CHAPTER 3

EFFECTS OF DIETARY FATS AND VITAMIN E ON EXPERIMENTAL ATHEROSCLEROSIS

3.1 INTRODUCTION

3.1.1 Malaysian Palm Oil

The Malaysian oil palm *Elaeis guineensis* is a tropical plant originating from West Africa. It was brought into Malaysia in the 1870's and initially grown as an ornamental plant; cultivation of the oil palm as a plantation crop was only started in 1910's [81]. The major oil palm variety grown in Malaysia is the *tenera*, a hybrid of *dura* and *pisifera* varieties. Two types of vegetable oils are produced from the oil palm fruits, crude palm oil from the fruit's mesocarp and palm kernel oil from the kernel. The crude palm oil can be fractionated into high melting point and low melting point fractions called crude palm stearin and crude palm olein, respectively [132]; these fractions have different fatty acid compositions as shown in Table 3.1. A multistage crystallization process of the palm oil can yield three fractions, i.e. super olein (60% yield, iodine value or IV 65), palm midfraction (30% yield, IV 36-38) and palm stearin (10% yield, IV 20) [116,132,337,642]. The major constituents of crude palm oil are triglycerides (ca. 94%) and free fatty acids (3-5%). Physical refining will remove most of the free fatty acids, oxidation products and other impurities. A combination of refining and fractionation of the crude palm oil will produce several finished palm oil products including refined-bleached-deodorized palm stearin (RBDPS), refined-bleached-deodorized palm olein (RBDPO) and others which have a wide range of versatile properties such as suitable for cooking oil, various food products and also non-food materials. About 1% of minor constituents are present in the palm oil including carotenoids (500-700 ppm), vitamin E (600-1000 ppm), triterpenoids (40-80 ppm), sterols (326-527 ppm), polar lipids, squalene and other hydrocarbons (200-500 ppm), and other natural products.

3.1.2 Nutritional Value of Palm Oil

Anti-palm oil nutrition campaign

Anti-palm oil campaigns have been initiated in the United States in 1986 because of the competition with the American soyabean oil. A series of adverse publicity have

Table 3.1 Fatty acid compositions of palm and other vegetable oils

Oil	6:0	8:0	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1
Palm Oils [43,116,117,622]															
Palm oil	-	-	-	0.1	1.0	43.7	0.1	4.4	39.9	10.3	0.3	0.3	-	-	-
Palm olein	-	-	-	0.1	1.0	39.8	0.2	4.4	42.5	11.2	0.4	0.4	-	-	-
Super olein	-	-	-	0.1	1.1	31.5	0.4	3.2	49.2	13.7	0.3	0.4	-	-	-
Palm stearin	-	-	-	0.1	1.3	54.0	-	4.7	32.3	7.0	-	-	-	-	-
Palm kernel oil	0.3	4.4	3.7	48.3	15.6	7.8	-	2.0	15.1	2.7	-	-	-	-	-
Other Vegetable Oils															
Cocoa butter	-	-	-	-	0.1	26.0	0.3	34.4	34.8	3.0	0.2	1.0	-	0.2	-
Coconut	1.3	12.2	8.0	48.8	14.8	6.9	-	2.0	4.5	1.4	-	0.1	-	-	-
Corn	-	-	-	0.1	0.2	13.0	-	2.5	30.5	52.0	1.0	0.5	0.2	-	-
Cottonseed	-	-	-	-	0.8	27.3	0.8	2.0	18.3	50.2	trace	0.3	-	-	-
Linseed	-	-	-	-	-	6.1	0.1	3.2	16.6	14.2	59.8	-	-	-	-
Olive	-	-	-	-	-	10.3	0.7	2.3	78.1	7.3	0.6	0.4	0.3	-	-
Peanut	-	-	-	-	-	12.5	-	2.5	37.9	41.1	0.3	0.5	0.7	2.5	1.0
Rapeseed (high erucic)	-	-	-	-	-	3.0	-	1.0	16.0	14.0	10.0	1.0	6.0	trace	49.0
Rapeseed (low erucic)	-	-	-	-	-	4.0	-	2.0	56.0	26.0	10.0	trace	2.0	trace	trace
Safflower (high 18:1)	-	-	-	-	0.1	5.7	-	2.3	73.6	15.8	-	0.7	0.3	-	-
Safflower (high 18:2)	-	-	-	-	0.1	6.5	0.1	2.9	13.8	75.3	-	0.4	0.2	-	-
Sesame	-	-	-	-	-	9.2	0.1	5.8	38.2	45.0	0.6	0.5	-	trace	-
Soyabean	-	-	-	-	-	11.0	0.5	4.0	22.0	53.0	7.5	1.0	1.0	-	-
Sunflower (high 18:1)	-	-	-	-	0.1	3.6	0.1	4.9	80.6	8.4	-	0.4	-	1.2	-
Sunflower (American)	-	-	-	-	0.1	5.5	0.1	4.7	19.5	68.5	-	0.3	-	0.9	-

been made against using palm oil in a variety of food products [612]. A few organizations have attempted to classify the palm oil as a saturated fat and tried to recommend it to be avoided in the American diets. Petitions to the United State Food and Drug Administration (FDA) to label palm oil and palm kernel oil as saturated fats [612] however failed, but the discriminatory labelling such as "No Tropical Oil" or "No Palm Oil" on several food products were only removed after a few years of misguided practice in the United States [453]. During the 1986 Annual Conference of the American Dietetics Association, participants involved in a questionnaire survey included several professional dietitians and nutritionists but most of them were unable to differentiate between the palm oil and palm kernel oil [612]. In fact, there are some misleading reports on the nutrition of palm oil in the previous years, particularly by classifying it as a saturated fat together with coconut oil which is 94% saturated and palm kernel oil which is 82% saturated. For example in a study [369], palm oil has been discriminated as an atherogenic oil where in fact palm kernel oil was used. Palm oil is the major oil derived from fruit mesocarp and palm kernel oil derived from fruit kernel and they actually have different physical properties and fatty acid compositions as shown in Table 3.1. A comparison of the fatty acid compositions of various palm oil products with other edible oils is also shown in Table 3.1.

Nutritional quality of palm oil

A number of studies have demonstrated that palm oil is not hypercholesterolemic but is a nutritious edible oil [9,116,117]. The unique chemical constitution and the excellent oxidative stability [20,293] of palm oil contribute to its nutritional values. Compared with other vegetable oils such as corn, soyabean and cotton seed oils which are too high in polyunsaturated fatty acids, palm oil is the most stable cooking oil with an excellent frying performance [19,169,703] since less peroxide, oxypolymers and other toxic oxidative products are formed during the cooking process [72]. The stability of palm oil has been attributed to its fatty acid composition, with only a moderate amount of

linoleic acid (ca. 11%) and a low level of linolenic acid (ca. 0.3%). Besides this, the antioxidant activity of tocopherols and tocotrienols also contribute to the stability of palm oil as vitamin E is capable in scavenging singlet oxygen and free radicals, thus will terminate free radical chain reactions which usually occur during the peroxidation of unsaturated fatty acids. A few reports have demonstrated that palm oil diets could promote the anti-clotting activity of prostacyclin or decrease the formation of thromboxane in animals [3,276,531]. Human studies show that palm oil diets could increase the beneficial high-density lipoprotein cholesterol, reduce the low density lipoprotein triglycerides [277] and cholesterol [336,400,408] levels in serum. In animal studies, animals fed palm oil diets have lower blood cholesterol levels as compared to tallow, lard and olive oils [491,609,614]. There are also reports that palm oil is equally as hypocholesterolaemic as olive oil [288]; palm oil and olive oil show similar lipidemic effects in hamsters [338,339] and guinea pigs [340].

