Lipidaemic effects of tocotrienols, tocopherols and squalene: studies in the hamster

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Syrian Golden hamsters have been widely used as a experimental model for the investigation of the aetiology and development of atherosclerosis and cardiovascular disease. The responses of the hamster to dietary fat manipulations are in many ways similar to that observed in humans. The lipidaemic effect of a tocotrienol rich fraction (TRF) from palm oil on human trials has not been consistent. In this study, the cholesterolaemic effect of tocotrienols and tocopherols were differentiated by using pure tocotrienols (that were isolated from palm oil fatty acid distillate) and pure commercial tocopherols and squalene. A palm oil triacylglycerol fraction (POTG), free of all unsaponifiable matter, was used as the dietary fat in different feeding experiments. Tocotrienols added at 162 ppm to POTG (POTG-T3L) significantly ($P<0.05$) lowered serum total cholesterol (TC) level as compared to that of the POTG group; but the serum LDL-C , HDL-C and TG levels of the POTG-T3L group were not significantly lower than that of the POTG group ($P>0.05$). Increasing the level of tocotrienol supplementation to the diet (POTG-T3H) appeared to raise rather then reduce the serum TC, LDL-C and HDL-C levels as compared to that of POTG-T3L group. This observation that lower level of tocotrienol supplementation appeared to exhibit stronger hypocholesterolaemic effect than a higher level of tocotrienol supplementation is interesting; but its explanation is not yet forthcoming. When tocopherols were supplemented at 72 ppm to the POTG diet it was observed that the serum TC, LDL-C and HDL-C levels were all somewhat increased when compared to that of the POTG group. These results suggest that tocotrienols and tocopherols may have opposite cholesterolaemic effects in the hamster, and further experiments need to clarify the mode of action of these vitamin E isomers. In our second series of experiments the cholesterolaemic effects of tocotrienols and tocopherols were studied in the presence of squalene, a key intermediate in the cholesterol synthesis pathway and a controversial cholesterol lowering agent. Squalene added to the diet at 0.1% level significantly lowered ($P<0.05$) serum TC level when compared to that of the POTG group. The LDL-C, HDL-C and TG levels appeared to be lowered by the squalene supplementation also but the differences between the POTG-SQ and POTG groups were not statistically significant ($P>0.05$). When tocotrienols or tocopherols were added to the squalene-containing POTG diets, the serum TC and LDL-C levels were further reduced ($P<0.01$) when compared to that of the POTG and POTG-SQ groups. The HDL-C and TG levels were not affected by tocotrienol or tocopherol supplementation in the presence of squalene. These results indicate that in the presence of tocotrienols and squalene POTG exhibit hypocholesterolaemic action whereas tocopherols may have a hypercholesterolaemic effect in the hamster.

Key words: Plasma lipids, vitamin E, tocotrienols, squalene

Introduction

Epidemiological studies reveal that plasma vitamin E level is inversely correlated with the risk of cardiovascular disease[1]. Two recent cohort studies provide further evidence of an association of high vitamin E intake and a lower risk of coronary heart disease in men and women[2,3]. All the above studies focus on tocopherols, the most widely distributed form of vitamin E in nature.

Tocotrienols, another form of vitamin E, were found to be present only in a few limited sources with palm oil as the richest source of tocotrienols in nature[4,5]. A tocotrienol concentrate, trade-named Palmvitee, was prepared from palm oil and capsulated; each capsule contains about 40 mg of tocotrienols and 20 mg of tocopherols. Human trials on the above tocopherol-tocotrienol-rich capsules with normal and hypercholesterolaemic subjects yielded inconsistent results[6-8]. The discrepancies in the above observations are not yet explained; however, one obvious difference in the above trials was the dosages used in the experiments. It appeared that experiments with lower dosages of Palmvitee tended to give positive hypo-cholesterolaemic effect whereas higher dosages of Palmvitee tended to give neutral effect.

