Original Article

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HISTOLOGICAL EFFECTS OF TOCOTRIENOL ON INTIMA THICKNESS IN AORTA OF DIABETIC MICE

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ABSTRACT

Objective: To compare the thickness of intima in aorta of diabetic mice being fed on normal laboratory diet, high fat diet and high fat diet with tocotrienol.

Study Design: Experimental study

Place and Duration of Study: Army Medical College, Rawalpindi and National Institute of Health, Islamabad from November 2009 to June 2010.

Material and Methods: Forty five female BALB/c mice were randomly divided into three groups. All the animals were made diabetic by intraperitoneal injection of streptozotocin (STZ) 40 mg/kg body weight. Group I was given normal laboratory diet, group II high fat diet and group III was given tocotrienol along with high fat diet for 32 weeks. At the end of experiment the mice were sacrificed. The hearts of animals were dissected out and ascending aortae were removed. The specimen was fixed in 10 % formol calcium and processed for paraffin embedding. Five micrometer thick sections were made. Haematoxylin & eosin and verhoeff staining was done. Thickness of intima and intracellular lipid depositions were noted.

Results: In contrast to group I, the intima thickness increased in groups II and III. Statistically significant increase in the thickness of intima was found in the aortae of diabetic animals in group II (high fat diet), when compared with group I (laboratory diet). The thickness of intima increased significantly in group III when compared with group I. When group II (high fat diet) and III (high fat diet + tocotrienol) were compared, a significant decrease in intima thickness was noted in group III.

Conclusion: In diabetics who consume high fat diet, there is a definite increase in the thickness of intima in aorta which can be prevented by giving tocotrienol.

Keywords: Aorta, Diabetes, Tocotrienol

INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disorder. There are 170 million cases worldwide, and this figure is expected to be more than double by 2030[1]. Diabetes is associated with major mortality and morbidity from late vascular complications, both microvascular (retinopathy, nephropathy, neuropathy) and macrovascular accelerated atherosclerosis (ATS)[2].

Atherosclerotic vascular disease is more prevalent in individuals with diabetes[3]. Atherosclerosis is accelerated by diabetes which increases the risk of cardiovascular disease[4]. Cardiovascular diseases secondary to atherosclerosis are a common cause of mortality and morbidity among subjects with type 1 diabetes mellitus[5]. In postmortem studies, atherosclerotic lesions from patients with type 1 or type 2 diabetes were found to have larger necrotic cores than lesions from non diabetic subjects[6].

Highlighting the factors that promote cardiovascular disease in diabetes is critical for the development of new therapeutic approaches. The prevalence of hyperglycemia, hypertension and dyslipidemia in association with systemic inflammation and oxidative stress accelerates the formation and propagation of atherosclerotic plaque[7]. Elevated levels of glucose are related to most of the complications secondary to diabetes[8]. The role of glucose in these complications is not completely understood and probably has a multifactorial origin. Although, an increase in oxidative stress seems to play a relevant role,

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since some of these complications including congenital malformations, neuropathy and retinopathy, can be prevented or ameliorated with the administration of antioxidants[9]. Therefore, the damage mediated by glucose could be secondary to the generation of free radicals.

Vitamin E is a mixture of tocopherols and tocotrienols (alpha, beta, gamma and delta tocopherol and alpha, beta, gamma and delta tocotrienol) synthesized only by plants[10]. Tocotrienols have potent cholesterol lowering[11], antioxidant[12] and anti-inflammatory properties[13]. A study carried out in mice showed that tocotrienols substantially reduced the growth of atheromatous plaques[14]. Vitamin E acts as a chain-breaking antioxidant for LDL lipids.

BALB/c is an albino, laboratory-bred strain that develops fatty streak lesions when hyperglycemia is also present[5]. Thus hyperglycemic BALB/c mouse provides a convenient model to identify genetic, dietary and diabetogenic factors contributing to accelerated fatty streak lesions of complex cellularity[15]. This study was planned to investigate the histological changes in intima in aorta of diabetic mice and their response to tocotrienol, a potent antioxidant.

MATERIAL AND METHODS

The study was conducted at Anatomy Department, Army Medical College Rawalpindi in collaboration with National Institute of Health (NIH), Islamabad from November 2009 to June 2010. Forty five, female BALB/c mice, six to eight weeks of age and weighing 20-30 grams were selected from the animal house of NIH. They were kept at standard temperature 21±2°C in a room maintained on 12 hour light/dark cycle. They had free access to standard NIH laboratory diet, high fat diet and water for 32 weeks. In this study only healthy, active and non diabetic animals were included. All animals were made diabetic by intraperitoneal injection of streptozotocin (STZ) 40 mg/kg body weight, diluted in 0.05 mmol/L citrate buffer (pH 4.5), given daily for five consecutive days during first week of experiment. The plasma glucose level was measured; at the start of experiment, 48 hours after administration of injection STZ and after seven weeks to ascertain diabetic status of the animals using Glucometer[16]. Injection STZ was repeated in the mice having plasma glucose level below 200 mg/dl.