Nutritional value of minor components in palm oil

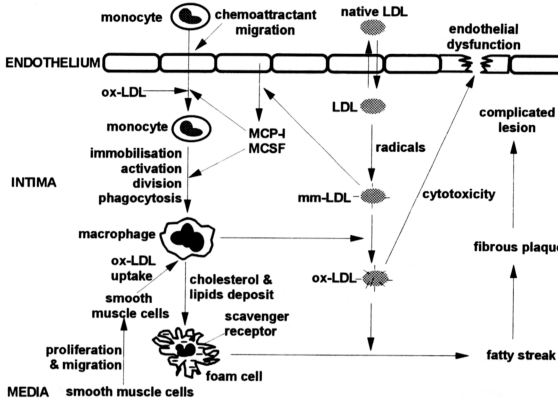
Numerous reports on the nutritional and beneficial properties of palm oil are mainly related to its minor components especially tocotrienols and carotenoids. For example, tocopherols and tocotrienols can reduce platelet aggregation and modulate prostanoid synthesis [99,395,529,603], and reduce the risk of certain experimental cancers [322,348,655]. Tocotrienols, but not tocopherols, have been reported to suppress cholesterol production [528-530,678]. β-Carotene is well known as a unique natural quencher of singlet oxygen [176,533] but it can also function as a radical-trapping antioxidant [86,424]. While having the advantage of being a precursor of vitamin A, dietary and supplemental β-carotene have been shown to possess various beneficial nutritional values [128,429,576]. For instance, the plasma level of β-carotene has been inversely related to the lower risk of certain cancer incidence [510,655,691]. In the present practice of palm oil refining, most of the carotenoids however are destroyed by the use of physical refining. In a modified refining process using lower temperature and better

vacuum it is now possible that these compounds can be retained to produce a carotene-enriched 'red palm oil' [103].

3.1.3 Atherosclerosis

Pathogenesis of atherosclerosis

Atherosclerosis is a metabolic disease which leads to reduction in the luminal diameter of the main arteries but it is a multifactorial process as illustrated in Fig. 3.1. Atherosclerosis has been recognized as one of the major factors for cardiovascular heart disease [377,541]; it can even be found in childhood [249,606] with spot injury to the artery wall. Atherosclerosis is histologically characterized by cellular proliferation, primarily of macrophages and smooth muscle cells with the accumulation of lipids, mainly cholesterol both intracellularly and extracellularly [144]. Atherosclerosis begins when blood constituents including lipoproteins leak into the artery wall through a damaged endothelium [143,659]. It has been shown by immunochemical techniques that large amounts of lipoproteins and other plasma proteins are present in atherosclerotic lesions [589,591,659,688]. An early event in atherogenesis may be adhesion of monocyte followed by migration of these cells through the endothelial junctions where they accumulate lipids and become foam cells [205,313]. Most of the cholesterol-loaded foam cells have been considered to be derived from resident tissue macrophages or from blood monocytes [178,204,206,411,558] which have entered the arterial intima in response to some chemotactic factors. In the presence of foreign materials (e.g. modified low density lipoprotein), smooth muscle cells from the middle layer of artery wall (i.e. media) are stimulated to proliferate and migrate into the inner layer (i.e. intima) in order to eliminate the foreign matters by phagocytosis [540]. After ingestion by the smooth muscle cells, all of the foreign constituents can virtually be hydrolyzed within lysosomes to yield small, relatively soluble materials such as amino acids, fatty acids and monosaccharides, which can then leave the cells and be transported out of the artery wall, but a relative deficiency in lysosomal acid lipase activity [130,568,569] will accelerate the atherogenesis.



LDL = low density lipoprotein, mm-LDL = minimally modified LDL, ox-LDL = oxidized LDL, MCP-1 = monocyte chemotactic protein-1, MCSF = macrophage colony-stimulating factors.

Fig. 3.1 The multistage events of atherosclerosis in the arterial wall.

Moreover, excessive cholesterol liberated from the hydrolysis of low density lipoprotein (LDL) may not be able to leave the smooth muscle cells unless it can be efficiently solubilized and transported into the plasma by a carrier, but this process is slow and hence the cholesterol accumulates within the cells. The smooth muscle cells esterify some of the unexcreted cholesterol for storage as cholesteryl esters [221]; a progressive accumulation of the cholesteryl esters in human atherosclerotic lesions has been documented [591]. The continuous macrophage activity and liberation of chemotactic and chemoattractant factors as well as oxygen free radical production lead to the continuous accumulation of macrophages, accelerate the proliferation and migration of smooth muscle cells to the intima [207]. Lipid-laden cells in the intima were mainly macrophages come from the blood stream and also smooth muscle cells which have migrated from the media [178,311]. Smooth muscle cells produce large amounts of collagen, elastin, and proteoglycans, all of these form atheromatous plaque [439]. In hypercholesterolaemia, macrophages will accumulate oxidized LDL to form foam cells and progress to fatty streaks [178,410,541], and then fibrous plaques by the uncontrolled accumulation of smooth muscle cells, connective tissue, both intracellular and extracellular lipids, necrotic debris and calcium [412]. The interaction of the vessel wall with platelets is also important in the progression of a fatty streak lesion to the complex proliferative lesion of severe atherosclerosis [671]. As a result of the multistep processes, the arteries become narrower, which causes decreased blood flow and eventually become completely blocked.

Risk factors for atherosclerosis

The known causes of atherosclerosis are generally classified into two major types, i.e. the endogenic and exogenic factors. The endogenic factors include hyperlipidemia such as hyperlipoproteinaemia [599], hypertension and diabetes mellitus; the exogenic factors are smoking, stress, overweight, lack of exercise, cholesterol intake and dietary fats [134,513]. All of these factors act in different ways or sometimes in combination to contribute to endothelial dysfunction or injury. Smoking can cause an increased extent of

atherosclerosis [605] because some components present in the cigarette smoke has adverse effects on platelet function, plasma lipoprotein levels, macrophage responses and hemodynamic regulation [241,251,307,565]. Hypertension could cause mechanical injury, or conceivably an alteration of the metabolic function of endothelial or other arterial cells. Hemodynamic factors [210] also lead to endothelial and platelet events which may contribute to the atherosclerosis. Endothelial injury and dysfunction have also been shown to occur in experimental animals subjected to manipulations such as diabetes mellitus and stressful stimulation which have been associated with increased risk of atherosclerosis [222]. Hyperlipidemia, or some component of hyperlipidemic serum, as well as other risk factors are thought to cause endothelial injury, resulting in adhesion of platelets or monocytes, release of platelet derived growth factor (PDGF) and other growth factors which leads to smooth muscle migration and proliferation [439].

