ORIGINAL ARTICLE

Protection by Tocotrienols Against Hypercholesterolaemia and Atheroma

M K Teoh, FRCSEd
J M K Chong, BSc (Hons)
J Mohamed, PhD
K S Phang, MRCPath
Faculty of Medicine, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur

Summary

Antioxidants such as tocotrienols may protect against atherosclerosis since tissue injury from free radicals is a final common pathway of damage in arterial disease. In this study, the effects of tocotrienols on serum cholesterol, lipid peroxides, and aorta atheroma were assessed in rabbits fed an atherogenic diet for 12 weeks. Tocotrienols were more effective than tocopherols in preventing increases in serum LDL ($p=0.03$) and total cholesterol ($p=0.008$) levels in the cholesterol-fed rabbits. Elevation of serum lipid peroxides was effectively suppressed by tocotrienols ($p=0.01$). Both tocopherols and tocotrienols offered significant protection against atheroma in the rabbit aorta, but tocotrienols had a stronger hypolipidaemic effect.

Key Words: Tocotrienols, Vitamin E, Atheroma, Antioxidants, Hypercholesterolaemia

Introduction

The harmful effects of free radicals arise from damage to lipids and proteins in living cells, either directly or by producing other more reactive species[1]. Free radical-mediated tissue injury is now identified as a final common pathway of damage in a large variety of diseases[2] including atherogenesis.

Damage by free radicals can to some extent be prevented by the body's multilevel defense system against free radicals, which comprises enzymes (superoxide dismutase, catalase, glutathione peroxidase) and antioxidant vitamins (beta-carotenes, vitamins A, C and E)[3]. Vitamin E is the only chain-breaking antioxidant in membranes. It is also the major if not the only lipid-soluble antioxidant in blood plasma[4]. It has the ability to break the chain reaction of lipid peroxidation by reacting with lipid peroxy radicals[5].

Lipid peroxides are themselves free radicals, produced when lipids (especially polyunsaturated fatty acids) are damaged by other oxygen radicals[5]. They play an important role in atherogenesis. Low density lipoproteins (LDL), after oxidation by lipid peroxides, is phagocytosed by macrophages forming foam cells, can early stage in atherogenesis[6]. Lipid peroxides an also activate platelet aggregation[7], modify vascular tone[8] and damage the endothelium[9]. The correlation between elevated levels of plasma lipid peroxides and atherosclerosis had been reported in rabbits[10] and in patients[11,12]. Many investigators have therefore looked into the potential role of free radical scavengers, such as vitamin E (tocopherols), in arterial disease. Although a causal link is difficult to prove, reports of beneficial effects from tocopherol supplements in arterial disease (especially heart disease) have been appearing in the medical literature since 1948[13-18].

Vitamin E is a group of compounds called tocopherols and tocotrienols[19]. In popular usage, vitamin E is synonymous with dlalpha-tocopherol acetate, which is widely regarded as the clinically predominant form. Although it may be the most biologically active, it is a weaker antioxidant than its isomers (gamma or delta tocopherol) or the tocotrienols[20]. Tocotrienols are the

predominant form of vitamin E in palm oil, a unique situation amongst the vegetable oils. The discovery that tocotrienols are stronger antioxidants[21] has raised great interest in view of the link between free radicals and arterial disease. Tocotrienol-rich vitamin E (TVE) improved the pain-free walking distance of patients with intermittent claudication after 12 weeks[22]. This was accompanied by a significant reduction in serum lipid peroxides in these patients. In addition, tocotrienols may also have a hypolipidaemic effect by inhibition of HMG-CoA reductase[23].

There is a need to know more about tocotrienols, especially their efficacy in arterial disease, since they are possibly stronger antioxidants than tocopherols. The aim of this study was to evaluate the role of tocotrienols as an antioxidant in atherogenesis. Their effects on serum cholesterol, lipid peroxides and atheroma in rabbits fed an atherogenic diet was assessed, and their activity compared with that of the tocopherols.

