The effects of a tocotrienol-rich fraction on experimentally induced atherosclerosis in the aorta of rabbits

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Abstract

This study investigated the effects of a tocotrienol-rich fraction (TTRF) on the microscopic development of atherosclerosis and lipid peroxidation in the aorta of rabbits. Group 1 was fed a normal diet, group 2 received a 2 % cholesterol diet and group 3 received a 2 % cholesterol diet plus daily oral administration of the TTRF. After 10 weeks, the aortic content of malondialdehyde (MDA) was measured as an index of lipid peroxidation. The MDA was lowest in rabbits that received the TTRF compared to the groups that did not. The degree of intimal thickening was higher in the cholesterol-fed rabbits without the TTRF compared to the cholesterol-fed rabbits with TTRF (P<0.05). The continuity of the internal elastic lamina (IEL) was noted to be preserved in the cholesterol-fed rabbits with TTRF but appeared disrupted in the cholesterol-fed rabbits without the TTRF. The disrupted and fragmented IEL may have resulted from the injury caused by lipid peroxidation that contributed to the more extensive intimal thickening. We conclude that the antioxidant activities of the TTRF can reduce experimental atherosclerosis.

Key words: high cholesterol diet, tocotrienol, tocopherol, lipid peroxidation, experimental atherosclerosis, rabbit, aorta

INTRODUCTION

The response to injury theory on the pathogenesis of atherosclerosis was one of the earliest theories proposed.' This theory states that injury to the endothelium initiates cellular and molecular interactions involving smooth muscle cell migration from the media to the intima and smooth muscle cell proliferation leading to intimal thickening.',' The accumulation of smooth muscle cells in the intima eventually leads to atherosclerosis.

In laboratory animals, a wide range of artificial exogenous stimuli such as heat shock,[3] viral infections,[4,5] electrical stimulation[6] and endothelial cell denudation[7][9] have been used to produce intimal thickening. These experimentally induced lesions do not however, reproduce the structure of human atherosclerotic lesions. When a high cholesterol diet is given to laboratory animals for a few months, the animal lesions tend to resemble atherosclerotic lesions in human subjects.[10,11]

Water soluble antioxidants (vitamin C, flavanoids) and lipid soluble antioxidants (vitamin E, carotenoids) are natural antioxidants that may have a role in preventing oxidation of lipoproteins in the blood and tissues respectively. Vitamin E is now considered to be to be a generic name describing bioactivities of both tocopherols and tocotrienols.[12] As reflected in their structural similarity, tocopherol and tocotrienol are recognized for their antioxidative effects and are suggested to reduce cardiovascular disease[13] and cancer by arresting free radical damage.[14]

The evidence provided by the present literature on oxidative atherosclerosis promotes the possibility that antioxidants may influence the progression of atherosclerosis. Research on the reduction of atherosclerosis by antioxidants, however, has generated conflicting results.[13, 15-18] In this study, we investigated the effects of a tocotrienol-rich-fraction that comprised of tocotrienols (70%) and tocopherols (30%). Tocotrienols are minor plant constituents especially abundant in palm oil and in cereal grains such as barley. They are structurally similar to tocopherols and differ only by possessing an un-

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saturated isoprenoid side chain. The tocotrienol-rich fraction found in palm oil contains a mixture of tocotrienols a-, γ- and 6- as well as a-tocopherol. Tocotrienol has been shown to be a more potent antioxidant compared to tocopherols.[19]

Some reports have dealt with the association between geometrical features of the aorta and atherosclerosis.[20,21] Others have reported regional variations in the antioxidant status of a particular vessel.[22] Although it has been shown repeatedly that in hypercholesterolemic states, there is an increase in circulating oxygen free radicals,[23-26] the number of studies describing the effects of antioxidants on vessel wall histology is not remarkable and there remains sparse information concerning possible association between variation in normal vessel wall histology and atherosclerosis. In this paper, we report the effects of a TTRF on the histology of the aorta and atherosclerosis.

MATERIALS AND METHODS

Induction of atherosclerosis

The study was carried out on 18 New Zealand White male rabbits with weights in the range of 2.2 – 2.8 kg. The rabbits were divided into three groups. One group received a normal diet (ND, n=6). The other groups received a high cholesterol diet (HC, n=6) or a high cholesterol diet with daily administration of the TTRF in the dose of 60mg/kg body weight given orally (HC + TTRF, n=6).