The animals were randomly divided into three groups labeled as group I (control) and group II (experimental) and group III (experimental). Group I was given standard laboratory diet, group II high fat diet comprising 15% butter, 1.25% cholesterol powder and 0.5% sodium cholate and in group III the high fat diet was enriched with tocotrienol 6 mg/kg body weight. Annato seedsderived tocotrienols (KABCO, USA) were obtained in the form of oil packed in capsules, each having 125 mg tocotrienols (mixture of 90% delta and 10% gamma tocotrienol). The concentration of tocotrienols was obtained by diluting the contents of one capsule in 5ml of olive oil and mixing 0.5 ml of oil in the diet of every mouse.

The animals were euthanized at the end of 32 weeks by giving ether by inhalation[17]. They were dissected by a longitudinal incision in midline extending from upper end of sternum to symphysis pubis. The abdominal viscera were retracted to one side. The ribcage was opened and lungs dissected out. Heart and upper section of aorta were removed. Peri-vascular fat was removed under a dissecting microscope. Upper section of aorta (from aortic valve to right carotid artery) was then taken out from the sample.

The specimen were put in numbered glass jars containing 10% formol calcium for fixation[18]. The ascending aorta was further processed for paraffin embedding. Five µm thick cross sections were cut transversely by rotary microtome. Hematoxylin and eosin, and verhoeff's elastic stains were used. Two consecutive sections of each stain showing maximum atherosclerotic changes were selected and studied for intima thickness using micrometer. For this morphometeric analysis, each slide was observed under 10 X objective and three points of maximum intimal

thickening were selected. Thickness of intima was measured at 400x magnification from endothelial cell surface to internal elastic lamina, with the help of ocular micrometer which was calibrated against stage micrometer. The mean of these three values was then taken[19].

The data was analyzed using statistical package for social sciences (SPSS) version 16. The results were expressed as Mean±SD. For comparison between blood glucose and intima thickness two way ANOVA (analysis of variance) was used. Group differences for categorical variable are expressed as percentages, while for comparison of numeric variables, Tukey's post hoc test was used. All the results were considered statistically significant at a *p* value equal to or less than 0.05.

RESULTS

Biochemical Analysis: The baseline blood glucose (mmol/l) was taken at first day of the experiment, 72 hours after injection STZ and finally after 32 weeks. The mean of plasma glucose (mean ± SD) at day 0 was 11.59 ± 0.433, 11.53 ± 0.380 and 11.36 ± 0.499 in groups I, II and III respectively. The mean plasma glucose (mean ± SD) at 72 hours after injection STZ was 17.77 ± 0.920, 16.7 ± 0.672 and 16.79 ± 1.038 in groups I, II and III respectively. The mean plasma glucose (mean ± SD) 32 weeks after the start of experiment was 21.04 ± 1.093, 19.24 ± 0.956 and 19.11 ± 2.451 in groups I, II and III respectively (Table.1). A statistically significant ($P<0.05$) increase in plasma glucose was noted when comparison was made among group I and II, in their mean glucose levels after 72 hours of injection STZ and after 32 weeks. Among groups II and III, the comparison of mean of plasma glucose at 72 hours after injection STZ and after 32 weeks revealed statistically insignificant difference ($P > 0.05$).

Gross Study: On gross examination, the aortae of animals in group I were without any notable peri-vascular fat deposition while most of the animals in group II were completely surrounded by fat. Severe fat deposition was found in 93.3% of group II animals and moderate in 6.7%. Group III had 53.3% cases with mild peri-vascular fat and 46.7% with moderate fat deposition (Table 2).

Qualitative findings: In group I, light microscopy on H & E slides showed a single continuous layer of flat, elongated basophilic nuclei of endothelial cells in intima. The endothelial cells were supported by a very thin layer of loose connective tissue (Fig. 1). Tunica media exhibited numerous pink, wavy and concentrically arranged elastic lamellae. Interposed between the lamellae were elongated basophilic nuclei of smooth muscle cells. In verhoeff stained slides, the elastic lamellae appeared black and clearly distinguished. In tunica adventitia few elastic fibers were present in the form of loose network, unlike the lamellar arrangement in media.