Materials and Methods

Tocotrienols

The pure tocotrienols and tocopherols used in this study were supplied by the Palm Oil Research Institute of Malaysia (PORIM). The vitamin E-rich fraction was extracted from palm oil and fractionated into its individual components by thin-layer chromatography. The tocotrienol fractions were further chromatographed until a purity of over 90% was obtained. The composition of tocotrienols used was as follows:

- 80% gamma-tocotrienol
- 20% alpha- and beta-tocotrienols

Rabbits

Single-strain local Malaysian rabbits of similar age and weight were divided randomly into three groups of 10 (5 males, 5 females) and were fed an identical atherogenic diet for 12 weeks. The average age was seven months. The mean weight of the rabbits at the start of the study was 2.03 kg (range 1.63 - 2.45 kg). The groups were:

- Cholesterol Group: on atherogenic diet alone.
- Tocopherol Group: atherogenic diet and 50mg tocopherol daily.
- Tocotrienol Group: atherogenic diet and 50mg tocotrienol daily.

The atherogenic diet consisted of standard feed pellets (Gold Coin, Malaysia) to which was added cholesterol powder 0.5% (Sigma) and coconut oil 3% by weight. Approximately 100g of the reconstituted pellests were given to each rabbit per day. Fresh water was freely available on demand.

A fasting blood sample (10ml obtained from the ear) was collected at 10 days, six weeks and again at 12 weeks. The blood was separated and tested for cholesterol profile and lipid peroxide levels. At the end of 12 weeks the animals were killed by an intravenous injection of pentobarbitone (1ml/2.5kg) and the aorta removed for estimation of aorta malondialdehyde (MDA) levels and assessment of atheroma lesions.

Atheroma assessment

After removal, a 2cm section of aorta was removed from the lower aorta just above the bifurcation and put in ice for processing and subsequent MDA estimation. The remaining proximal aorta was preserved in formalin and then stained with Oil Red-O. The aorta assessment for atheroma and fatty streaks was performed "blind" by three observers independently. The specimens were all photographed after staining and evaluation was based on a visual analogue scale of one to four, where one is mild and four is severe. To minimise between-assessor error, repeated evaluations of standard specimens chosen to represent each of the different grades was preliminarily carried out by the assessors and discussed together.

Measurement of serum lipid peroxides

The method used here is based on the thiobarbituric acid (TBA) reaction[24] and assayed by fluorometry (515nm excitation, 553nm emission) in a luminescence spectrometer (Shimadzu Digital Spectrofluorometer RF-510, 204-25000-02). A standard curve of fluorescence versus known MDA concentrations was created using measured quantities of 1,1,3,3-tetraethoxypropane (a MDA standard) in the TBA reaction. The fluorescence intensity of sample serum compared to that of the standard solution of MDA was used to calculate the concentration of lipid peroxide in µmol MDA/l serum.

Assessment of aorta lipid peroxides

The standard 2cm sample of sectioned lower aorta removed for lipid peroxide estimation was transported in

ice for immediate processing. The specimen was first cleaned using 0.15M NaCl before weighing and being cut into small segments. It was then added to phosphate buffer saline (pH 7.4) in the ratio of one part specimen: four parts of buffer by weight, and homogenised in ice for two minutes. The homogenate, kept at a temperature range of 1-4°C, was then centrifuged at 2500rpm for 15 minutes. The sediment was discarded, and the supernatant kept in aliquots and stored at below -20°C for MDA assay using the thiobarbituric acid reaction.

Statistical analysis

The differences in the means were tested by one-way analysis of variance (ANOVA) where data was normally distributed. For non-parametric data, or where group numbers were small with outliers, the Mann Whitney U test was used. Chi-square tests were done to analyse differences in frequencies and proportions between groups. The statistical p value of less of less than 0.05 ($p<0.05$) was taken as significant.

Results

All the rabbits survived to the end of the 12 weeks except one early death in the group on atherogenic diet alone (Group 1). The food consumption patterns of the three groups were very similar. Although each rabbit was given the same quantity (100g) of fresh feed daily, and their feeding habits closely monitored, it was impossible to accurately measure the amount of food consumed by each rabbit daily.

