenytoin increase the synthesis of basic-fibroblast growth factor (bFGF), transforming growth factor beta (TGF-β) and heparan sulphate glycosaminoglycon (HsgG) which probably induce gingival overgrowth.

In another study Sooriyamoorthy, Gower, Eley [25] concluded that an increase in androgen metabolism is an important factor for drug-induced gingival overgrowth and indicated that either the respective drugs or their metabolites can be cytotoxic for less active gingival fibroblasts and facilitate highly active populations of fibroblasts which are involved in the matrix synthesis.

Clinical studies performed by Seymour, Thomson, Ellis [26] indicate that gingival inflammation increases the incidence and severity of gingival overgrowth in nifedipine and/or cyclosporine A-mediated patients.

Nurmenniemi, Pernu, Knuuttila [27] found that that this increased epithelial thickness in both the groups, nifedipine and cyclosporine A is due to increased mitotic activity, especially in the oral epithelium.

Clinical appearance

The clinical appearance of drug-induced gingival growth is mostly characteristic; however, variation is possible depending upon the site, nature of irritant and extent of inflammation. Usually there are no differences in clinical characteristics of gingival overgrowth induced by different drugs but more severe degree of gingival enlargement is seen in patient treated with phenytoin [28]. Growth normally starts from the inter-dental papilla and may favor the appearance of clinical symptoms, signs that include pain, bleeding and friability of tissue, abnormal movement of the teeth, changes of appearance, phonetics, and occlusion, as well as appearance of dental caries and other periodontal disorders (Figure 1) [29,30].

With the progress of the disease the gingivae grow into massive tissue. This extent of growth may progress to the

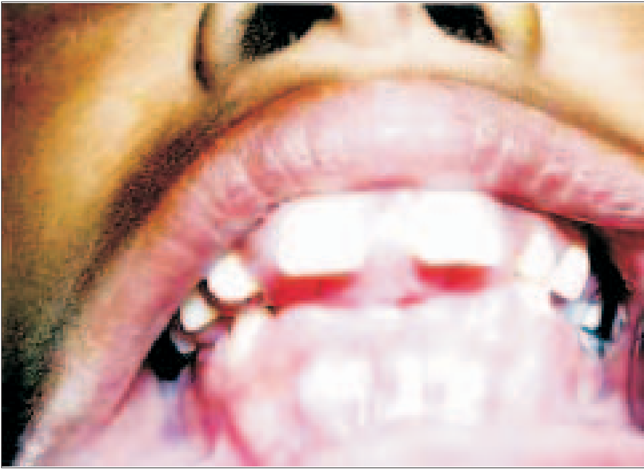

Figure 1: *Phenytoin sodium induced gingival hyperplasia in maxillary and mandibular arch*

extent that it may interfere with the common functions like speech and mastication or mastication alone. The pre-existing periodontal infection, the chemotherapy and subgingival microbiodata affect the periodontal infection and general health. If such cases are not treated, the disease leads to more severe destruction of periodontium and bone.

Treatment/prevention

The preventive measures of drug-induced gingival hyperplasia include recognition of the potential patients and rendering early intervention. Proper oral prophylaxis and good oral hygiene can prevent the phenytoin-induced gingival hyperplasia [31]. Further, a correlation exists between three variables and an increased risk of developing DIGH in patients receiving phenytoin therapy: gingivitis, a visible plaque index and duration of phenytoin therapy [32].

Prevention of recurrence after hyperplasic gingivectomy also deserves consideration. Many attempts have been made in this direction and chlorhexidine mouth wash has achieved some success. Chlorhexidine gluconate (0.1%) mouthwash is used three times daily in addition to aggressive plaque control measures. Meticulous plaque control by thorough and frequent tooth brushing and dental flossing appears to be an effective method. However, the unwanted effects of chlorhexidine of bacterial resistance and taste disturbance limit its long term use. Since phenytoin inhibits the folic acid metabolism, oral folic acid supplementation and folic acid mouthwash 1mg/ml has also been attempted, but with uncertain efficacy [33]. It has been suggested that folic acid mouthwash may reduce gingival inflammation by binding to the plaque-derived endotoxins which may ultimately reduce gingival overgrowth. This hypothesis is, however, yet to be verified.