In group II, light microscopic examination of sections showed extensive thickening of intima, disorganization and intracellular lipid deposition in media (Fig.2). The endothelial cells were rounded at many places, lacking the normal squamous pattern. Intima was rich in large vacuolated cells having central round basophilic nucleus, with abundant foamy cytoplasm in H & E stained sections (Fig.3).

In groups III the atherosclerotic changes were less marked with few foam cells and droplets of extracellular lipids.

Quantitative findings: Mean ± SD thickness of intima (µm) in group I, II and III was 3.1±0.4, 52.1±8.1, and 40.9±1.8 respectively (Table 3). Difference of mean between group I and II as well as among groups I and III was statistically significant ($p <0.05$). The difference of means between group II and III was also statistically significant ($p <0.05$) (Table 4).

DISCUSSION

A lot of literature is available on tocopherols, but little is known about tocotrienols. There is some evidence, however, that tocotrienols may be superior in their biological properties, and that its anti-inflammatory and antioxidant activities could prevent diabetes and cardiovascular diseases[20].

Kunjathoor et al. developed the first diabetic atherosclerosis murine model using

Table.1: Showing blood glucose (mmol/l) of mice in groups 1,II and III, at day '0', 72 hours after injection streptozotocin (STZ) and after 32 weeks

Subjects	Blood Sugar (mmol/l)								
	Control Group I Diabetic on standard diet			Experimental Group II Diabetic on athrogenic diet			Experimental Group III Diabetic on athrogenic diet with Tocotrenol (6mg/kg/day)		
	Day 0	After 72 Hrs	After 32 Wks	Day 0	After 72 Hrs	After 32 Wks	Day 0	After 72 Hrs	After 32 Wks
Mean	11.67	17.74	21.08	11.53	16.73	19.3	11.41	16.82	19.39
Std. Dev	0.392	0.899	1.064	0.379	0.658	0.890	0.536	1.043	2.082

Table.2: Percentage (%) scoring of fat around aorta in groups I, II and III

Groups	Percentage (%) scoring of fat around aorta			
	Negligible	Mild	Moderate	Severe
Group I (Control)	100%	0%	0%	0%
Group II (Experimental)	0%	0%	6.7%	93.3%
Group III (Experimental)	0%	53.3%	46.7%	0%

Table.3: Showing mean ± SD thickness of intima (µm) in groups I, II and III

Groups	Subject	Thickness of Intima (µm)
I	Mean	3.1±0.4
II	Mean	52.1±8.1
III	Mean	40.9±1.88

Table.4: Anova followed by post hoc tukey's test

Parameter	Group I (Control) Group II (Experimental)	Group I (Control) Group III (Experimental)	Group II (Experimental) Group II (Experimental)	Level of Significance
Intima Thickness	0.000	0.000	0.000	P<0.05

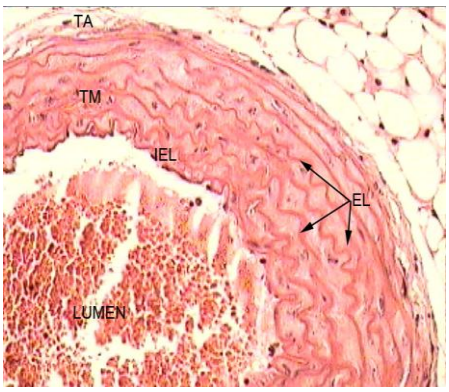

Fig.1 Photomicrograph of a section from ascending aorta of animal in group I showing internal elastic lamina (IEL), elastic lamina (EL) in tunica media. H&E stain. Approx X400.

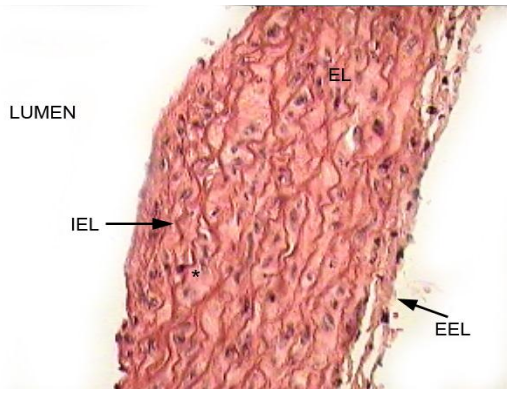

Fig.2 Photomicrograph of a section from ascending aorta of animal in group 11, showing internal elastic lamina (IEL), elastic lamina (EL) and external elastic lamina (EEL).Intracellular lipid deposition (*) is also seen. Verhoeff stain Approx. X400.