All groups received 100g of food daily and the period of feeding for all rabbits was 10 weeks. The standard basic laboratory diet and the 2 % cholesterol diet were obtained from ICN Biomedicals, U.S.A. The standard basic laboratory diet contained crude protein 15.6 %, crude fat 3.1%, carbohydrates 46%, vitamins (carotene, vitamin K, thiamine, riboflavin, niacin, pantothenic acid, choline, folic acid, pyridoxine, biotin, vitamin B,,, vitamin A, vitamin D, vitamin E) and minerals (ash, calcium, phosphorous, potassium, magnesium, sulphur, sodium, chlorine, fluorine, ferum, zinc, manganese, copper, cobalt, iodine, chromium, selenium). Both diets were identical in their chemical composition except that 2% sucrose was substituted for 2% cholesterol in the cholesterol diet.

The rabbits were killed at the end of the study. The aortas between the origin and bifurcation into iliac arteries were removed and cleaned of visible adventitial tissue. The aorta was cut longitudinally. Part of the aorta (3mm of the ascending aorta) was processed for the microscopic assessment of atherosclerosis and the rest were quickly frozen at -70° C for the determination of malondialdehyde content.

Determination of aortic tissue content of malondialdehyde

The aortic tissue was added to 5 volumes (wt/vol) of phosphate buffer and homogenized with an Ultra-Turrax (T25 Janke and Kunkel, IKA Labor Technic). The homogenate was centrifuged at 3000xg for 15 minutes and the supernatent was taken for the determination of the aortic tissue content of malondialdehyde (MDA) using a method described previously[2'] with some modifications. A sample of 0.5 ml was acidified with 2.5 ml of 1.22 M trichloroacetic acid 10.6 M HCl and left to stand at room temperature for 15 min after which 1.5 ml of 0.67% thiobarbituric acid/0.05 M NaOH was added. The samples were incubated in a 100∞C water bath for 30 minutes. Subsequently it was left to cool at room temperature before the addition of 4 ml of n-butanol. After thorough mixing, the mixture was centrifuged at 1500 x g for 10 minutes. The absorbency of the upper phase was read at 535 nm. Various amounts of malondialdehyde standard, freshly prepared by acidification of 1,1,3,3-tetraethoxypropane were subjected to the identical test procedure as the basis for constructing a standard curve of thiobarbituric acid reactivity as malondialdehyde equivalent. The content of MDA is expressed as nmol/mg protein. Protein was measured by the method descibed by Lowry.[28]

Microscopic appearance of aortic wall

Aortic tissue was taken from consistent segments of the ascending aorta for histopathological examination 10 weeks after induction of atherosclerosis. The aortic tissue, prepared according to the methods described by Clayden[29] was fixed by immersion in 10 % formalin for 2 days. The specimens were sectioned transversely and stained with haematoxylin and eosin for light microscopy. Tissue sections of aorta were also stained with Verhoeff van Gieson for ease of identification of different fibres such as elastic, collagen and muscle.

Histomorphometric assessment of the aorta

Measurements of the thickness of intima was calculated using a Zeiss computerised image analyser (KS400, Carl Zeiss, Germany). The

image of each slide was captured and analysed with a stereomicroscope and a camera that the image analyser was equipped with. The mean intimal thickness was calculated from the lumen to the IEL.

Statistical analysis

Results are presented as the mean ± S.E.M. Statistical analysis was done by using the student's t-test. A P-value <0.05 was considered significant.

RESULTS

Aortic tissue MDA

Rabbits (n=6) from all three groups completed the study. MDA contents of aortic tissue in the three experimental groups are shown in Table 1. The aortic content of MDA was lowest in the HC+TTRF group compared to the HC (P<0.005) and the ND (P<0.003) groups. The group with the highest content of MDA was the HC group. The TTRF prevented the increase in MDA content in the aortic tissue in rabbits given a high cholesterol diet and the ND group.

Description and histomorphometric assessment of the atherosclerotic plaques

The histological sections of the aortas from the three experimental groups stained with H & E or Verhoeff van Gieson are shown in Figs. A (ND),

TABLE 1: Aortic content of MDA (mean ± S.E.M.) in all three experimental groups.