3.1.4 Effect of Dietary Factors on Atherosclerosis

Diet has been the major focus of research in relation to the exogenic factors for cardiovascular diseases. Some dietary factors affecting atherogenesis include cholesterol, dietary fats, micronutrients and other natural products. The development of atherosclerosis is an important aspect of cardiovascular disease and is influenced by the plasma cholesterol level [630] which can be modulated by the amount and type of dietary fats and various micronutrients, e.g. tocotrienols [663].

Cholesterol

High level intakes of dietary cholesterol have been demonstrated to cause a substantial increase in serum cholesterol and has been used to induce atherosclerosis in experimental animals [260,334,405,527]. The level of esterified cholesterol in the aorta also increased in human atherosclerosis [588] as well as experimental atherosclerosis [247,456,594]. Despite the large number of accumulated reports for the effect of dietary factors on plasma cholesterol and atherosclerosis, the effect of dietary cholesterol on the

changes of plasma lipids and the mechanism of action are still poorly understood. Most of the studies have only reported in isolation the effects of various dietary fats on the lipid metabolism or the development of atherosclerosis.

Saturation and unsaturation of fatty acids

The fatty acid compositions of plasma [613] and lipoprotein [428,570] lipids have a direct relation with the nature of dietary fats. Particularly, an unsaturated dietary fat is known to increase the content of unsaturated fatty acids in the plasma lipoproteins [73,325,428]. Polyunsaturated fatty acids are known to exert a hypocholesterolaemic effect in humans and animals; however, high levels of polyunsaturated fatty acids in plasma and tissues may also pose a deleterious effect resulting from lipid peroxidation apart from problems associated with lowering of the immune system function. Atherogenicity with regard to the saturation and unsaturation of fatty acids has been indirectly predicted by the risk factors of atherosclerosis which are commonly determined by the cholesterol levels of plasma or lipoprotein fractions. It is generally accepted that diets with high content of saturated fatty acids such as lauric and myristic acids can elevate serum cholesterol level [260], promote thrombosis [305] and enhance atherogenesis. Lauric acid however has also been reported to exert only small effect in raising plasma cholesterol level [232]. Dietary replacements of saturated fats by monounsaturated [32,230,260,332] or polyunsaturated [26,47,148,229,332,334,406,415, 569] fats have been consistently reported to exhibit a significant hypocholesterolaemic effect. However, when added to a cholesterol-free commercial feed for rabbits, cocoa butter [112] rich in stearic acid as well as other saturated fats [366] are not atherogenic. Despite of various discrepancies in reports on the hypercholesterolaemic effect of other saturated fatty acids [133,260,333], stearic acid (18:0) was consistently reported to have a neutral cholesterolemic action. When saturated fats were added to a commercial rabbit chow rather than to a synthetic or semi-synthetic diet, hypercholesterolaemia and atherosclerosis do not develop in the animals [368].

Unlike lauric and myristic acids, palmitic acid does not raise the level of blood cholesterol in monkeys [252].

Other Factors

There are many other dietary factors associated with atherosclerosis and cardiovascular diseases. For example, there is increasing evidence that *trans* fatty acids may increase the levels of LDL cholesterol [46] and lipoprotein(a) (Lp(a)) [417]. Lp(a) is now widely accepted as another important risk factor for cardiovascular disease, high levels of Lp(a) have been associated with heart attacks and stroke [375]. Furthermore, there is considerable heterogeneity among lipoproteins and recent evidence indicates that high density forms of LDL and Lp(a) may be more atherogenic [98].

3.1.5 Lipoproteins and Atherosclerosis

Among various plasma lipoproteins, LDL is the major carrier of cholesterol in circulation, it has been identified as the principal pro-atherogenic lipoprotein [600,602] and most often linked to the risk of coronary heart disease in epidemiological studies [93,386]. An increase in plasma levels of LDL or other lipids may increase penetration of the LDL into the artery wall and subsequently subjected to uptake and degradation by vascular smooth muscle cells and infiltrating macrophages [74,577,590]. Foam cell formation can be promoted by intimal proteoglycan-LDL complexes [555,654], and modification of LDL by malondialdehyde [174] or free radical oxidation [500]. Uptake of cholesterol by the classical LDL receptor pathway is subjected to feedback inhibition by cellular cholesterol content [75]. It has been shown that monocyte migration leads to increased LDL transport across the aortic endothelial cell monolayer in culture [628]. LDL can cause smooth muscle injury [266], and smooth muscle cell proliferation is stimulated by some hyperlipidemic components in the serum [172]. Platelet aggregation, with subsequent release of PDGF and smooth muscle replication, is favoured by higher LDL levels [94]. Cellularly modified LDL causes monocyte recruitment by chemotactic

mechanisms, inhibition of macrophage motility, and enhances the rate for formation of foam cells [525,526].

Effects of oxidized LDL

A high level of LDL-cholesterol is not a sufficient condition for atherosclerosis as the incidence of coronary heart disease among hypercholesterolaemic individuals remains variable [274]. This variation is a consequence of different biochemistry among individuals of which modification of LDL and its metabolism are probably more important. There is fast growing evidence to support the hypothesis that oxidative modification of plasma LDL enhances its atherogenic properties [21,95,264,427,600]. The major targets of peroxidation of LDL are polyunsaturated fatty acids [159,315,600,602,690]. A diet rich in monounsaturated fatty acids enhance the oxidative stability of animal [501] or human [47] plasma LDL when compared with a diet high in polyunsaturated fatty acids. The accumulation of LDL-derived cholesterol in cells of the arterial wall is now widely recognized as a result of the modification of LDL, especially since oxidatively modified LDL has been detected in atherosclerotic lesions [71,690]. Oxidized and mildly oxidized LDL, wholly or partially inclusive of oxidized products leaving the particles, can have a number of deleterious effects, e.g. being chemotactic for monocytes, immunogenic and inhibitory of nitric oxide-mediated relaxation of coronary tone, causative of the accumulation of inflammatory cells, disturbance of homeostasis, etc. [526,539,597]. Recognition and uncontrolled uptake of the oxidized LDL by macrophages cause a series of biochemical events which may then lead to the formation of foam cells, fatty streaks and complicated atherosclerotic lesions. The free radical-mediated oxidation products of LDL polyunsaturated fatty acids include a variety of reactive hydroperoxides and aldehydic fragments which can modify the LDL [159,314] by forming complex products presumably with epsilon-amino groups of lysine residue [159,174]. The oxidative degradation of LDL may essentially occur at the unsaturated bonds of polyunsaturated fatty acids [159,161,380] or cholesterol [704], this destructive process can be initiated either by

exogenous oxidizing compounds or prooxidants [164] and endogenously cell-derived radicals such as superoxide [269,310]. Modified lipoproteins, especially the oxidized LDL become more atherogenic than the native form [600] and has been related to an unregulated enhanced uptake by macrophages *via* a scavenger receptor pathway [265,601], and this has been widely implicated in the atherogenesis [92,240,496]. The oxidized LDL can also act as a potent chemoattractant for the circulating monocytes [315,600,602,690]. Cytotoxicity due to oxidized LDL may induce endothelial cell dysfunction and promote the evolution of the fatty streak to a more complex and advanced lesion. In the early phase of oxidation, minimally modified LDL is formed in the subendothelial space, such mildly oxidized LDL could induce the endothelium to express adhesion molecules for monocytes, and to secrete monocyte chemotactic protein (MCP-1) and macrophage colony-stimulating factors (MCSF) [46,125]. These molecular events (as depicted in Fig. 3.1) result in monocyte binding to the endothelium and its subsequent migration into the subendothelial space where minimally modified LDL promotes its differentiation into macrophages. Because the oxidized LDL is a potent inhibitor of macrophage motility, it could also promote the retention of macrophages in the arterial wall [525]. These macrophages further modify the minimally modified LDL to a more oxidized form [447] which is then taken up by smooth muscle cells *via* a scavenger receptor mechanism and thus accelerate the production of macrophages.