In order to study the cholesterolaemic effect of tocotrienols and tocopherols separately we have isolated pure palm oil tocotrienols from palm oil fatty distillate, a by-product of the palm oil refining process. Tocopherols and squalene were obtained from commercial sources. The tocotrienols, tocopherols and squalene were added to the dietary fat, palm oil triacylglycerols, isolated free of all unsaponifiable components from palm oil. The Hamster was used as the experimental model because it has many similar features of lipid metabolism as that seen in humans including the responses to dietary lipid manipulations. Hamsters have been extensively used in studies of lipid metabolism in relation to atherosclerosis and cardiovascular disease [9-12].

Materials and methods

Animals and diets

Male Golden Syrian hamsters of body weights ranging from 100 to 150 g were obtained from the animal Research Centre, University of Malaya. They were divided into groups of approximately equal body weights and housed individually in stainless cages in a temperature-regulated, 25 ± 2° C, and light-controlled room with a 12-hr of dark and light cycle. All groups were given cholesterol-free, high fat (20%, w/w) semisynthetic diets supplemented either with tocotrienols, tocopherols or squalene. Tocotrienols were isolated from palm oil fatty distillate by solvent extraction and column chromatography[13]. The tocotrienols were chromatographically pure (99.9%) by HPLC and Capillary GLC and were composed of α-tocotrienol (43%) g-tocotrienol (50%) and δ-tocotrienol (7%). Tocopherols (containing mainly α-tocopherol) and squalene were obtained from commercial sources (Sigma Chemical Co, St Louis, USA) The general formula of the semisynthetic diet is given in Table I. The animals were fed these experimental diets for 45 days. Water was given *ad libitum* and diets were given daily. At the end of the

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experimental period, the animals were fasted overnight. Blood and liver were taken while the animals were under ether anaesthesia. Serum was prepared by centrifugation at 2500 rpm for 10 minutes at room temperature. Serum and liver were stored at -20° C until analysis.

Table 1. Composition of the semisynthetic diets

Ingredients	g/100g	Ingredients	g/100g
Cornflour	29.0	DL-methionine	0.3
Dextrose	18.0	Choline bitartrate	0.2
Cellulose	5.0	Squalene	0 - 0.1
Casein	22.0	Mineral mix	4.5
Oil*	20.0	Vitamin mix	1.0

* palm oil triglycerides isolated free of all unsaponifiable matters from commercial palm oil.

Experimental protocols

Study 1. Effect of dietary tocotrienols and tocopherols on serum and liver lipids. Four groups of hamsters were used. One group was fed on semisynthetic diet containing POTG as the dietary fat. The second group was fed on semisynthetic diet containing POTG supplemented with 72 ppm of tocopherols; the third group was fed on semisynthetic diet containing POTG supplemented with 162 ppm of tocotrienols, and the fourth group was fed on semisynthetic diet containing POTG supplemented with 1000 ppm of tocotrienols..

Study 2. Effect of squalene, tocotrienols and tocopherols in serum and liver lipids. Four groups of hamsters were used. Group 1 was fed on a control semisynthetic diet which contained POTG as the dietary fat. Group 2 was fed on the control semisynthetic diet supplemented with 0.1% of squalene. Group 3 was fed on the control semisynthetic diet supplemented with 0.1% of squalene and 162 ppm of tocotrienols. Group 4 was fed on the control semisynthetic diet supplemented with 0.1% of squalene and 72 ppm of tocopherols.

Analysis of serum lipids

Serum total cholesterol, HDL-cholesterol and TG levels were analysed by enzymic methods using Sigma diagnostic kits (Sigma Chemical Co., St Louis, USA)[7]. LDL-cholesterol level was determined from the supernatant of the HDL fraction according to that described by Pearce *et al*[14].