Drug substitution/withdrawal

This is the most effective treatment of drug-induced gingival enlargement. It usually takes few weeks to resolve the gingival lesions [34,35] but response to this method varies [36].

Usually reduction in gingival overgrowth is noticed where a structurally different calcium channel blocker is used. Same therapeutic goals can be archived by using structurally different medications. However, Linderman, Lumerman, Reuben, Freedman [37] had reported the same effect where verapamil was substituted for nifedipine.

With the obvious side effects produced by phenytoin sodium, the trend of using this medication is changing. Now carbamazepine and sodium valproate are preferably used as alternatives to phenytoin sodium since they have not shown to cause drug-induced gingival hyperplasia.

The prevalence and severity of drug-induced gingival hyperplasia in patients receiving tacrolimus has been shown to be approximately half of the cyclosporine. However, even after the change in immunosuppressant medication from cyclosporine to tacrolimus, calcium channel blocker may be continued. The concomitant use of calcium channel blocker increases both the severity and prevalence of drug-induced gingival hyperplasia [38]. Thus the change in medication from cyclosporine to tacrolimus neither reduces the severity nor leads to complete resolution of overgrowth [39].

Drug therapy

Gingival hyperplasia is a known complication of cyclosporine therapy with a frequency between 21-25% in renal transplant patients [40]. A study of clinical trials revealed benefits of systemic azithromycin in such conditions [41] Hooda and Narula [13] gave azithromycin to their patients with gingival hyperplasia and symptomatic relief was noticed in all patients. They proposed that treatment with azithromycin is inexpensive and effective in short period of time, avoiding gingival surgery in large number of patients. However, patients' refractory to treatment, discontinued cyclosporine and replacing with tacrolimus might be appropriate as gingival hyperplasia is rarely observed in tacrolimus-treated patients [13].

Systemic administration of azithromycin was found to be more effective in reducing overgrowth than the local delivery preparation [42].

The role of metronidazole in drug-induced gingival overgrowth is being regularly studied. The reported results

vary. Wong, Hodge, Lewis [43] reported a total resolution of high grade cyclosporine A-induced gingival hyperplasia in each of their four patients.

Mesa, Osuna, Aneiros, Gonzalez-Jaranay, Bravo, Junco, Del Moral, O'Valle [44] conducted a double-blinded controlled randomised study image digital analysis to determine the effect of metronidazole or azithromycin on the regression of incipient cyclosporine A-induced gingival overgrowth in 40 adult renal transplanted patients. None of their patients with gingival overgrowth showed complete remission after 30 days.

However, Montebugnoli, Servidio, Prati [45] suggested that the repeated use of locally delivered metronidazole gel associated with periodontal conventional therapy as simply an additional procedure, probably providing a greater long- term efficacy in the overall management of gingival overgrowth induced by cyclosporine than conventional therapy alone. In another study, Aufricht, Hogan, Ettenger [46] observed no improvement in the gingival hyperplasia of their serially observed children after metronidazole treatment. The results differed with Wong, Hodge, Lewis, Shrpstone, Kingswood [43] for which they suggested that the variation might be due to differences in age of the patients.

However, studies on larger samples size are needed to confirm their role. The current studies have small sample size and were short term too. Further, statistical analysis is also required to decipher the significance of results. Azithromycin appeared to be more effective than metronidazole [47].

Mouth rinses

The role of antiseptic mouth rinses in the management of drug-induced gingival hyperplasia is not established. O'Neil and Figures [39] have shown that regular use of chlorhexidine mouth rinse helps reduce chances of recurrence after gingival surgery. We could not find out if any other mouthwash has been used for prophylactic or therapeutic purpose in such cases.