Groups	Aortic content of MDA (nmol/mg protein)
Normal diet	0.099 ± 0.007
High cholesterol diet	0.168 ± 0.0026
High cholesterol diet + TTRF	*0.048 ± 0.006

*p < 0.05 compared to the other 2 groups

B (HC) and C (HC + TTRF). Fig. 1A is the aortic ring from the D group. There was no intimal thickening in the normal diet group and the intima was seen as a thin layer adjacent to the clean lumen. At x 100 magnification (Fig. 2A), the tunica intima was very thin and thus well distinguished from the tunica media, which appeared as a thick band.

The aortic ring from the HC group is presented in Fig. 1B. In this section, the intima is thickened and easily differentiated from the media. The intimal thickening involved almost the whole of the inner circumference of the aortic ring, protruding into the lumen. In this group, the distribution of the atherosclerotic plaques was extensive.
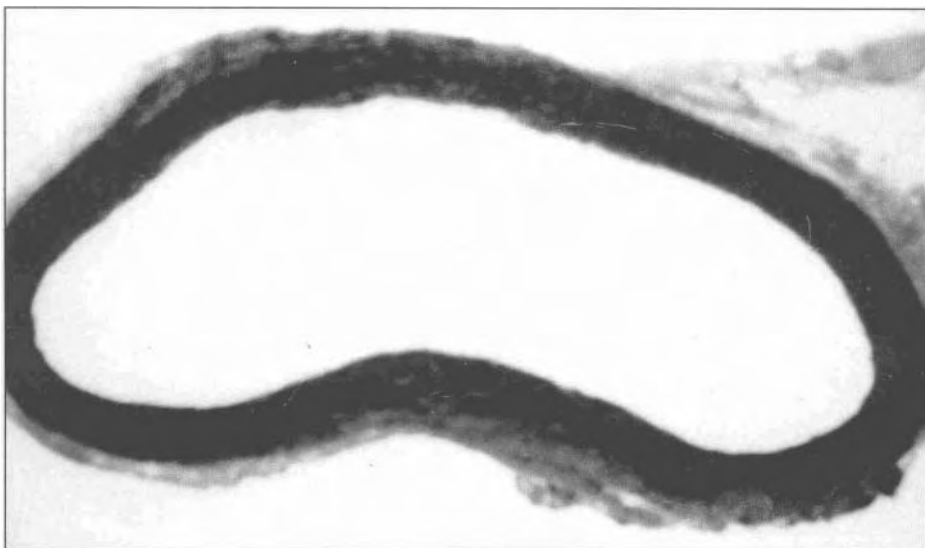

FIG. 1A: Cross section of aortic ring from rabbit fed a normal diet. Stained with Verhoeff van Gieson (magnification x 20).

Fig. 1C represents the aortic ring from the HC + TTRF group. In this section, the intimal thickening was appreciably less extensive. In this treated group, the distribution of the atherosclerotic plaques was clearly more focal in nature and did not involve the whole inner circumference of the aortic ring.

Fig. 3 shows that the rabbits fed with a normal diet had the least intimal thickening and the cholesterol fed rabbits without TTRF had the highest degree of intimal thickness. Morphometric analyses showed that the cholesterol-fed rabbits with TTRF had a lesser degree of intimal thickening compared to the cholesterol-fed group without TTRF. This indicated that the TTRF has got the potential to retard the formation of atherosclerotic plaques.
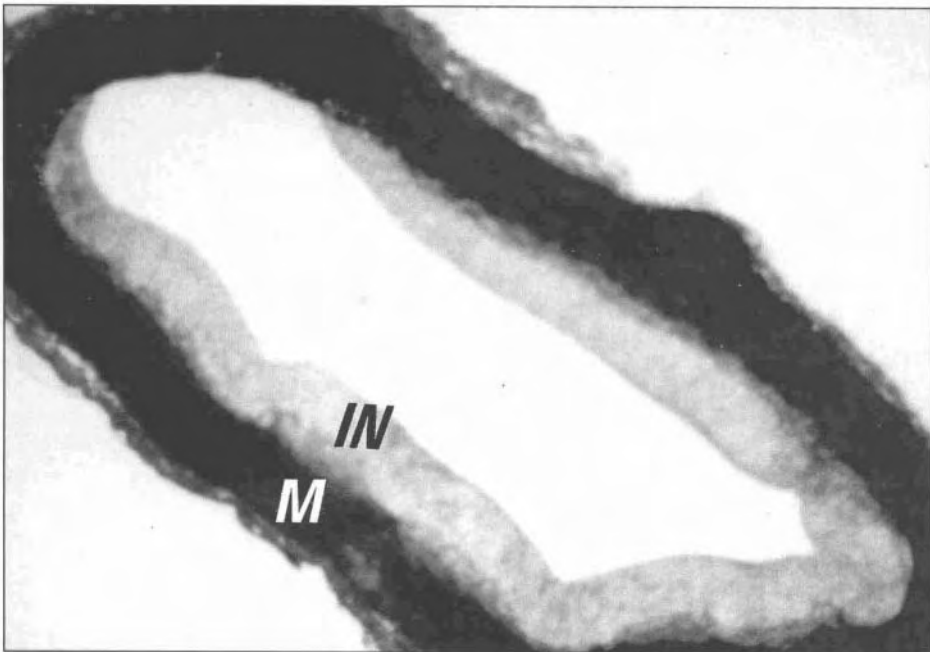