Effects of antioxidants

There has been accumulated evidence in the literature for the protective effects of natural antioxidants especially vitamin E and β-carotene in atherosclerosis and cardiovascular diseases [656]. Epidemiologic data suggests that persons with high intake of antioxidant vitamins have a lower risk of cardiovascular disease [202]; a significant inverse correlation between cardiovascular disease and vitamin C, vitamin E or β-carotene has been observed. A growing body of evidence shows that smoking can decrease the

plasma levels of vitamin C, vitamin E and β-carotene [655] making smoking the most important risk factor in cardiovascular disease.

The role of oxidized LDL in atherogenesis is supported by the observations that antioxidants such as probucol and butylated hydroxytoluene can inhibit the development of atherosclerotic lesions in Watanabe heritable hyperlipidemic rabbits (WHHL) and cholesterol-fed rabbits [55,92,344]. Chain-breaking antioxidants such as vitamin E has been reported to play a profound role in inhibiting the oxidation of LDL *in vitro* [161,310]. While a number of studies demonstrated that vitamin E (especially α-tocopherol) and other antioxidants like probucol [92,163,344] have suppressive effects on atherosclerosis [679,686], some studies indicated that vitamin E lacked of this beneficial activity, and it is noteworthy that a high dietary level of vitamin E can even promote the potentiation of atherosclerotic lesions [213]. Although the actual initiation process of the peroxidation of LDL is not precisely known, accumulated evidence indicates the end products are derived from lipid peroxidation. Among the various factors determining the susceptibility of LDL particles to oxidation, it is generally considered that antioxidants are most important in inhibiting oxidation, and the polyunsaturated fatty acids in LDL particles will be the targets of attack by radicals once the antioxidative defense systems are exhausted. However, diets rich in monounsaturated fatty acids have been reported to increase the resistance of plasma LDL to oxidative modification, independent of the content of antioxidants [65]. The oxidation of LDL induced by radical initiators have been demonstrated to proceed by a free radical-mediated chain mechanism [557]. Copper ion-induced oxidation of LDL can be suppressed by antioxidants such as α-T, γ-T, and carotenoids including retinyl stearate, lycopene and β-carotene [158,160,163,315]. The predominant antioxidant in the LDL particle is vitamin E [162,393] which can significantly reduce the susceptibility of plasma LDL to oxidation [137]. Vitamin E supplementation has been shown to suppress the increased lipid peroxidation in cigarette smokers [289], and inhibit oxidative LDL modification induced by cigarette smoking [163]. In fact, some

studies in animal models suggest that dietary vitamin E can retard the progression of atherosclerosis [282].

3.1.6 Studies on Palm Oil and Atherosclerosis

In order to counteract the adverse anti-palm oil campaign in the U.S. [612], the nutrition research on palm oil has been embarked upon promptly all over the world. It has been found that palm oil does not increase arterial thrombotic tendency, but it decreases the platelet aggregation when compared with safflower oil [275]. From the earlier literature it was reported that when rabbits were fed 2% cholesterol and 6% test fat for two months, the average serum cholesterol and severity of atherosclerosis (using 0 to 4 scale) in the palm oil-fed rabbits were less than those in the coconut oil-fed rabbits [367]. Studies carried out on human subjects also showed that palm oil and olive oil lowered plasma cholesterol level as compared to coconut oil, and that palm oil was more anti-thrombotic than olive oil [459]. In an animal study, palm oil may behave like other polyunsaturated oils in that it did not promote experimental atherosclerosis as compared to the coconut oil [347]. Despite of some reviews that palm oil has been demonstrated to be a non-hypercholesterolemic oil [9,116,147,234] there are also contradicting reports [231,532] about the nutritional effect of the oil.

3.1.7 Objectives of the Present Research

One of the aims of the present study is to correlate the experimentally induced atherosclerosis in rabbits with changes of plasma lipids in response to the feeding with atherogenic diets containing a range of dietary fat types. This study attempts to investigate the importance of palm oil's fatty acid constitution and/or the minor components with respect to the experimental atherosclerosis in rabbits. Studies were made to determine the effects of various antioxidants and dietary fats with different degrees of unsaturation on the oxidative susceptibility of LDL isolated from the treated rabbits, and the development of atheroma in their aorta was also examined.

3.2 EXPERIMENTAL

3.2.1 Diets and Animals

New Zealand White rabbits (six-month old) with body weight 1.3-1.7 kg, were randomly divided into groups and fed atherogenic semi-synthetic diets containing 0.5% (w/w) of cholesterol and 15% (w/w) of various dietary fats with or without various vitamin E supplementation. The semi-synthetic diets consisted of 5% of commercial rabbit pellets and the composition of the synthetic substances is shown in Table 3.2. All purified diet components and cholesterol were purchased from United States Biochemical Corporation (USB, Cleveland, Ohio).

3.2.2 Experimental Atherosclerosis in Rabbits

Experiment 1: Effects of various dietary fats

Rabbits were randomly divided into five groups and fed *ad libitum* semi-synthetic diets (Table 3.3) containing 15% dietary fat as follows:- (a) 13% coconut oil plus 2% corn oil (CNO), (b) 15% refined-bleached-deodorized palm olein (PO), (c) 15% crude palm olein (CPO), or (d) 15% soyabean oil (SO), all of the above diets were supplemented with 5 g of cholesterol per kg diet, (e) 15% refined-bleached-deodorized palm olein without supplementation of cholesterol (POWC). All of the rabbits were fed with these respective dietary regimes for a duration of 12 weeks.

Experiment 2: Effects of various antioxidants

Rabbits were divided into groups and fed with palm-oil based diets supplemented with various antioxidants as follows:- (a) *dl*-α-tocopheryl acetate (diet denoted as PO+α-T), (b) γ-tocotrienol (PO+γ-T_3), (c) δ-tocopherol (PO+δ-T), (d) without supplementary vitamin E (PO–E), (e) vitamin C but without supplementary vitamin E (PO+C), (f) vitamin C and *dl*-α-tocopheryl acetate (PO+CE). The compositions of various vitamin E in the diets are shown in Table 3.3. All of the rabbits were fed with the respective semi-synthetic diets for a period of 12 weeks.

Table 3.2 Formulation of semi-synthetic feed*

Component	Composition g/kg	% Energy
Casein-VITAFREE	200	22
DL-methionine	3	-
Dextrose monohydrate	150	16
Corn-starch	240	27
CELUFIL (non-nutritive bulk)	200	-
AIN mineral mixture #	45	-
AIN vitamin mixture 76 ‡	10	-
Choline dihydrogen citrate	2	-
Dietary fat	150	35

* 50 g of commercial pellets (fat content 2.5%), 950 g of a mixture of feed materials and dietary fats, and 5 g of cholesterol were blended and repelletized.