streptozotocin[21]. In this study, BALB/c mice were treated with STZ to destroy pancreatic ß-cells, which caused insulin deficiency, hyperglycemia (diabetes), and atherosclerosis. BALB/c mice develop atherosclerosis which can be accelerated on a high cholesterol diet;

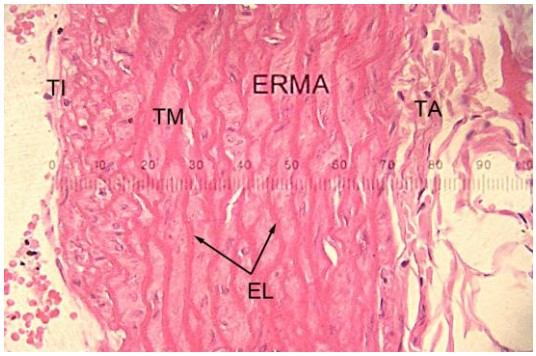

Fig.3 Photomicrograph of a section from aorta of mice ib group II, showing measurement of tunica intima (TI) by micrometer. Tunica adventitia (TA) and elastic lamina (EL) are also visible. H&E stain. Approx. X800.

however, the lesions in mice occur at sites different from human lesions, for example, the aortic root and thoracic aorta. They do not exhibit the single most important event in human atherosclerosis, that of plaque rupture leading to vessel occlusion.

Tocotrienols exhibit antioxidant activities, and most of their effects can be linked to antioxidant function. The antioxidant activities of tocotrienols are mediated through induction of antioxidant enzymes such as superoxide dismutase[22], NADPH quinone oxidoreductase[23], and glutathione peroxidase[24], which quench free radicals such as superoxide radicals[25]. Tocotrienols are known to suppress the activity the endoplasmic reticulum enzyme, HMG-CoA reductase through a post transcriptional mechanism involving accelerated degradation of enzyme. This hepatic enzyme produces mevalonate which is converted to sterols and other products responsible for cholesterol synthesis[26]. Thus, the results of our study can be contributed to the hypocholesterolemic properties of tocotrienols. Paola et al investigated antioxidant efficiency of different tocotrienols isoforms by evaluating their ability to inhibit lipid peroxidiation and reactive oxygen species (ROS) production linking the results of our study to oxidative stress in diabetes[27].

The safe dose of tocotrienols for human consumption (200-1000 mg/day) has been used by researchers earlier[28]. Husain et al used tocotrienol in dose of 100mg/kg [29].

There was an insignificant decrease in blood glucose of diabetic mice fed on athrogenic diet and given tocotrienols in addition to athrogenic diet in our study, which was in correspondence with Otero et al where the administration of either the atherogenic diet or vitamin E had no effect on plasma levels of glucose in the diabetic BALB/c mice[5].

Histomorphologic examination of verhoeff stained slides in group I demonstrated a single continuous layer of flat, elongated basophilic nuclei of endothelial cells in intima. The endothelial cells were supported by a very thin layer of loose connective tissue. The intimal thickness increase in mice on high fat diet, with disorganization and discontinuous epithelium was also observed by Mohammadi et al[30].Wagenknecht et al supported this in their study when they observed an increased rate of progression of carotid atherosclerosis in persons with diabetes[31].

Thickening of intima is the hallmark of atherosclerosis[15]. We measured thickness of intima in verhoeff stained slides on account of clear demonstration of internal elastic lamina which was difficult to appreciate in H&E stained slides[32]. Intimal thickening was present in both the experimental groups, however the prevention of initmal thickening occurred in experimental group III by tocotrienol, 6 mg/kg. These findings are consistent with morphometeric analysis of Nafeeza et al in experimentally induced atherosclerosis in the aorta of rabbits through high cholesterol diet which showed that tocotrienol rich fraction had significant potential ($p < 0.05$) to minimize the progression in intimal thickening, although not completely preventing it[33].

Overall, the results of this study allow us to suggest that subjects with diabetes might need tocotrienols to reduce the development and progression of atherosclerosis.

CONCLUSION

In this study carried on diabetic mice, comparison of control group on regular diet with other experimental groups on high fat diet revealed increased deposition of fat around aorta on gross study in experimental groups.

On microscopic examination, intimal thickening with intracellular and extracellular lipid deposition, smooth muscle proliferations and endothelial cell disruptions was observed in experimental groups. Quantitative analysis showed statistically increased thickness of intima in experimental groups. When effect of tocotrienols, a powerful antioxidant, on histomorphologic changes in aorta of diabetic mice was evaluated, it was found that tocotrienols reduce the intimal thickening.

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