FIG.1B: Cross section of aortic ring from rabbit fed a high cholesterol diet. Stained with Verhoeff van Gieson (magnification x 20). IN = intima, M = media.
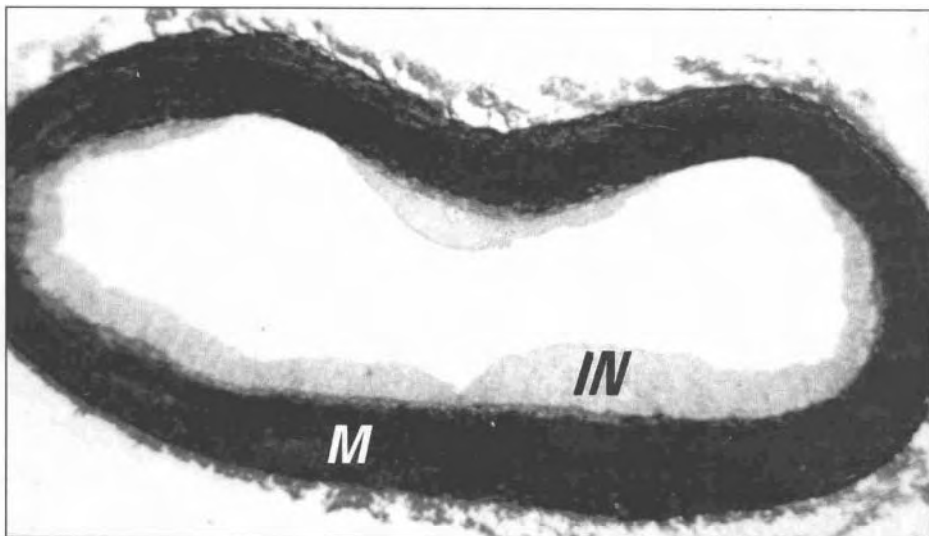

FIG. 1C: Cross section of aortic ring from rabbit fed a high cholesterol diet with supplementation TTRF. Stained with Verhoeff van Gieson (magnification x 20). IN = intima, M = media.

Description of aorta histology

Fig. **2A** represents the aortic section from the ND group stained with Verhoeff van Gieson (VHVG) at **x**100 magnification. The internal elastic lamina (IEL) could be clearly visualised. The IEL was intact and appeared as a dark line that separated the thin intimal layer from the media.