Serum cholesterol profile (Table I)

Total cholesterol: Mean serum levels were lower in the Tocotrienol group than either the Tocopherol or Cholesterol Groups at 12 weeks (ANOVA, $p=0.008$). Even as early as 10 days the differences between the mean total cholesterol levels were significant (ANOVA, $p=0.003$). In the Cholesterol and Tocotrienol Groups the levels peaked at six weeks and declined to a lower level at 12 weeks. (Figure 1)

Table I
Serum lipid profile of rabbits

	Cholesterol	Tocopherol	Tocotrienol
Total Cholesterol	**mean (sd)**		
10 days	8.04 (3.87)	3.79 (2.20)	3.46 (2.79)
6 weeks	18.77 (7.50)	8.81 (5.97)	7.88 (6.15)
12 weeks	14.01 (8.29)	9.66 (7.94)	3.11 (2.21)
Triglyceride	**median (range)**		
10 days	1.30 (1.48)	0.69 (0.63)	0.54 (1.69)
6 weeks	1.04 (2.42)	0.71 (0.52)	0.66 (0.30)
12 weeks	0.60 (2.41)	0.52 (1.60)	0.47 (0.25)
HDL	**mean (sd)**		
10 days	0.88 (0.23)	1.22 (0.48)	1.07 (0.45)
6 weeks	1.41 (0.52)	1.72 (0.77)	1.56 (0.77)
12 weeks	1.14 (0.34)	0.85 (0.31)	1.14 (0.48)
LDL	**median (range)**		
10 days	6.18 (13.27)	1.33 (7.04)	2.79 (7.77)
6 weeks	20.05 (21.53)	5.16 (18.72)	3.59 (16.87)
12 weeks	8.91 (21.58)	7.21 (23.95)	1.47 (4.97)

Median (range) used here for non-parametric data
Units are mmol/l serum.

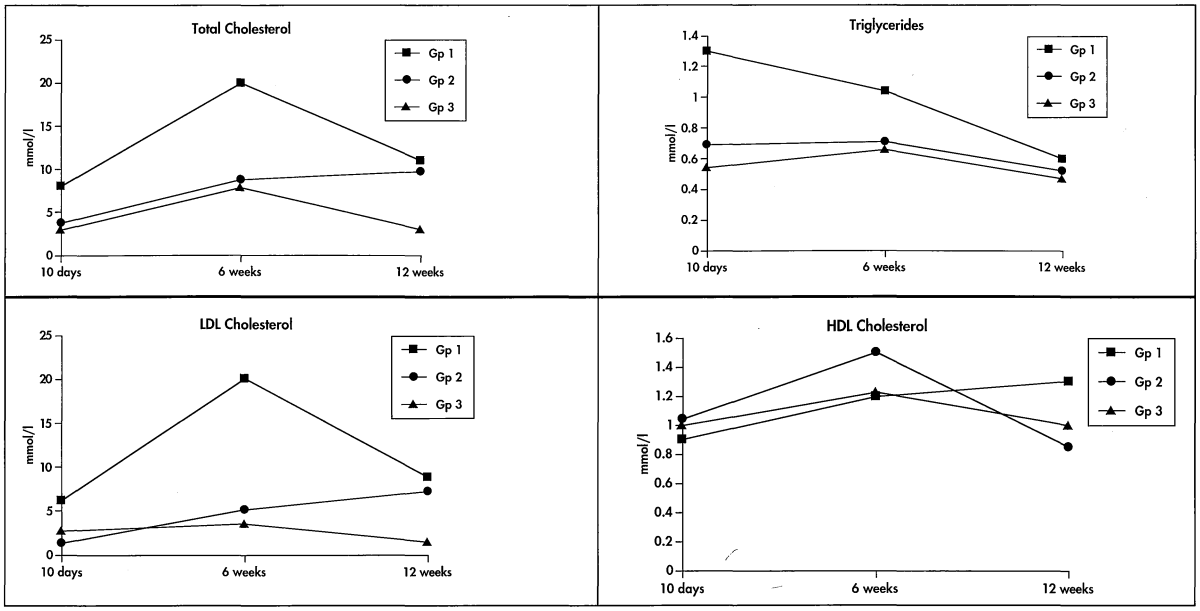

Fig. 1: Serum lipid profile changes in cholesterol-fed rabbits
Gp 1 = Cholesterol only, Gp 2 = Cholesterol + Tocopherol, Gp 3 = Cholesterol + Tocotrienol

Triglycerides: There were only small changes after 12 weeks and the differences between the groups were not significant. (Mann-Whitney U test: Tocotrienol vs Tocopherol, p=0.52; Tocotrienol vs Cholesterol, p=0.36; Tocopherol vs Cholesterol, p=0.15)

High density lipoproteins (HDL): The difference in HDL between the groups was not significant (ANOVA, p=0.173 at 10 days, p=0.180 at 12 weeks).

