EFFECT OF TOCOTRIENOL ON AORTIC ATHEROSCLEROSIS IN DIABETIC MICE

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ABSTRACT

Objective: To study the histomorphological effect of tocotrienol on aortic atherosclerosis in diabetic mice having high fat diet.

Study Design: Lab based randomized controlled trial.

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. The diabetic mice model was established by intraperitoneal injection of streptozotocin (STZ) 40 mg/kg body weight. Group A was given normal laboratory diet, group B high fat diet and group C 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 taken out. The specimen was fixed in 10% formol calcium and processed for paraffin embedding. Five micrometer thick sections were made for haematoxylin and eosin, and Verhoeff's staining. After staining, histomorphologic changes in slides were noted.

Results: In contrast to group A, atheroscelrosis developed in groups B and C. Statistically significant atherosclerotic changes were found in the aortae of diabetic mice in group B when compared to group A. On comparison of group A to C, atherosclerotic changes were statistically insignificant. However when group B was compared with group C, the aortic atherosclerotic changes decreased significantly in group C.

Conclusion: In diabetics with high fat diet intake, there is an increase in development of atherosclerosis in aorta which can be reduced by tocotrienol.

Keywords: Aortic atherosclerosis, Diabetes, Tocotrienol.

INTRODUCTION

Atherosclerosis is a complex chronic disease characterized by the accumulation of lipids within arterial walls that eventually goes on to form plaques that cause narrowing, hardening and complete blockage of arteries. One well known risk factor in humans is hypercholesterolemia (i.e. elevated total cholesterol and low-density lipoprotein cholesterol[1], and other important contributors to this disease include inflammation, oxidative stress, and insulin resistance[2,3].

Endothelial dysfunction is induced by a variety of atherogenic stimuli which promote the adhesion and transendothelial migration of blood circulating leukocytes that accumulate within the subendothelial space to form so-called fatty streak, an early atheromatous lesion which contains mostly highly proliferative macrophages. These macrophages take up the lipoproteins and become lipid-laden foam cells[4]. Smooth muscle cells are primarily located in the arterial tunica media in a non-proliferative state during homeostasis. However, activated leukocytes in growing atheromas produce inflammatory chemokines and cytokines that promote smooth muscle cell proliferation and migration from the tunica media towards the intimal lesion, thus contributing to plaque development[5]. In addition to cellular components, atheromatous lesions contain cholesterol and other fatty materials, and increased content of specific extracellular matrix components[6].

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It is a well-established fact that diabetes is a risk factor for cardiovascular disease[7]. There is growing evidence that excess generation of highly reactive free radicals, largely due to hyperglycemia, cause oxidative stress, which further exacerbates the development and progression of diabetes and its complications. Overproduction and insufficient removal of these free radicals results in vascular dysfunction, damage to cellular proteins, membrane lipids and nucleic acids. Oxidative stress is defined in general as excess formation and/or insufficient removal of highly reactive molecules such as reactive oxygen species and reactive nitrogen species[8].

Natural 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[9]. Recent studies have demonstrated that tocotrienols have potent cholesterol lowering[10] antioxidant[11] and anti-inflammatory properties[12]. Study carried out in mice showed that tocotrienols substantially reduced the growth of atheromatous plaques[13]. Vitamin E acts as a chain-breaking antioxidant for low density lipoprotein lipids.

This study will help us to evaluate the histomorphological response of atherosclerosis to tocotrienols in diabetes and thus it will contribute in better management of atherosclerosis in diabetic patients.

MATERIAL AND METHODS

This lab based randomized controlled trial was conducted at Anatomy Department, Army Medical College Rawalpindi in collaboration with the National Institute of Health (NIH), Islamabad from November 2009 to June 2010. Forty five, female BALB/c mice, six to eight weeks of age, 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. The mice had free access to standard NIH laboratory diet, high fat diet and water *ad libitum* 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 32 weeks to ascertain diabetic status of the animals by using glucometer[14]. 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 A (control), group B (experimental) and group C (experimental). Group A was given standard laboratory diet, group B high fat diet comprising 15% butter, 1.25% cholesterol powder and 0.5% sodium cholate and in group C the high fat diet was enriched with tocotrienol 6 mg/kg body weight. The animals were euthanized at the end of 32 weeks by giving ether inhalation. They were dissected by a longitudinal incision made 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 was 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 was put in numbered glass jars containing 10% formol calcium for fixation. The ascending aorta was further processed for paraffin embedding. Five micro-meter thick sections were made by transversely cutting the sample using rotary microtome. Staining with hematoxylin and eosin, (H & E) and Verhoeff's elastic stains, was done.

Each slide was observed at 10 X and at 40 X. Two consecutive sections of each stain showing

maximum atherosclerotic changes were selected. In H&E stained slides, intima was evaluated for lipid accumulation, its site and pattern of deposition. Histological changes in arterial wall were graded according to criteria modified from American Heart Association (AHA) classification of atheromatous lesions[15].

The data was analyzed using statistical package for social sciences (SPSS) version 16. Descriptive statistics were used to describe the results. AHA classification between the three groups was compared using chi-square test. A *p*-value < 0.05 was considered as significant.