Fig. 2B (VHVG stain, **x**100 magnification) is a section of the aorta from the HC group. This section shows that the intima is filled with large vacuolated cells or foam cells. The unicellular endothelial layer was not disrupted and appeared to cover the area of atherosclerotic plaque. The IEL, however did not appear normal compared to the IEL as was seen in the ND group. It was
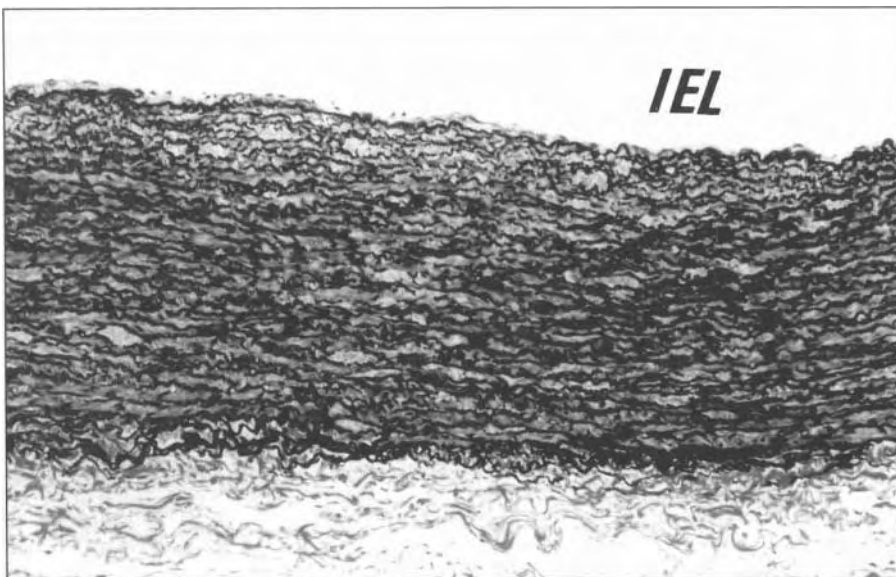

FIG. **2A:** Histological section of aorta from rabbit fed a normal diet. Stained with Verhoeff van Gieson (magnification x 100). IEL = internal elastic lamina.
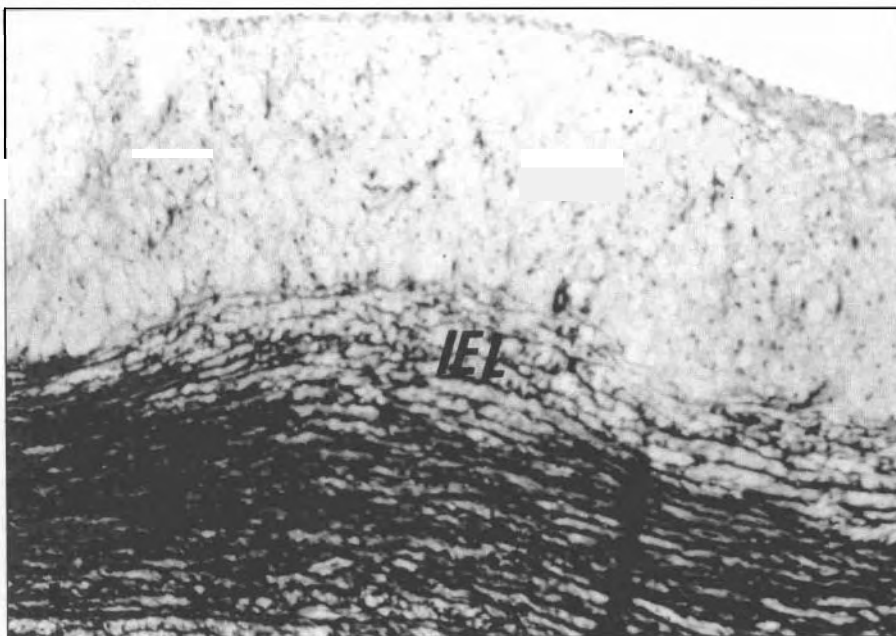

FIG. 2B: Histological section of aorta from rabbit fed a high cholesterol diet. Stained with Verhoeff van Gieson (magnification x 100). IEL = internal elastic lamina.

fragmented and appeared as several layers with very fine folds. In numerous places, the IEL appeared disrupted and lack the normal continuity with evident breaks in the lamina.

Fig. 2C (VHVG stain, x100 magnification) is a section from the HC + TTRF group. At the areas of the focal atherosclerosis, the intima had features similar to the HC group. The folds of the E L appeared more shallow compared to the ND group. The IEL, however had a more folded appearance compared to the HC group. The IEL in the treated group remained intact and was not fragmented nor disrupted and had normal continuity.

DISCUSSION

The dose of cholesterol used in this study was similar to previous studies including that by our group.[25, 30-33] Other investigators have used lower doses of cholesterol.[23, 24, 34, 35] The atherosclerosis was triggered by cholesterol feeding. The rabbits on normal diet did not have much evidence of atherosclerosis. Although the study period was not a prolonged one and the feeding period was standardised in all the experimental groups, the absence of atherosclerosis in the normal diet group eliminated the factor of aging that is known to have a role in atherogenesis.