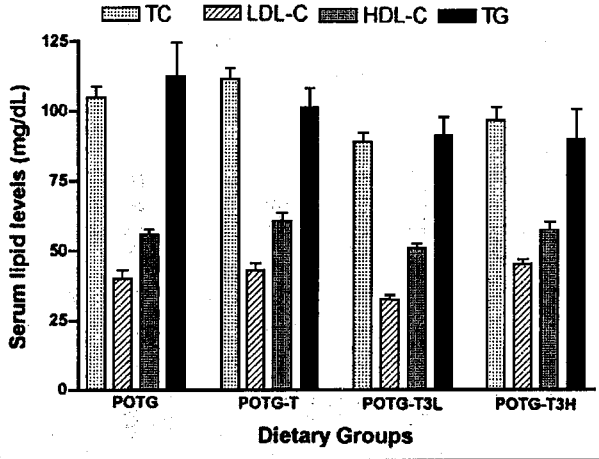
Analysis of liver lipids

Liver lipids were extracted with chloroform-methanol (2:1,v/v) as described by Folch *et al*[15]. Liver lipids were fractionated into neutral and polar lipid fraction on a acid-treated Florisil column[16]. The neutral lipids were then separated into different lipid classes on HPLC using a 10 μ silica column and quantified with a evaporative light scattering detector[17].

Results

The hamsters grew well and appeared healthy on these semisynthetic diets and no significant difference in body weights was evident at the end of the feeding period. Supplementation of the POTG diets with different levels of tocotrienols and tocopherols had significant effect on the serum lipids. As shown in Figure 1, the serum total cholesterol (TC), LDL-C and HDL-C levels were somewhat elevated, though not significantly, when the hamsters received a diet supplemented with 72 ppm of tocopherols (POTG-T) as compared to the control, POTG, group which received no tocopherol supplementation.. When tocotrienols were supplemented at 162 ppm the serum TC, LDL-C and HDL-C levels were lower as compared to that of the control POTG group, but statistical significance was attained only between TC differences of the control (POTG) and tocotrienol-treated (POTG-T3L) groups. When the level of tocotrienol supplementation was raised from 162 ppm to 1000 ppm in the diet, the serum TC, LDL-C and HDLC levels were not further reduced but on the contrary somewhat raised as compared to the POTG-T3L group.

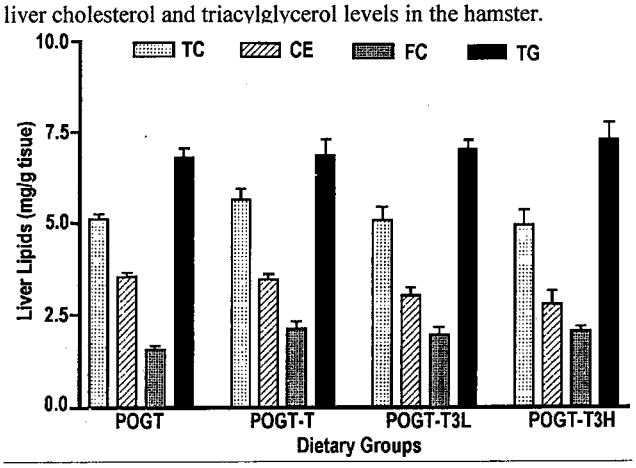
Figure 1. Effect of tocopherol and tocotrienol supplementation on the serum lipid levels in the hamster.



Supplementation with either tocopherols or tocotrienols appeared to lower the serum TG level as compared with the control but due to variability in TG values among groups there was no statistically significant effect. There also appeared to be no differential effect between the tocopherols and the tocotrienols.

Supplementation of the diets with tocopherols and tocotrienols produced no effect on the liver total lipids. Similarly tocopherol supplementation caused only a slight elevation whereas tocotrienol supplementation caused a slight reduction in liver TC when compared to the control (Figure 2). The slight elevation in liver TC in POTG-T, POTG-T3L and POTG-T3H groups as compared to the control was due to substantial increases in free cholesterol (FC) levels. the liver TG level was also appeared to be slightly elevated by tocopherol and tocotrienol supplementation (Figure 2). The other liver lipids such as diacylglycerols (DAG), monacylglycerols (MAG) and free fatty acids (FFA) were not much affected.