LDL: The pattern of changes followed that of Total Cholesterol. The median LDL level at 12 weeks in the tocotrienol group was significantly lower than either the Cholesterol and Tocopherol groups. Differences between groups using the Mann-Whitney U test were: Tocotrienol vs Cholesterol (p=0.001), Tocotrienol vs Tocopherol (p=0.030), Tocopherol vs Cholesterol (p=0.270).

Serum lipid peroxides

The results at 10 days, six weeks and 12 weeks can be compared between the groups (Table II). The Tocotrienol Group consistently had the lowest levels. Even at 10 days the two groups receiving vitamin E had significantly lower MDA levels than the group on atherogenic diet alone. This suggests that there was an early antioxidant protection from vitamin E. The difference in lipid peroxide levels at 12 weeks between the Tocotrienol Group and the other groups were highly significant (Figure 2).

Rabbit aorta lipid peroxides

One rabbit died prematurely and processing delays made another three aorta specimens unsuitable for MDA assay, leaving 26 aorta specimens for analysis (Table III). The mean lipid peroxide level was lowest in the Tocotrienol Group (0.61 µmol/l; ANOVA, p=0.038). The serum/aorta MDA ratio was lowest in the Tocotrienol Group (0.82) and highest in the Cholesterol Group (2.76).

Aorta atheroma assessment scores

There was close agreement of the scores generally among the three assessors. The difference in the mean score between the Cholesterol Group (3.00) and the other two groups (1.40 and 1.63 for the Tocopherol and Tocotrienol groups respectively) was highly significant (ANOVA, p<0.0001). There were more grades 1 and 2 in the groups receiving tocopherol or tocotrienol compared to the group on atherogenic diet only (Table IV). When

Table II
Serum lipid peroxide changes in the rabbit groups

	Cholesterol	Tocopherol	Tocotrienol
10 days	1.17 (0.57)	0.57 (0.31)	0.51 (0.40)
6 weeks	2.71 (1.09)	1.24 (0.81)	1.10 (0.81)
12 weeks	1.88 (1.02)	1.37 (1.08)	0.50 (0.32)

Values are means (standard deviations)
Units are in µmol MDA/litre serum
Differences between groups (ANOVA): at 10 days, p=0.005; at 12 weeks, p=0.010

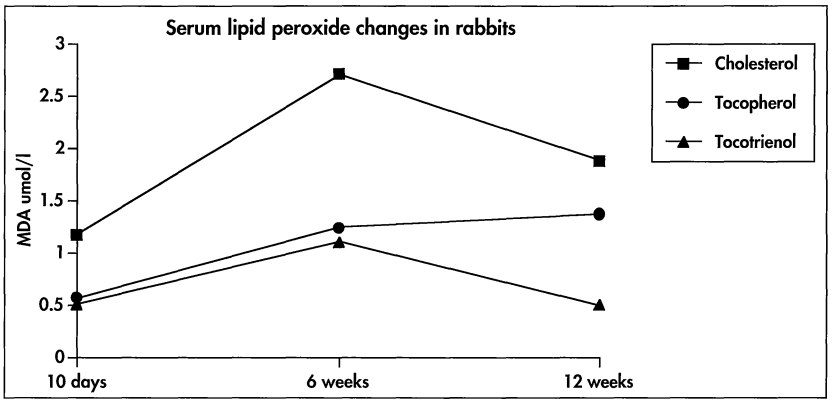

Fig. 2: Serum lipid peroxide changes in rabbits

the grades 1 and 2 scores were combined as "Mild" scores and the grades 3 and 4 combined as "Severe", the differences between the groups was even clearer (see Figure 3). The Chi-square test showed that the differences were highly significant with $p<0.0001$. Fatty streaks and atheroma induced in the rabbits fed on the high cholesterol diet for 12 weeks were especially severe in the untreated group of animals. In the rabbits receiving tocopherols or tocotrienols a protective effect against the formation of fatty streaks and atheroma was evident in spite of the same atherogenic diet.