Similar to the findings of our previous studies,[25, 26] we found that rabbits fed with a high cholesterol diet had very high aortic content of MDA. This reflected an intensification of the lipid peroxidation process that occurred in the aorta. The TTRF was able to prevent the rise in the aortic content of MDA in rabbits fed with this a similar high cholesterol diet. Presently, to our knowledge there is no other data besides that of ours which illustrates the efficacy of TTRF on retarding the lipid peroxidation process in the aorta from rabbits subjected to such high intake of cholesterol. The high intake of cholesterol produced a stress-oxidative-like condition whereby the lipid peroxidation process is enhanced with increased production of MDA. The ability of tocopherols to reduce MDA has been reported in rabbits fed with a lower dietary content of cholesterol such as 0.5% or 1%.[23, 24, 34, 35] In this study, the TTRF was able to reduce lipid peroxidation to an even lower degree compared to rabbits fed with a normal diet. The significantly lower MDA content in the aorta reflects the substantial antioxidant status of the vessel.

The most important finding in this study is that 10 weeks of treatment with TTRF inhibited the extent of intimal thickening. This indicated that the extent of the atherosclerotic lesion occurred to a lesser extent with TTRF compared
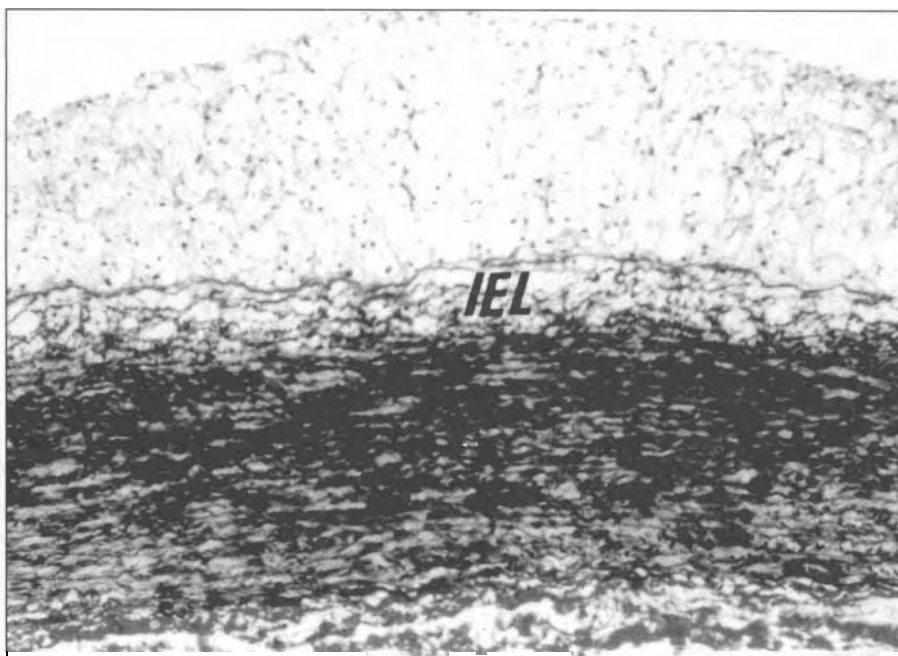

FIG. 2C: Htstological section of aorta from rabbit ted a htgh cholesterol diet with supplementation of vitamin E mixture. Stained with Verhoeff van Gieson (magnification x 100). IEL - internal elastic lamina.
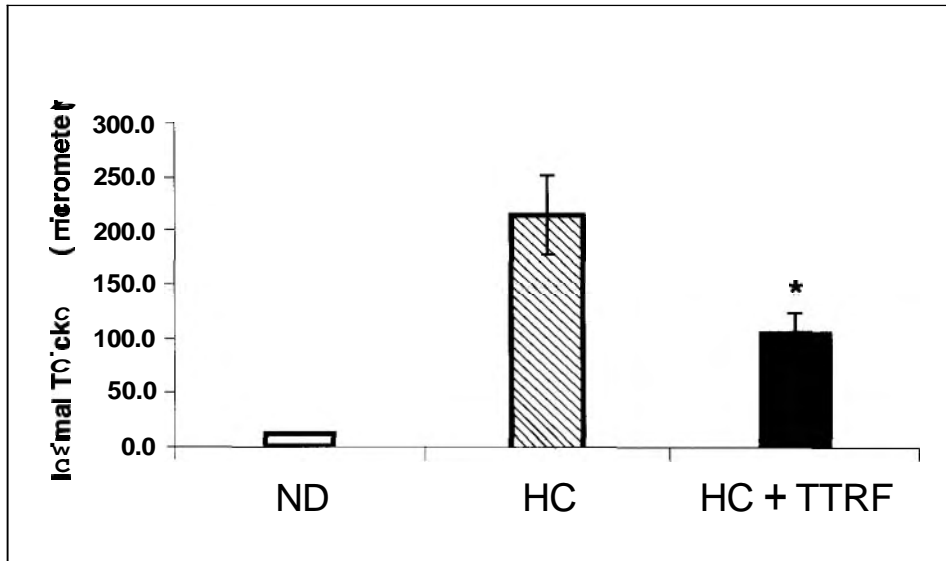
FIG. 3: Intimal thickness in the aorta of rabbits treated with a Tocotrienol-rich Fraction. ND = Normal Diet; HC = High Cholesterol Diet; TTRF = Tocotrienol-rich Fraction. There is a significant difference (*p<0.05) in the intimal thickness of the aorta in rabbits fed a high cholesterol diet with and without supplementation with the TTRF. Results are expressed as mean **+** S.E.M.