Composition of the AIN mineral mixture (in g per kg of mixture): calcium phosphate, dibasic, 500.0; sodium chloride, 74.0; potassium citrate (monohydrate), 220.0; potassium sulfate, 52.0; magnesium oxide, 24.0; manganous carbonate, 3.5; ferric citrate (16-18% Fe), 6.0; zinc carbonate, 0.6; cupric carbonate, 0.3; potassium iodate, 0.01; sodium selenite (1.5 H_2O), 0.0075; chromium potassium sulfate, 0.55; sucrose (finely powdered), 118.0.

‡ Composition of the AIN vitamin mixture 76 (in gram per kg mixture, based on the NAS-NRC recommendation): thiamine hydrochloride, 0.6; riboflavin, 0.6; pyridoxine hydrochloride, 0.7; nicotinic acid, 3.0; D-calcium pantothenate, 1.6; folic acid, 0.2; D-biotin, 0.02; cyanocobalamin (Vitamin B_{12}), 0.001; retinyl palmitate (Vitamin A), 0.8 (as stabilized powder (pre-mix) containing 500,000 I.U./g); DL-α-tocopheryl acetate, 20.0 (as stabilized powder); cholecalciferol (Vitamin D_3), 0.0025 (100,000 I.U.); Vitamin K (menadione), 0.005; sucrose (finely powdered), 972.9.

Table 3.3 Antioxidant contents in the palm oil diets (mg/kg diet)

Vitamin	Dietary groups					
	PO–E	PO+α-T	PO+γ-T_3	PO+δ-T	PO+C	PO+CE
α-TAc[#]	0	176	0	0	0	176
α-T	18	20	6	15	16	10
α-T_3	10	14	3	9	11	8
γ-T	0	0	0	34	0	0
γ-T_3	13	22	186	32	14	10
δ-T	0	0	0	863	0	0
δ-T_3	5	8	14	12	6	6
Total vitamin E	46	240	209	965	47	210
Vitamin C*	<20	<20	<20	<20	1000	1000

[#] The weight of *dl*-α-tocopheryl acetate (α-TAc) is given in α-T equivalent.

* Vitamin C was only supplemented in the semi-synthetic diets for PO+C and PO+CE groups.

3.2.3 Analyses of Lipids and Atherosclerosis

Analyses of lipids

Overnight-fasted blood samples were collected before dietary treatment and after feeding with cholesterolemic diets for 12 weeks. Plasma samples were analyzed for total cholesterol and triglycerides by a Kodak Ektachem DT60 autoanalyzer. The lipids were extracted from 1 mL plasma or LDL fractions using chloroform-methanol according to the method of Folch *et al.* [175]. The vitamin E contents of the lipids samples, redisolved in hexane, were analyzed by a modified method [372] using normal phase HPLC equipped with a silica column (4 mm x 250 mm) and a fluorescence detector, the mobile phase being hexane-tetrahydrofuran-isopropanol (v/v 973.5:25:1.5). The plasma and LDL lipids were saponified with 2M ethanolic potassium hydroxide containing 1 mg/mL of butylated hydroxy-toluene in vacuum-sealed tubes for 2 hours at 100°C. After acidification, free fatty acids were extracted with hexane and esterified with BF_3-methanol [60]. The total fatty acid composition was analyzed by a HP5970A gas chromatograph equipped with a 0.5 μm DB-23 capillary column (30 m) from J&W Scientific.

Gross examination of atherosclerosis

For the examination of atheroma in the experimental rabbits, aortas from aortic valve to the iliac bifurcation were removed from the anaesthetized rabbits and carefully freed from adventitial tissues. The aortas were opened longitudinally, pinned on wax board, fixed in 1% calcium acetate-10% formalin for more than 24 hours. The atherosclerotic aortas were stained with Oil Red O in isopropyl alcohol [6] and after rinsing with 70% alcohol preserved in 10% formalin. The areas of atheromas were grossly estimated.

Statistical Test

Student's t test was used for statistical evaluation of the results, differences at $p < 0.05$ were considered significant.

3.2.4 Experiments on the Oxidative Susceptibility of Low Density Lipoproteins

Isolation of LDL

Plasma lipoproteins were isolated by a modified method of Havel *et al.* [250] using a Beckman L8-80M ultracentrifuge equipped with a fixed-angle rotor model 45Ti. 15 mL of plasma, layered with 1.006 g/mL KBr solution, was centrifuged at 45000 rpm at 4°C for 20 hours. After removal of the chylomicron and VLDL fraction, 0.3 g KBr was dissolved in the 15 mL residue and layered with 1.019 g/mL KBr solution, centrifuged for 24 hours and the IDL was collected. The residue (20 mL) was mixed with 0.6 g of KBr, layered with 1.063 g/mL KBr solution, centrifuged for 30 hours, and the LDL (density 1.019-1.063 g/mL) was obtained from the supernatant fraction, the remainder was taken as HDL fractions.

Oxidation of LDL

The UV-absorption method as described by Esterbauer and his co-workers [163,524] was used to evaluate the oxidative stability of LDL. The LDL samples were dialyzed at 4°C for 24 hours against four changes of 100 volumes of deoxygenated phosphate-buffered saline (0.01 M, pH 7.4). Dialyzed LDL samples were diluted with oxygen-saturated phosphate-buffered solution (0.01 M pH 7.4) to obtain a solution containing 0.1 mg protein per mL solution. These LDL samples were incubated at 37°C, and oxidation was initiated by addition of copper (II) chloride solution to a final concentration of 4.0 μM. The progress of oxidation was continuously monitored using a U-200 Hitachi spectrophotometer by measuring the absorbance at 234 nm, which is characteristic for the conjugated hydroperoxy dienes produced during the lipid peroxidation.

3.3 RESULTS AND DISCUSSION

3.3.1 Lipidemic Effects of Various Dietary Fats

Effect on fatty acid compositions of plasma and low-density lipoprotein lipids

After a 12-week feeding of the high-fat semi-synthetic diets, the body weights of all groups of rabbits were about 2.0 ± 0.3 kg and no significant difference was observed among the groups. Three types of vegetable oils used for the present study are coconut-corn oil (86.7% & 13.3%) (denoted as CNO), soyabean oil (SO) and palm oils including refined-bleached-deodorized palm olein (PO) and crude palm olein (CPO), all of which contributed about 35% of caloric energy to the respective diets. The fatty acid compositions of the dietary fats are given in Table 3.4. The coconut oil was mixed with corn oil so that the blended oil contained 8.6% linoleic acid as the essential fatty acid. The CNO-based diet is rich in medium chain saturated fatty acids (i.e. lauric 12:0 and myristic 14:0), the palm oil diets (PO and CPO) are rich in palmitic (16:0) and oleic (18:1) acids, and the SO diet is rich in polyunsaturated fatty acids mainly linoleic acid (18:2).

