TOCOTRIENOLS;
EFFECTS ON INTIMAL THICKENING IN ASCENDING AORTA OF CHOLESTEROL-FED RABBITS

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ABSTRACT... Objective: To evaluate the effect of tocotrienols on intimal thickening in ascending aorta of cholesterol-fed rabbits. **Study Design:** Randomized control trial. **Place and duration of study:** The Anatomy department of Army Medical College, Rawalpindi, from March 2009 to February 2010. **Material and Methods:** Thirty, male, New Zealand white rabbits were randomly divided into three equal groups. Group-I was fed normal lab diet for six weeks. For the similar period, group-II & III were given 2% high cholesterol diet. However, group-III diet was also supplemented with tocotrienols (6 mg/kg body weight/day). By the end of study, aorta was removed from each animal. Cross sections from ascending aorta were processed and embedded in paraffin. Light microscopic examination was performed in H & E and Verhoeff elastic stained slides. **Results:** Tunica intima in group-I appeared as single layer of squamous endothelial cells, lying on a thin layer of loose connective tissue. High cholesterol diet in group-II induced marked atherosclerotic changes which were characterized by extensive intimal thickening with raised fatty streaks, pools of extracellular lipids, proliferation of smooth muscles and deposition of connective tissue matrix. Intimal thickening was also observed in group-III, but lesions were of lesser degree than group-II ($P<0.05$). Histomorphometric analysis revealed significantly ($P<0.001$) higher thickness of intima in group-II and in group-III when either was compared with group-I. However, thickness of intima was 35% lesser ($P<0.05$) in group-III than group-II. **Conclusions:** Tocotrienols has significant potential in suppressing the intimal thickening of aorta in cholesterol-fed rabbits.

Key words: Tocotrienols, intimal thickening, rabbits

INTRODUCTION

Atherosclerosis is the leading cause of morbidity and mortality in developed as well as in developing countries. Intimal thickening characterized by lipid deposition, proliferation of smooth muscle cells and extracellular connective tissue elements; is the hallmark of atherosclerotic lesions[1]. High blood cholesterol levels are considered to be an obligatory and sufficient factor for the development of this enormous health problem[2].

Nutritional recommendations for the prevention of atherosclerosis aim at increasing the dietary antioxidants[3]. Vitamin E comprises tocopherols and tocotrienols, each with four analogues: alpha (α), beta (β), gamma (γ) and delta (δ). About 95% of vitamin E studies address α-tocopherol while its other isomers remain unexplored. Even though, some emerging reports have shown that tocotrienols exhibited more potent hypolipidemic and antioxidant properties than tocopherols and δ-tocotrienol was found to be the most potent isoform[4]. The evidence offered by the fore-mentioned literature promotes the possibility that tocotrienols may thus suppress the intimal thickening in aorta of cholesterol-fed rabbits.

MATERIAL AND METHODS

The present study was carried out in the department of Anatomy, Army Medical College, Rawalpindi (from March 2009 to February 2010), in cooperation with National Institute of Health (NIH), Islamabad. All procedures were approved by the institutional animal ethical committee. Thirty male New Zealand white rabbits, weighing 1.5 to 2.5 kg were obtained from NIH. Animals were housed individually in wire-bottomed cages. Each rabbit was given 100 g/day powdered regular lab diet (NIH). Water was accessible ad libitum. Rabbits were acclimatized to experimental environment for one week and then randomly divided into three study groups (n=10/group). Group-I continued the regular rabbit diet for next six weeks. For the similar period, group-II & III were fed 2% high cholesterol diet [2g cholesterol powder (Applichem, Germany) mixed with

100g regular lab diet /head/day], however, group-III diet was also supplemented with annatto tocotrienols [mixture of 90% δ- and 10% γ-tocotrienols (Kabco, USA)] in a dose of 6 mg/kg body weight/day.