Discussion

There was a very significant early cholesterol response to the atherogenic diet in all three groups. An early protective effect (at 10 days) was seen in the groups receiving vitamin E (either tocopherols or tocotrienols). Tocotrienol was clearly more effective than tocopherol in suppressing elevation of cholesterol levels. The present finding that tocotrienols have a stronger hypolipidaemic effect is consistent with previous studies[25]. The hypolipidaemic action of tocotrienols may involve suppression of HMG-CoA reductase thereby inhibiting cholesterol biosynthesis[26]. Gamma-tocotrienol had a 30-fold greater activity in inhibition of cholesterol biosynthesis than alpha-tocotrienol.

Large variations in total cholesterol values in rabbits, like those seen here, had been reported in other studies. A number of these rabbits had levels of serum total cholesterol of over 20mmol/l. Similar huge increases were seen in another animal study using rabbits[27]. In humans it is most unusual for total cholesterol to be elevated as high as 15mmol/l. In another study[28], rabbits fed on a diet with 1% cholesterol for three months produced mean plasma cholesterol concentrations of 22.8mmol/l compared to 0.61mmol/l in controls.

Both tocopherols and tocotrienols had kept the lipid peroxide levels low in spite of cholesterol feeding and elevated serum cholesterol levels. Tocotrienols consistently showed a stronger antioxidant effect than tocopherols in this study. Estimation of serum lipid peroxide by fluorometric assay of the reaction product of MDA with

Table III
Rabbit aorta lipid peroxides after 12 weeks (μmol MDA/l)

	Cholesterol	Chol.+Tocopherol	Chol.+Tocotrienol
n	8	10	8
Mean*	0.68	0.82	0.61
s.d.	*0.11*	*0.21*	*0.14*
serum/aorta MDA	2.76	1.67	0.82

**p=0.038, ANOVA overall difference between groups*

Table IV
Summary of rabbit aorta assessment scores

Scores	Cholesterol n=27	Tocopherol n=30	Tocotrienol n=30
Grade 1	3	20	17
Grade 2	4	8	8
Grade 3	10	2	4
Grade 4	10	0	1
Chi2=37.80, 6df, p<0.001			
Grades 1+2	7	28	25
Grades 3+4	20	2	5