Fatty acid compositions of the rabbit plasma before and after feeding with various diets are given in Table 3.5. The fatty acid compositions of dietary fats in semi-synthetic diets were not fully reflected in the total fatty acid compositions of plasma lipids from all groups of rabbits. Although high levels of lauric (40.4%) and myristic (16.4%) acids were fed to the CNO rabbits, compositions of these medium chain fatty acids (0.8% and 6.3%, respectively) appeared to be low in their plasma lipids. However, present results indicate that all rabbits fed cholesterolemic diets showed significant changes in the compositions of long chain fatty acids. High intakes of fat and cholesterol resulted in a relatively lower percentage of palmitic and stearic acids in the plasma lipids of rabbits as compared to the baseline values. For POWC rabbits fed with palm olein but without supplementary cholesterol, their plasma fatty acid compositions appeared to be similar as before dietary treatment. The compositions of palmitic acid in the plasma of rabbits fed CNO-, PO- and

Table 3.4 Fatty acid compositions of dietary fats* (% of total fatty acids)

Fatty acid	CNO	PO	CPO	SO
6:0	3.4	-	-	-
8:0	5.1	-	-	-
10:0	4.6	-	-	-
12:0	40.4	0.2	0.2	0.1
14:0	16.4	0.9	1.0	0.1
16:0	8.8	36.1	38.3	11.0
16:1	-	0.2	0.2	0.1
18:0	3.0	3.8	4.1	3.7
18:1	9.7	46.8	45.0	19.8
18:2	8.6	11.2	10.5	55.9
18:3	-	0.5	0.4	8.6
20:0	-	0.2	0.3	0.2
22:0	-	-	-	0.4
Saturates	81.7	41.2	43.9	15.5
C14 saturates	16.4	0.9	1.0	0.1
C14+C16 saturates	25.2	37.0	39.3	11.1
Monounsaturates	9.7	47.0	45.2	19.9
Polyunsaturates	8.6	11.7	10.9	64.5

* CNO = coconut oil (86.7%) and corn oil (13.3%), PO = refined-bleached-deodorized palm olein, CPO = crude palm olein, SO = soyabean oil.

Table 3.5 Fatty acid composition of plasma lipids (% of total fatty acids)

Fatty acid	Baseline*	CNO n = 5	PO n = 6	CPO n = 4	SO n = 6	POWC n = 5
12:0	0.8±0.4	1.5±0.6	0.1±0.1	0.1±0.1	0.2±0.1	0.6±0.3
14:0	6.3±2.1	6.3±3.5	2.2±0.2	1.2±1.0	2.1±0.9	9.7±3.2
16:0	46.7±6.0	29.5±3.2^{a}	30.2±2.8^{a}	31.6±3.9^{a}	20.8±2.8^{b}	44.8±8.8^{c}
16:1	1.4±1.0	3.7±1.2	4.2±1.7	3.7±0.8	3.4±1.1	0.6±0.1
18:0	10.3±2.0	6.8±1.2	5.7±1.2	5.8±1.5	5.9±1.3	5.3±1.4
18:1	18.4±4.1	33.7±3.3^{a}	43.7±3.9^{b}	44.5±5.4^{b}	32.5±5.1^{a}	27.8±3.0^{a}
18:2	14.2±4.0	18.9±1.4^{a}	13.6±6.4^{a}	12.7±2.4^{a}	33.2±2.4^{b}	10.8±1.9^{a}
18:3	1.3±1.0	0.4±0.2	0.3±0.1	0.2±0.1	1.0±0.5	0.3±0.1
20:4	0.1±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.3±0.2	0.1±0.1

* Values are means ± standard deviations, n = number of rabbits. Baseline values are means for all rabbits fed commercial rabbit pellets before switching to semi-synthetic diets. CNO = coconut oil (86.7%) and corn oil (13.3%); PO = refined-bleached-deodorized palm olein; CPO = crude palm olein; SO = soyabean oil. POWC = PO-based diet but without cholesterol supplementation. ^{a,b,c}Means in the same row with different superscripts are significantly different ($p < 0.05$).

CPO-diets are relatively high, i.e. 29.5%, 30.2% and 31.6%, respectively; but only 20.8% palmitic acid were present in the plasma lipids of the rabbits fed SO. The plasma levels of oleic acid for all rabbits fed high-fat diets supplemented with cholesterol increased significantly after a 12-week feeding; while drastic increases in the percentage of oleic acid were observed for rabbits fed PO and CPO, only small increases were observed for rabbits fed CNO as well as SO. The POWC rabbits fed refined-bleached-deodorized palm olein without supplementary cholesterol also showed an increased level of oleic acid but at a relatively smaller extent. Only SO-fed rabbits showed a significant increase in the percentage of plasma linoleic acid to 33.2%, other groups of rabbits showed small differences from the baseline values.

The fatty acid compositions of LDL are shown in Table 3.6. The present results are in agreement with the previous reports that caprylic (8:0) and capric (10:0) acids do not contribute to the fatty acid compositions of plasma lipoproteins [22,232]; this reflects that metabolic pathways for the short chain fatty acids are different from those for long chain fatty acids. Generally, the fatty acid profiles of LDL of the respective groups are similar to those of their plasma lipids. The percentage of palmitic acid in the LDL of CNO, PO, CPO and SO rabbits are 39.8%, 37.7%, 33.5% and 37.4%, respectively; these values are significantly lower than those of palm oil diet without supplementary cholesterol, i.e. 46.3% for the POWC rabbits. The percentages of oleic acid in the LDL of rabbits fed PO- and CPO-diets were significantly higher than those fed SO and CNO. The levels of linoleic acid in the rabbits fed with SO-diet were significantly higher than those from other groups.

Despite many reports demonstrating that lauric [397] and myristic [227,252,260] acids are the most hypercholesterolaemic fatty acids, it is a fact that only small quantities of the medium chain saturated fatty acids are found in the plasma or deposited in adipose tissue [235]. The fatty acid distributions of the diets have undergone considerable modulation by the time they are incorporated into the lipoprotein particles so that their

Table 3.6 Fatty acid composition of LDL lipids (% of total fatty acids)*

Fatty acid	CNO n = 5	PO n = 6	CPO n = 4	SO n = 6	POWC n = 5
12:0	1.7±0.4	1.0±0.6	0.3±0.1	0.6±0.4	0.3±0.1
14:0	7.0±0.7	3.7±0.6	4.5±1.0	1.2±0.1	7.2±2.9
16:0	39.8±2.3	37.7±2.4	33.5±2.9	37.4±2.5	46.3±5.3
16:1	2.2±1.8	3.4±1.7	5.4±0.8	2.6±2.3	3.7±1.3
18:0	7.7±1.1	5.9±1.1	4.3±0.3	8.6±1.3	4.0±1.0
18:1	28.6±2.6	36.9±0.8	43.7±2.5	29.8±3.5	27.7±1.5
18:2	11.9±1.1	12.2±2.1	8.0±1.9	21.2±4.5[a]	10.3±0.1
18:3	0.4±0.5	0.3±0.4	0.2±0.1	0.6±0.1	0.2±0.1
20:4	1.4±0.8	0.8±0.2	0.2±0.1	0.6±0.1	0.2±0.2

* Values are means ± standard deviations. CNO = coconut oil (86.7%) and corn oil (13.3%); PO = refined-bleached-deodorized palm olein; CPO = crude palm olein; SO = soyabean oil. POWC = PO-based diet but without cholesterol supplementation. n = number of rabbits. [a] Value significantly different ($p < 0.05$) from other groups.

distribution in the plasma only partially reflect the fatty acid composition in the diets. In the present experiments, the plasma composition of lauric and myristic acids appeared to be low although 56.8% of these fatty acids were present in the diets for the CNO-rabbits. The CNO-diet contained mainly short and medium chain fatty acids, but incorporation of these fatty acids was found to be minimal in the LDL of the CNO group as well as other groups of rabbits. These results indicate that a rapid metabolism of the short and medium chain fatty acids has occurred in the liver before incorporation in the LDL, *viz.* saturated fatty acids such as myristic and palmitic acids can undergo elongation and desaturation to oleic acid which become the major fatty acid in the LDL.