By the end of six weeks, all animals were euthanized with ether anesthesia. Aorta together with heart was removed from each rabbit and placed in 10% formol calcium. Following 48 hours of fixation, ascending aorta was divided into three (approximately 3 mm wide) aortic rings. These aortic rings from each aorta were further processed in ascending series of alcohol, cleared in xyelene, infiltrated and embedded in paraffin wax. Five µm thick cross sections were taken using rotary microtome (LEICA-RM-2255, Germany). Hematoxylin and Eosin (H & E) staining was done for histomorphological study of aorta. Verhoeff's elastic stain was used for histomorphometric analysis. At a magnification X200, atherosclerotic changes in arterial wall were graded according to American Heart Association (AHA) classification of atherosclerotic lesions (Table-I)[5].

Table-I. Classification for atherosclerotic lesions

No lesions	**Type-0: No intimal thickening**
Early lesions	Type-**I**: Foam cells Type-**II**: Fatty streak Type-**III**: Raised fatty streak, small and sparse pools of extracellular lipid
Advanced lesions	Type-**IV**: Large pool of extracellular lipid covered by a proteoglycan rich layer infiltrated with foam cells & smooth muscle cells with & without lipid droplets Type-**V**: Calcifications, fibrous plaque without lipid deposits Type-**VI**: Endothelial surface defect/ complicated lesion

Following scoring criteria was used: No lesions = 0, Early atherosclerotic lesions = +, Advanced atherosclerotic lesions = ++. For each section, under light microscope (Olympus CX21 FS1), at a magnification X400, thicknesses of tunica intima (from endothelial margin to the internal elastic lamina) was measured with the help of a calibrated ocular micrometer. Measurements were made at the point of smallest minimal luminal diameter[6,7].

Statistical analysis: Data were entered in a database using SPSS soft ware, version 16. All the results were expressed as means ± SE (Standard error of mean). Categoric data among the three groups were compared by chi-square test while numeric data were analyzed by one way analysis of variance (ANOVA) followed by Tukey's post-hoc test. All the results were considered significant at a P-value < 0.05.

RESULTS

Light microscopic examination of group-I showed intima as a single continuous layer of squamous endothelial cells, lying on a thin layer of loose connective tissue (Fig. I-a). Internal elastic lamina was closely applied to the endothelium. High cholesterol diet in group-II induced marked intimal thickening and disorganization with large gaps between a discontinuous endothelial lining and internal elastic lamina. Intimal thickening was characterized by raised fatty streaks, pools of extracellular lipids in the deeper layers, enhanced nuclear densities, proliferation of smooth muscles, calcifications and deposition of connective tissue matrix (Fig. I-b, I-c). In group-III, intimal thickening was observed but lesions were of lesser degree than group-II (Fig. I-d). Percentage of early and advanced atherosclerotic changes, observed in each group, is given in Table-II.

Table-II. Percentage of atherosclerotic changes in ascending aorta of each group

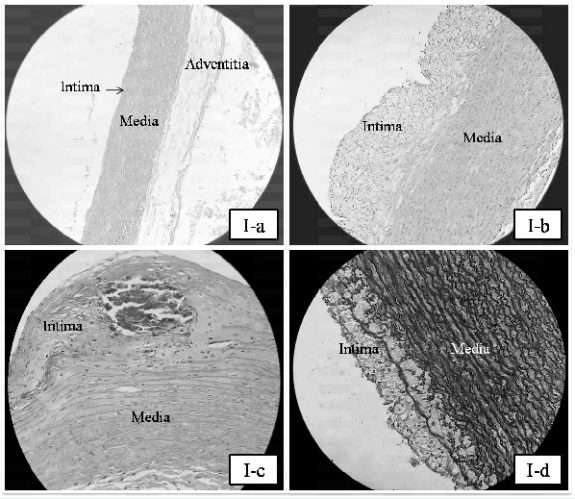
Groups	Atherosclerotic changes			P-value among the three groups
	None	Early	Advanced	
I	100%	-	-	
II	-	40%	60%	0.000
III	-	90%	10%	

Histomorphometric analysis revealed significantly (P<0.001) higher thickness of intima in group-II and in group-III when either was compared with group-I. However, thickness of intima was 35% lesser (P = 0.03) in group-III in contrast to group-II (Table III).