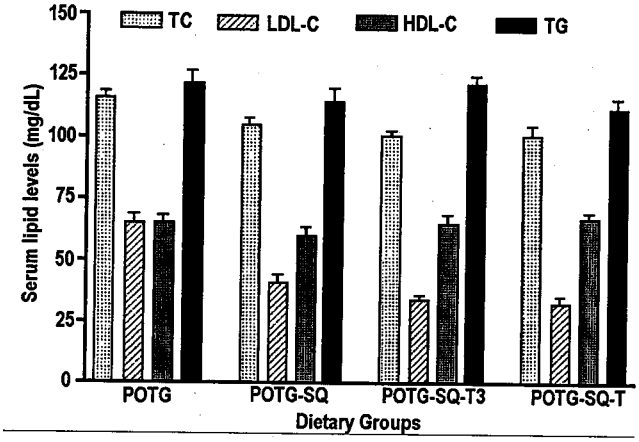
Figure 2. Effect of tocopherol and tocotrienol supplementation on liver cholesterol and triacylglycerol levels in the hamster.



The effect of squalene supplementation (0.1%) to a cholesterol-free, high fat semi-synthetic diet on serum lipids is shown in Figure 3. These results show that squalene supplementation significantly lowered ($P<0.05$) serum TC level between the control and the squalene-supplemented groups; the HDL-C, LDL-C and TG levels also appeared to be somewhat lower as compared to the control POTG group. When tocotrienols (162 ppm) were added to the squalene-supplemented diet the serum TC and LDL-C ($P<0.01$) levels were further lowered as

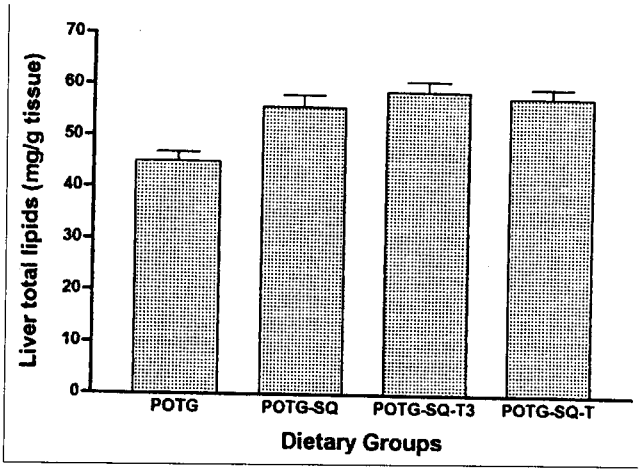
compared to the control POTG group. The HDL-C and TG levels were not affected as compared to the POTG group. Addition of tocopherols (72 ppm) to the squalene-supplemented diet produced significantly lower serum TC and LDL-C levels ($P<0.01$) than that of the control POTG group. The HDL-C and TAG levels were not much affected by the tocopherol addition.

Figure 3. Effect of squalene, tocopherol, and tocotrienol supplementation on serum lipid levels in the hamster.



Squalene supplementation in the diet also produced significant changes in the liver lipid profiles. Squalene supplementation in the diet resulted in significant increase in liver total lipids (TL) as compared to the control group (Figure 4). The increase in liver TL in the squalene-supplemented group was due to significant increase in liver cholesterol content which was attributed to a significant increase in cholesterol esters; the free cholesterol content was not affected. There was a slight increase in liver TG content of squalene -supplemented group as compared to the control (Figure 5). The other liver lipids, namely DAG, MAG, and FFA, were not much affected by squalene or tocotrienol or tocopherol supplementation.