Effect on plasma cholesterol and triglycerides

The data on plasma lipids of various rabbits after dietary treatment are shown in Table 3.7. High fat intakes significantly increased the TG level from 0.64 mmol/L (baseline) to 2.2, 2.6, 2.8 and 1.8 mmol/L for CNO, PO, CPO and SO rabbits, respectively. The cholesterolemic diets also obviously elevated plasma cholesterol levels from 2.3 mmol/L (baseline) to 29.2, 28.6, 34.3 and 23.8 mmol/L for the CNO, PO, CPO and SO rabbits, respectively. However, the high-fat POWC-diet only increased the TG level but did not influence the plasma cholesterol level of the rabbits fed palm oil without supplementary cholesterol. The high-density lipoprotein cholesterol levels for all groups of rabbits fed cholesterolemic diets (CNO, PO, CPO and SO) did not show significant difference from that for POWC without cholesterol supplementation. However, low-density lipoprotein cholesterol levels of the rabbits fed cholesterolemic diets are remarkably elevated to 2.4-3.4 mmol/L as compared to 0.99 mmol/L for the POWC rabbits. The plasma vitamin E levels were also remarkably increased from 3.0 μg/mL (baseline) to 17.7, 25.2, 25.4 and 12.2 μg/mL for the CNO, PO, CPO and SO rabbits, respectively; however, the plasma vitamin E levels from the POWC rabbits seem to be unaffected by a high fat intake without supplementary cholesterol.

Table 3.7 Plasma lipids and the susceptibility parameters for LDL oxidation

	Baseline	CNO	PO	CPO	SO	POWC
Plasma Lipids						
TG (mmol/L)	0.64±0.30	2.2±1.3	2.6±1.2	2.8±1.1	1.8±1.5	1.5±0.4
Cholesterol (mmol/L)	2.3±1.8	29.2±6.3	28.6±14.9	34.3±7.9	23.8±11.4	3.8±0.9
LDL-Cho (mmol/L)	-	3.0±0.4	2.4±1.1	3.4±0.4	2.6±0.2	0.99±0.29
HDL-Cho (mmol/L)	-	0.60±0.47	0.74±0.50	1.7±0.8	0.89±0.64	0.79±0.32
Vitamin E (μg/mL)	3.0±1.0^a	17.7±4.3^b	25.2±5.6^b	25.4±10.8^b	12.2±5.1^b	3.8±1.6^a
LDL PUFA (%)	-	13.7±2.4^a	13.3±2.7^a	8.4±2.1^a	22.4±4.7^b	10.7±0.4^a
LDL MUFA (%)	-	30.8±4.4	40.3±2.5	49.1±3.3	32.4±5.8	31.4±2.8
LDL SFA (%)	-	56.2±4.5	48.3±4.7	42.6±4.3	47.8±4.3	57.8±9.3
LDL Oxidation						
Lag time (min)	-	56±6^a	111±23^b	103±25^b	54±15^a	110±8^b
Lag-phase slope (10^{-4}/min)	-	20.0±9.0	2.9±0.9	2.3±1.3	18.0±7.0	3.5±0.5
Propagation-phase slope (10^{-3}/min)	-	15±4	19±4	15±6	45±4	19±5
Maximum absorbance at 234 nm	-	1.52±0.35	1.35±0.08	1.19±0.02	2.18±0.10	1.26±0.17

Values are means ± standard deviations. CNO = coconut oil (86.7%) and corn oil (13.3%); PO = refined-bleached-deodorized palm olein; CPO = crude palm olein; SO = soyabean oil. POWC = PO-based diet but without cholesterol supplementation. LDL-Cho = Low density lipoprotein cholesterol, HDL-Cho = High density lipoprotein cholesterol, TG = triglycerides, PUFA = polyunsaturated fatty acids, MUFA = monounsaturated fatty acids, SFA = saturated fatty acids. Lag-phase slope is the increase of absorbance at 234 nm per min during the lag phase. Propagation-phase slope is the increase of absorbance at 234 nm per min during the propagation phase. ^{a,b}Values in the same row with different superscripts are significantly different ($p < 0.05$).

Some saturated fats (e.g. myristic acid) have now been widely accepted as one of the major dietary factors which could raise plasma cholesterol level in humans. For instance, coconut oil may raise plasma cholesterol more effectively than monounsaturated and polyunsaturated oils as well as palm oil [260]. However, among the saturated fats, there are considerable controversies in the literature regarding the hypercholesterolaemic effect in response to the dietary lauric, myristic and palmitic acids. Apart from various explanations for the hypercholesterolaemic effect of medium chain fatty acids, an indirect influence through their metabolic action may be crucial in manipulating the hepatic and plasma cholesterol levels.

Epidemiological studies show that in the Mediterranean region such as Greece and Southern Italy where the traditional diet is high in olive oil which is rich in oleic acid, the rates of coronary heart disease in these countries are relatively low [335,598]. Oleic acid has been demonstrated to exhibit a neutral cholesterolemic action [261,341,527] or hypocholesterolaemic effect in hypercholesterolaemic patients [369,406], in normocholesterolaemic men [47] and monkeys [549]. This monounsaturated fatty acid has been shown to lower plasma cholesterol [32,260,332] and cause reduction in LDL-cholesterol as effectively as polyunsaturated fatty acids [230]. In other report studies, rats [286,288,364,378] and hamsters [338] fed with cholesterol plus high-oleic fats have higher plasma or serum cholesterol level than animals fed cholesterol plus fats with lower oleic acid (i.e. palm oil). The dietary regimes used in the present experiments are from three types of dietary fats, i.e. highly saturated (coconut oil), moderately saturated and monounsaturated (palm oil), and highly polyunsaturated (soyabean oil) fatty acids. Palm olein and olive oil, both rich in oleic acid could lower the cholesterol level when compared to coconut oil [459]. Although the oleic acid level in olive oil is about twice of that in palm olein, both these oils appeared to exhibit similar cholesterolemic effects in guinea pigs fed cholesterol [340]. The present results indicate that concomitantly high intakes of oleic acid and cholesterol appeared to be effective in increasing the plasma cholesterol

level. The cholesterolemic action of the monounsaturated fatty acid together with the supplementary cholesterol may involve a preferential hepatic synthesis, accumulation and redistribution of cholesteryl oleate.