RESULTS

In group A, on light microscopy, there was a continuous single layer of elongated, flat basophilic nuclei in endothelium in H & E stained slides. The endothelial cells were supported by a thin layer of loose connective tissue. Tunica media exhibited numerous pink, wavy and concentrically arranged elastic lamellae. Elongated basophilic nuclei of smooth muscle cells were interposed between these lamellae. Tunica media was separated from intima by internal elastic lamella and it was separated from tunica adventitia by external elastic lamella. In Verhoeff's stained slides, the elastic lamellae were clearly distinguishable and appeared black. In tunica adventitia some elastic fibers were present in the form of loose network in contrast to the lamellar arrangement in media. The scoring criteria of atherosclerosis modified from American Heart Association (AHA) showed 100% of animals with AHA-0.

In group B, light microscopy revealed extensive thickening of intima and disorganization with large gaps between endothelial lining and internal elastic lamina. Normal squamous pattern of endothelial cells was not present and the endothelial cells were rounded at these places. The intima was rich in large vacuolated cells having central round basophilic nucleus, with abundant foamy cytoplasm in H & E stained sections. Extracellular lipids were also visible in deeper layers in addition to intracellular lipid deposition seen in foam cells present mostly towards the lumen. The internal elastic lamina was fragmented at many locations and appeared in more than one layers. In severely affected mice, vacuolated lipid laden cells were also seen in the outer layer of media. The AHA scoring showed no animal with AHA-0, 53.33% with AHA-I, 26.7% with AHA-II and 20% with AHA-III.

In group C, although atherosclerotic changes were present but foam cells and droplets of extracellular lipids decreased. The AHA scoring showed 66.7% with AHA-0, 6.7% with AHA-I, 13.33% with AHA-II and 13.33% with AHA-III.

In contrast to the mice of group A, aortic atheroscelrosis developed in groups B and C. Statistically significant ($p = 0.01$) atherosclerotic changes were found in the aortae of diabetic mice in group B when compared to group A. On comparison of group A to C, atherosclerotic changes were statistically insignificant ($p = 0.8$). However when group B was compared with group C, the aortic atherosclerosis decreased significantly ($p = 0.04$) in group C.

DISCUSSION

In diabetes blood glucose level remains persistently high because of insufficient insulin production or insulin resistance. Studies have been carried out showing that tocotrienols have antidiabetic potential[15].

In present study, BALB/c mice were selected as animal model which have been used earlier as an experimental model of atherosclerosis secondary to both hypercholesterolemia and hyperglycemia, where the administration of vitamin E decreased both the fatty deposits and the accumulation of macrophages in arterial wall[16]. The feeding period and diet for generation of atherosclerotic lesions in diabetes[17] as well as the safe dose of tocotrienols were standardized with earlier studies[18]. Studies conducted on isolated vascular

cells suggest that elevated glucose levels cause a plethora of pro-atherogenic responses[19].

In this study, there was disorganization and discontinuation of endothelium in aorta alongwith an increase in thickness of intima in aorta of diabetic mice fed on high fat diet with which was also observed by Mohammadi et al. in their study[20]. This was supported by Wagenknecht et al. who observed an increased rate of progression of carotid atherosclerosis in persons with diabetes mellitus[21]. The continuous smooth surface of intima with few defects and squamous pattern of endothelial cells in tocotrienol treated experimental groups was also observed by Budin et al. where histomorphological effects of palm oil tocotrienol-rich fraction on thoracic aorta in diabetic rats produced similar findings. In their study streptozotocin induced diabetes caused severe alterations in the structure of the vascular wall in diabetic mice fed on high fat diet alone[22] which is also supported by our results. In present study, the tunica intima of the aorta was thickened and the endothelial cells were rounded at many places showing atrophic characteristics, with infiltrating mononuclear cells. Similar changes were appreciated by Budin et al. in their study where oral administration of tocotrienol-rich fractions (200 mg/kg body weight) given daily for eight weeks to streptozotocin induced diabetic rats, on electron microscopy, showed disruption in normal morphology of thoracic aorta[22].

According to American Heart Association, foam cell formation is a widely recognized phenomenon for the early lesion detection of atherosclerosis[23]. This supported the results of our study where foam cells appeared in intima of all the mice who counsumed high fat diet. A high percentage of foam cells, found in the aorta of diabetic mice fed only on high fat diet in this study was supported by study of Zulkhairi et al[23]. Foam cells are large vacuolated cells having central round basophilic nuclei, with abundant foamy cytoplasm and their number decreased in group C which was given tocotrienol in addition to high fat diet. This was in accordance with the study of Black et al. who demonstrated 98 % reduction in the size of atheromatous lesions through palm tocotrienol supplementation[24]. In aorta of animals of group B, H & E stained slides displayed that in addition to intracellular lipid accumulation in foam cells present mostly in intima, pools of extra cellular lipids were also visible in deeper layers. Kunjathoor et al. also found that in BALB/c mice oil red O staining of fatty streaks showed intra and extra cellular lipids thus confirming findings of present study[18].

Tocotrienol treatment of diabetic mice reduced vascular smooth muscle cells proliferation and disruptions of elastic lamella in media which was also supported by Budin et al. in their study carried out to evaluate the effects of palm oil tocotrienol-rich fraction supplementation on biochemical parameters, oxidative stress and the vascular wall of streptozotocin-induced diabetic rats[22].

CONCLUSION

It is concluded that tocotrienol reduces the atherosclerotic plaque formation in aorta of diabetic mice fed on high fat diet. So Tocotrienol can be given as a regular supplement to those diabetics who have a high risk of developing atherosclerosis and its complications.

Conflict of Interest

This study has no conflict of interest to declare by any author.

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