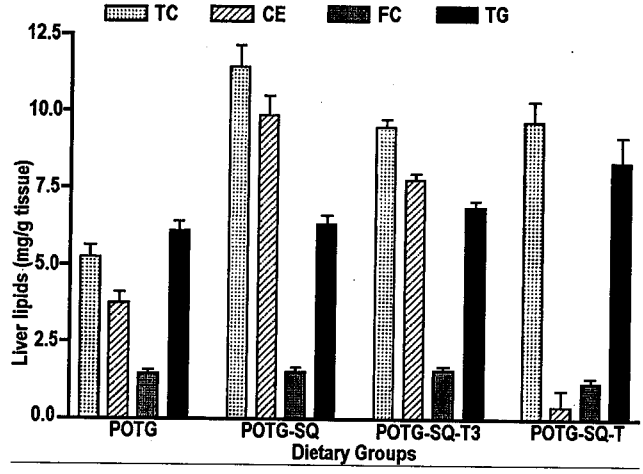
Figure 4. Effect of squalene, tocopherol and tocotrienol supplementation on liver total lipids in the hamster.



Discussion

The antioxidant effect of vitamin E, both tocotrienols (only *in vitro*) and tocopherols, in biological systems is well established[18] but the cholesterolaemic effect of these vitamins is still controversial. Previous studies in humans showed that tocopherol supplementation had no effect on serum cholesterol level[19-24] or actually showed a slight lowering effect on serum cholesterol in some individuals[25,26]. In animal models, Khor and Chieng[27] reported that short-term (6 days) treatment of male guinea pigs with tocopherols produced no effect on serum cholesterol levels, but Chen *et al*[28] reported that adding 100 ppm tocopherols to a semipurified diet containing vitamin E-stripped corn oil significantly increased serum total cholesterol and HDL-C levels in male Sprague-Dawley rats. Our present results (Figure 1) which show that hamsters fed on semipurified diet containing 72 ppm of tocopherols for 45 days had a nonsignificant rise in serum total cholesterol level suggests that tocopherols are probably neutral.

Figure 5. Effect of squalene, tocopherol and tocotrienol supplementation on liver cholesterol and triacylglycerol levels in the hamster.



Tocotrienols isolated from barley was shown to inhibit HMG CoA reductase activity in broilers[29]. More recently Khor *et al*[13] reported that tocotrienols isolated from palm oil fatty acid distillate inhibited HMG CoA reductase activity in the guinea pig after 6 consecutive treatments. A tocopherol-tocotrienol rich fraction from palm oil, Palmvitee, however, produced inconsistent results on human trials; both positive[6,7] and neutral effect[8] were observed. The discrepancy in observations on Palmvitee effect on serum cholesterol levels was ascribed partly to the dosages of Palmvitee used in the above experiments. Our present results (Figure 1) which show that tocotrienols were more effective at lower dosages in lowering serum cholesterol level than at higher dosages and that low dosage (5 mg/day for 6 days) of tocopherols showed slight inhibitory effect whereas high dosage (50 mg/day for 6 days) of tocopherols showed strong enhancing effect on HMG CoA reductase activity[27] may explain the above observed discrepancy with Palmvitee trials. However, 6 days is not long enough to be of clinical trial importance. More recently Qureshi *et al*[30] reported that small dosage (21 nmol/g) of α-tocopherol attenuated the inhibitory effect of γ-tocotrienol on HMG CoA reductase activity in chickens. Moreover, further experiments are required to establish the differential dose-dependent cholesterolaemic effect of tocotrienols and tocopherols.

Cholesterol biosynthesis in the mammalian body is subjected to feedback regulation by its intermediates. Squalene is a key intermediate in the biosynthesis of cholesterol and its cellular level is believed to play a regulatory role in cholesterol biosynthesis[31]. Squalene is present in many food products in different amounts[32] and its daily intake for an individual varies according to the types of foods consumed. In America the squalene intake can vary from 25 mg to 200 mg[32]. Previous studies with nonhyper-cholesterolaemic subjects showed that squalene feeding (900 mg/day) for 30 days did not raise serum cholesterol levels[33]. On the other hand, studies with hypercholesterolaemic subjects showed that low squalene supplementation (0.5 g/day) in the diet had no serum cholesterol-raising effect; whereas large supplementation (1 g/day) elevated the serum cholesterol levels[34]. In laboratory animal studies, Tilvis and Miettinen[35] observed that supplementation of 1% of squalene in the diet did not significantly raise serum cholesterol levels in male Sprague-Dawley rats whereas Huang *et al*[36] reported that