Non-surgical treatment

Wherever possible, non-surgical techniques should be adapted [48]. However, before rendering such treatment, local factors that enhance plaque accumulation should be eliminated. Such techniques can limit the occurrence of gingival overgrowth, reduce the plaque-induced gingival inflammation and also reduce the rate of its occurrence [49]. Professional debridement with scaling and root planning as needed had been shown to provide some relief in such cases [50]. A few individual case reports revealed that self administered oral hygiene procedures, scaling and root planning resulted in reduced drug-induced gingival over growth [51].

In extreme, medically-compromised patients in whom surgery bears more disadvantages rather than advantages, self-administered oral hygiene measures in addition to professional oral prophylaxis and root planning prove beneficial. They reduce gingival inflammation and may make surgical correction possible.

Surgical treatment

Surgical treatment is the definitive therapy of the drug-induced gingival overgrowth. However, the recurrence cannot be avoided. Since the labial gingiva of anterior region is commonly involved, surgery is frequently performed, primarily for aesthetic reasons, before any functional consequences are seen. Seymour *et al*. [52,53] suggested that surgical interventions should be carried out when clinical overgrowth index score is evident.

External bevel gingivectomy and internal bevel gingivectomy are two commonly practised approaches. External bevel gingivectomy is the simple excision of the excessive gingival tissue with secondary healing. Internal (reverse) bevel gingivectomy is often used in place of external bevel technique, where the tissue to be excised is thick, and a long bevel incision would be required to create a knife edge margin [54]. This highly technical approach has benefit of limiting the large denuded connective tissue wound that results from external gingivectomy, thereby minimizing the post operative pain and bleeding [55]. Both the techniques of gingivectomy are essentially limited to the treatment of pseudo-pockets. The common surgical technique is the simple excision of excessive gingival tissue with secondary healing. Peri-operative haemorrhage is main intra-operative complication or disadvantage of gingivectomy [56]. There are few studies on recurrence rates.

The apically repositioned full thickness flap is a better technique and bears many advantages. It eliminates periodontal pocket, improves the alveolar bone morphology, preserves the attached gingiva and improves aesthetics in the labial region when moderate periodontitis with gingival overgrowth is treated. This technique is specially indicated in the cases with lack of keratinized tissues or in the presence of osseous defect [57].

The role of periodontal flap surgery in drug-induced gingival overgrowth has not been established. Mavrogiannis,

Thomason, Seymour, Ellis [58] found no difference between the treatments with respect to gingival overgrowth. Further, flap surgery may be complicated by excessive gingival enlargement.

Lasers

There are many types of lasers available to treat gingival overgrowth. Lasers possess remarkable tissue-cutting ability and provide rapid haemostasis. Lasers with the cutting of tissues also generate a coagulated tissue layer along the surgical wound which promotes healing [59]. These properties of lasers make the management of drug-induced gingival overgrowth accurate, bloodless operative phase, less painful post-operative phase, minimal oedema and scarring [60] and avoid need of periodontal dressing. Thus CO2 laser had been used to excise gingival over growth and studies demonstrated the technique significantly lowered the rate of recurrence and patients' preference of the technique [61]. Although the technique bears all the desired benefits, it involves costly equipment.

Conclusion

Gingival enlargement is a clinical condition which has been widely studied and is directly associated with specific local or systemic conditions. It is very rare that gingival enlargement presents without a clear underlying aetiology. Recently, McLeod *et al.* reported gingival enlargement in post-partum phase, treated with non-surgical and surgical periodontal therapy [62].

We have covered almost all the aspects of drug-induced gingival growth presented in literature. Clinicians would find the paper helpful in deciding the treatment strategy in drug induced gingival overgrowth. It should always be kept in mind that when drugs which cause gingival enlargement are started, the patient should be evaluated for gingivitis, educated on dental and oral hygiene, motivated to brush thoroughly, use dental floss regularly and advised for regular dental check-ups. On the other hand, physicians should also visually examine oral cavity on every visit of the patient and if any changes are noticed, consider therapy and the required dental treatment. Whatever strategy is decided, focus should be on the prevention of growth and recurrence.

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Correspondence:
Syed Sayeed Ahmed,
Department of Oral and Maxillofacial Surgery,
Dr. Ziauddin Ahmed Dental College,
Aligarh Muslim University, Aligarh-202 002, India.
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