to without TTRF whereby the intimal thickening was clearly more. The implication can be extended to mean that TTRF had the ability to minimise the progression of the intimal thickening in the aorta. It is possible that this is one of the protective manifestations of vitamin E on atherosclerosis. As these atherosclerotic plaques protrude into the lumen, the impact of this finding is in relation to the degree of patency of the affected vessel. An increase in the intimal thickness would lead to a greater degree of narrowing of the vessel.

The other distinct finding in our study is that the IEL remained intact in the presence of TTRF. In the absence of TTRF, the IEL was disrupted with loss of continuity and appeared fragmented. The fragmentation of the IEL is likely to be caused by injury as a result of lipid peroxidation. The IEL demarcates the end of intima and the beginning of media. The normal organisation of this membrane is beyond doubt an important feature that denotes the integrity of the vessel. The intima is a thin layer that is normally composed of endothelial cells and collagen fibres. It contains minimal smooth muscle cells compared to the media which is, a muscular layer. In the present study, it was noted that smooth muscle cells were present in the intima of both the cholesterol fed groups with or without TTRF. We could not, however quantify whether the

number of smooth muscle cells were present to a greater extent in the group that did not receive TTRF compared to the treated group. This was because, based on fore knowledge, we know that the smooth muscle cells, on entering the intima in the environment defined by our study, would be filled with lipids and be transformed into foam cells. It is however, possible that because of the discontinuity in the IEL induced by the atherogenic diet, the smooth muscle cells migrated into the intima with greater ease at the fragmented areas that occurred throughout the aortic ring. The now lipid laden smooth muscle cells contribute to the atherosclerotic lesions seen along the whole circumference of the aortic ring. Hence, in this study we showed that the disrupted IEL accompanied the presence of a more extensive lesion shown by a greater degree of intimal thickening whereas an intact IEL was associated with less intimal thickening. In view of this, we hypothesise that the TTRF preserved the integrity of the IEL and this deterred the migration of smooth muscle cells into the intima. The lack of opportunity for the smooth muscle cells to migrate to the intima reduced the extent of intimal thickening.

The present study highlighted the influence of TTRF on the vascular histology. The TTRF protected the IEL from extensive injury from lipid peroxidation and the integrity was con-

served. This protection is probably in part, due to its ability as an antioxidant to retard lipid peroxidation. The intact IEL retarded the migration of smooth muscle cells while fragmented IEL may allow for the migration of smooth muscle cells. This study also demonstrated that the development of more extensive lesions can be prevented in the presence of exogenous protective factors. The migration of smooth muscle cells and intimal injury have both been identified as key factors in atherogenesis. TTRF have an impact on these key factors through their protective effects on IEL from the insults of cytotoxic by products of lipid peroxidation.

In conclusion, TTRF is able to suppress intimal thickening in a rabbit model of cholesterol induced atherosclerosis. One of the likely mechanisms involved is the inhibition of lipid peroxidation that preserved the vascular architecture of cholesterol-fed rabbits as outlined by this study. Taken together, this study illustrated a link between lipid peroxidation process inducing a variation in normal vessel wall histology that contributed to the formation of atherosclerosis.

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