adding 1% of squalene to the diet significantly increased serum cholesterol levels in the rat. In our present study adding 0.1% squalene in the diet significantly lowered serum total cholesterol level in the hamster (Figure 3). The differences in the above observation could be due to differences in the animal models and the level of squalene supplementation. In our study the lowering of serum cholesterol levels was associated with a significant increase in liver cholesterol esters (Figure 5). It appears that squalene may enhance the cholesterol esterase activity in the liver resulting in accumulation of cholesterol esters in the tissue. Further experiments are in progress to confirm this assumption.

Addition of tocotrienols to squalene-supplemented diet resulted in further reduction of serum cholesterol levels (Figure 3). This result confirms earlier observation that tocotrienols possessed hypocholesterolaemic effect in the hamster. Unexpectedly addition of tocopherols to squalene supplemented also resulted in further reduction in serum cholesterol levels in the hamster (Figure 3). Nakabayshi *et al*[37] also observed that α-tocopherol enhances the hypocholesterolaemic action of sesamin in rats.

In conclusion, our results show that tocotrienols possess hypocholesterolaemic effect while tocopherols may have hypercholesterolaemic action in the hamster. Squalene at 0.1% supplementation shows hypocholesterolaemic effect and tocotrienols and tocopherols may enhance the hypocholesterolaemic action of squalene. The hypocholesterolaemic action of squalene appears to be mediated by an accumulation of cholesterol esters in the liver.

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生育三烯酚，生育酚和鯊烯的催脂血作用

摘要

Syrian金倉鼠已廣泛用作實驗模型，去研究動脈粥樣硬化和心血管疾病的形成和病因．倉鼠對膳食脂肪的反應在許多方面與人類相似．由于從棕櫚油提取的富含生育三烯酚碎片（TRF）的催脂血作用在人類實驗中仍未得到一致的效果．因此該研究用一種缺所有非皂化物質的棕櫚油三酰基甘油碎片（POTG）作爲膳食脂肪喂養動物．我們第一個實驗結果顯示，加入162ppm生育三烯酚于POTG（POTG－T3L）膳中，與POTG膳組比較可明顯降低血清總膽固醇（Tc）（P<0.05）；但POTG－T3L組的血清LDL－c，HDL－c和TG水平較之POTG組没有明顯下降（P<0.05）．增加補充生育三烯酚于膳食中（POTG－T3H）與原來低生育三烯酚組（POTG－T3L）比較似可增加血清Tc，LDL－c和HDL－c水平．這種觀察是有趣的，但仍得不到較好的解釋．當生育酚加至72ppm于POTG膳中，可觀察到血清Tc，LDL－c和HDL－c水平．與原來POTG組比較均有某些程度的增加．這些結果指出了生育三烯酚和生育酚在倉鼠體内也許有增加血液膽固醇的作用，對這些維生素E异構體的作用進一步實驗證明是需要的．．我們第二個實驗加入鯊烯去研究生育三烯酚和生育酚的催膽固醇血的作用．鯊烯是膽固醇合成代謝中的重要中間產物，同時又是一種爭論性的降膽固醇制劑．我們的結果顯示：加入0．1％鯊烯于膳食，與POTG組比較，可明顯降低血清Tc（P<0.05）．鯊烯的加入似可降低LDL－c，HDL－c和TG的水平，但是POTG－SO組與POTG組比較没有統計學上的顯著性（P<0.05）．當加入生育三烯酚和生育酚于含有鯊烯的POTG膳中，與POTG組和POTG－SQ組比較，可進一步降低Tc和LDL－c水平（P<0.01）．但HDL－c和TG水平不受影響．這些結果指出了生育三烯酚和鯊烯在倉鼠體内可降低血液膽固醇的作用．而生育酚也許有增高膽固醇的效果．

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