Table-III. Comparison of intimal thickening (mean ± SE) among the three groups of rabbits

	Group-I (n=10)	Group-II (n=10)	Group-III (n=10)
Thickness of intima (µm)	9.25±0.75	221.00±29.89	143.50±19.99
P-value	Group-I versus Group-II = 0.000, Group-I versus Group-III = 0.000, Group-II versus Group-III = 0.03,		

Figure-1. Aortic cross sections of rabbits fed normal diet (I-a) or 2% high cholesterol diet (I-b, I-c) or 2% high cholesterol diet with tocotrienols (I-d) for 6 weeks.



H&E stain: I-a,I-b, I.c; Verhoeff stain = I-d; Magnification = X200: I-a, I-b; X400: I-c, I-d.

DISCUSSION

Anitschkow established the cholesterol-fed NZW rabbit as a model for atherosclerosis research showing that high cholesterol diet in rabbits was enough to create atherosclerotic lesions quite similar to and with the association of same molecular mechanisms as in humans[8].

Suseeptibility of different parts of rabbit aorta, for the development of atherosclerosis through high cholesterol diet, has already been compared in out previous study[9].

Ascending aorta was analyzed as this aortic part in New Zealand White rabbits is exceptionally prone to develop advanced atherosclerotic lesions while lesions in descending thoracic and abdominal aorta develops only in the form of foam cells or fatty streaks[9]. Lin and colleagues stated that descending thoracic and abdominal aorta in New Zealand White rabbits had relative athero-protective properties like lesser permeability for monocytes, higher laminar shear stress, and greater mRNA expression of glutathione peroxides[10]. Thickness of intima was measured in verhoeff elastic stained slides as internal elastic lamina was clearly appreciated in this stain[11].

The present study uncovered the effect of annatto-tocotrienols on aortic intimal thickening. Formerly, tocotrienols either in the form of rice bran oil or palm oil were used. Moreover, their anti-atherogenic effects were reported with conflicting results. Worthy to note, rice bran oil comprises 50% tocopherols in addition to 50% tocotrienols. Crude palm oil consists of 70% tocotrienols and 25% tocopherols4. Annatto (Bixa orellana L.) seeds extract is the only source containing 100% tocotrienols (free of tocopherols)[12]. Nafeeza and colleagues induced aortic atherosclerosis in rabbits by feeding high cholesterol diet and found a substantial decline in intimal thickening with palm oil[13]. Qureshi et al[14] highlighted the atheroprotective properties of novel tocotrienols of rice bran by substantial reduction in growth of atheromatous plaque in three genotypes of mice. Their study also suggested that dietary intervention of tocotrienols was more important before the formation of plaque or in the initial stages of their development. Contrarily, no beneficial effects on atheroma formation in six rabbits given palm tocotrienols plus 2% cholesterol were also viewed[15]. Vitamin E preparations containing 100% tocotrienols especially desmethyl tocotrienols (δ- and γ-) have significantly greater antioxidant and anti-inflammatory properties as compared to fully methylated rest of its isoforms[4].

Protective role of tocotrienols against intimal thickening can be ascribed to its known biochemical properties like alteration of steroid metabolism; reduction of intestinal cholesterol absorption and thus persuading its clearance from intestine[16]. Moreover, γ- and δ-tocotrienols improve endothelial nitric oxide synthetase which plays a vital role in protection from arterial wall thickening[17,18].

CONCLUSIONS

In conclusion, tocotrienols has significant potential in suppressing the intimal thickening characterized by raised fatty streaks, pools of extracellular lipids, enhanced nuclear densities, proliferation of smooth muscles, calcifications and deposition of connective tissue matrix in a rabbit model for atherosclerosis. However, human trials are essential for final elucidation of tocotrienols as atheroma-suppressive agents.

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Article received on: 03/12/2011 Accepted for Publication: 10/02/2012 Received after proof reading: 10/05/2012

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Article Citation:
Shahid U, Butt SA, Shaukat F. Tocotrienols; effects on intimal thickening in ascending aorta of cholesterol-fed rabbits. Professional Med J Jun 2012;19(3): 341-345.