Chi2=34.58, 2df, p<0.0001
Scores: Grade 1 = mild, to Grade 4 = severe

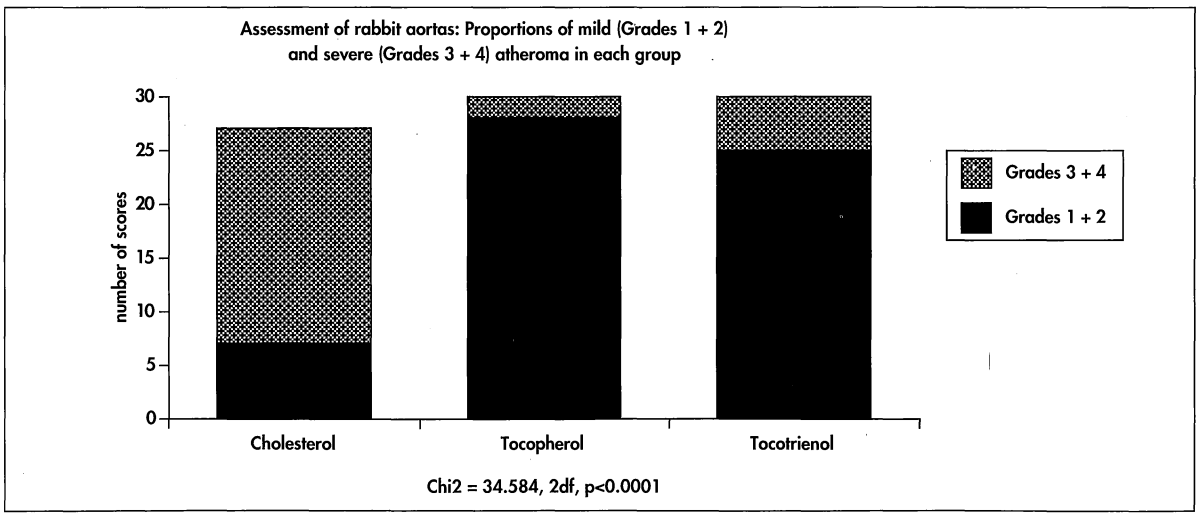

Fig. 3: Rabbit aorta atheroma assessment

TBA is well established[24]. Despite the presence of other sources of MDA in plasma, the MDA assay is in fact an estimation of the lipid peroxide level. The test actually measures the MDA that is formed from decomposition of the lipid peroxides. Any free MDA produced in vivo is very unstable and is rapidly metabolised[5].

The mean aorta lipid peroxide level was lowest in the group receiving tocotrienols, but was higher in the tocopherol group than the cholesterol group. Interpretion of this data is difficult. It is possible that tocopherol had little effect in suppressing lipid peroxidation in rabbit aorta. The serum/aorta lipid peroxide ratios provided a slightly different view. The rations in the groups taking Tocotrienol (0.82) and Tocopherol (1.67) were both lower than the Cholesterol Group (2.76). The lower ratios may reflect a more effective antioxidant activity in serum compared to aorta.

Direct comparison was not possible between serum and aorta lipid peroxide levels as their units were different (μmol/l serum vs μmol/l protein). Studies have reported an increase in lipid peroxides both in the blood and aorta of rabbits fed on an atherogenic diet[27,10]. The significance of aorta lipid peroxide levels is unclear, and so is their precise relationship with serum levels. It is possible that aorta lipid peroxide levels merely reflect the degree of damage from free radical activity in the blood. The levels will therefore be highest in areas of the aorta that are most affected by atheroma. This may explain the relatively low lipid peroxide levels in the specimens studied. The protocol dictated that specimens be taken from the lower aorta just above the bifurcation. This segment of rabbit aorta, unlike human aorta, was least affected by atheroma.

Tocotrienols and tocopherols were equally effective in offering significant protection against atheroma developing in rabbits fed a high cholesterol diet. There was good agreement between the 3 independent observers who made the assessment blind, thus underlining the reliability of these results. Other investigators have found that the scores correlated well with aorta cholesterol levels[29]. To avoid subjective errors, the precautions used in a similar visual grading system reported in the International Atherosclerotic Project[30] were observed.

Lipid peroxides in the blood oxidise LDL and injure endothelial cells, contributing to platelet aggregation and atherogenesis. Following endothelial injury, lipid peroxides and LDL may enter the medial layer and stimulate smooth muscle cells[10], an important early step in atherogenesis. The accumulation of lipid peroxides in atheroma plaques and their pathological potential had been described[31]. Further lipid peroxidation and damage may be propagated by these free radicals within the atheroma. The primary function of vitamin E in vivo appears to be the protection against lipid peroxidation of polyunsaturated fatty acids in phospholipids, and thereby the prevention of damage to membranes by free radicals[4]. Although tocotrienols showed a strong effect in suppressing elevation of LDL cholesterol, an equally important activity may be the prevention of oxidative damage to LDL. Another antioxidant, Probucol, can reduce atherosclerosis without changes in cholesterol levels[32].

The use of pure tocotrienols in animal studies needs to be repeated in view of the positive results in this study. We need to look in particular at platelet thrombosis as well as measuring free radical activity in vivo using more sophisticated methods such as electron spin resonance. This should help to unravel the pathological mechanisms linking free radical activity in LDL, endothelium and platelets to atherogenesis.

In conclusion, tocotrienols were effective in blocking the rise in lipid peroxide levels in cholesterol-fed rabbits. The elevation of serum cholesterol levels in these rabbits were also effectively suppressed. This may have resulted from the inhibition of HMG-CoA reductase by tocotrienols. Tocopherols had a weaker antioxidant action and hardly any lipid-lowering effect compared with tocotrienols. However, both tocotrienols and tocopherols equally protected against the development of fatty streaks and atheroma in the rabbit aorta. Antioxidant activity was likely to have been instrumental in conferring protection against atherogenesis.

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