Effect on oxidative susceptibility of LDL

The oxidative stability of LDL derived from rabbits fed various dietary fats have been studied by using the Cu^{2+}-catalyzed oxidation, a method established by Esterbauer *et al.* [163]. The main targets of this oxidation are the polyunsaturated fatty acids *viz.* linoleic, linolenic and arachidonic acids and antioxidants to a small extent. Conjugated diene hydroperoxides were produced during the oxidation. The concentrations of these compounds were measured by UV absorption at 234 nm and each reaction provided a profile consisting of lag, propagation and decomposition phases. A typical plot of absorption *versus* time is shown in Fig. 3.2. The lag time which precedes the onset of rapid lipid autoxidation is an indicator of the oxidative susceptibility of the LDL. After the depletion of the antioxidants a rapid oxidation occurs and finally a plateau is reached when the breakdown of peroxides occurs. The lag time (and also the lag-phase slope) which precedes the onset of rapid lipid autoxidation is important as it reflects the induction period of radical oxidation and is an indicator of the oxidative susceptibility of the LDL.

The results on the oxidative susceptibility of LDL isolated from rabbits fed with various dietary fats are presented as lag times, lag-phase slopes, and UV-absorption maxima as given in Table 3.7. A slow increase in the absorption at 234 nm at the initial phase of the oxidation is indicative of a longer lag phase. Palm oil groups (PO and CPO) showed longer lag times (i.e. 111 and 103 min) than CNO and SO groups (i.e. 56 and 54 min). As determined by the slope of the lag phase, the rates for the oxidation of LDL obtained from CNO and SO groups were shown to be about 6-fold faster than that for LDL from rabbits fed palm oil. During the propagation phase, the oxidation rates for the LDL from the SO group were about 3-fold higher than the other groups. The high

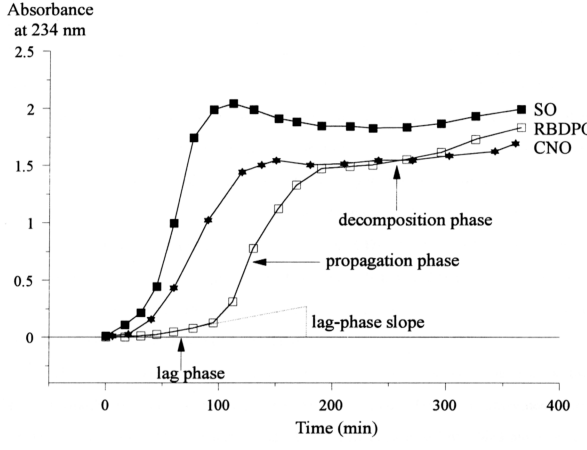


Fig. 3.2 Time course of absorption at 234 nm for the Cu^{2+}-catalyzed oxidation of LDL isolated from the rabbits fed atherogenic diets containing coconut oil and corn oil (CNO), refined-bleached-deodorized palm olein (RBDPO) and soyabean oil (SO).

polyunsaturated fatty acid proportion in the plasma and LDL lipids of SO rabbits was high as expected from the dietary soyabean oil. The final UV-absorptions for the SO group were relatively higher than others, confirming that larger amounts of conjugated dienes formed because of the high PUFA in these LDL samples. The lag times and lag-phase slopes were reflective of the type of dietary fats, with the LDL from palm oil diets having the highest stability. The propagation phase for the oxidation of LDL from SO-diet is much more rapid and appeared to be dependent directly on the polyunsaturation. CNO-diet is closer to palm oil diets in terms of the degree of polyunsaturation and show approximately similar propagation-phase slopes. As high levels of monounsaturated and saturated fatty acids offer oxidative resistance to the LDL [501], the oleic acid has been shown to posses resistance to lipid peroxidation [29]. It is noted that the high oleic and palmitic acid compositions for the LDL derived from palm oil-fed rabbits appears to be partly responsible for its resistance to oxidation.

The present data indicate that oxidative stability of LDL is also dependent on the level of vitamin E which is the major antioxidant in LDL. This is in agreement with previous *in vitro* work [162] which indicated that increasing levels of added antioxidants (vitamin E, ubiquinols, carotenes, etc.) can effectively prolong the lag times of the oxidation of LDL. While the level of antioxidants may be an important factor in the oxidative stability, it is noted that the degree of polyunsaturation of the LDL is also important. It has been observed that an increased intake of polyunsaturated fatty acids in soyabean oil-fed rats required a higher supplement of antioxidants to protect the polyunsaturated fatty acid from lipid peroxidation [445]. There may be some simple explanations, e.g. the coconut-oil diet provides low levels of vitamin E whereas soyabean oil with high PUFA places a greater demand on the antioxidants resulting in a reduced amount of vitamin E in the plasma. The relatively low amount of PUFA in palm oil diets leads to the excellent stability of the LDL. However, the explanations may be a little simplistic for at least two reasons. Firstly, good stability of LDL was observed for rabbits

fed palm oil diet even though the vitamin E level of LDL is low. Secondly, palm oil diets may provide tocotrienols but these are not accumulated in the plasma or LDL. However, only α-tocopherol accumulates in the LDL and the liver (see Chapter 4).

The present observations may likely be due to endogenous peroxides in the LDL. The role of all the antioxidants is likely to be in the prevention of peroxide formation. For the results from rabbits fed CPO which is unrefined palm olein, it is noteworthy that although the oil is rich in carotenes in addition to vitamin E (in contrast to the refined PO which does not contain carotenes) it did not confer increased stability to the LDL. The most direct reason is that very little carotene is incorporated into LDL, most of the carotenes are metabolized. Although many studies have focused on the oxidation of LDL, not much is known about the actual chemistry under *in vivo* conditions. The observed relationship between the oxidative stability of LDL and PUFA may be indicative of preformed or endogenous peroxides in the LDL, it is believed that oxidative damage may have occurred in its earlier intermediates (e.g. VLDL and IDL). The shorter lag times and higher lag-phase slopes for LDL from coconut oil and soyabean oil diets are the results of oxidative stress from a combination of low vitamin E and high PUFA levels. While there was a high saturation of fatty acids in the CNO diet, the oxidative susceptibility of LDL derived from CNO-rabbits was lower than that of PO-rabbits, this might be a reflection of its lower antioxidant levels.

3.3.2 Lipidemic Effects of Various Antioxidants

Fatty acid compositions for the plasma lipids of rabbits fed palm oil diets enhanced with various vitamin E components and vitamin C are shown in Table 3.8. Generally, palmitic and oleic acids are present as the major fatty acids in the plasma of all of the rabbits because of the palm oil diets. As compared to the baseline values, significant increases in the oleic acid levels were observed apparently contributed by the high intake of this fatty acid, i.e. about 47% of the total fatty acids present in the palm oil (Table 3.2), although a decrease in palmitic acid percentage is also seen. The present results

Table 3.8 Fatty acid compositions (%) and vitamin E contents of plasma lipids of rabbits fed palm oil diets enhanced with various antioxidants*

Fatty acid	Baseline	PO–E n = 5	PO+α-T n = 10	PO+γ-T_3 n = 6	PO+δ-T n = 6	PO+C n = 8	PO+CE n = 8
12:0	0.9±0.6	0.1±0.0	0.2±0.1	0.4±0.2	0.4±0.8	0.5±0.3	0.3±0.2
14:0	6.0±2.3	2.7±0.8	3.4±3.0	5.4±2.5	6.5±4.5	4.7±1.7	3.2±1.6
16:0	47.3±7.0	28.5±1.7	25.2±4.9	27.9±2.8	28.3±8.1	18.8±5.1	33.0±18.6
16:1	1.3±1.0	3.9±1.3	2.9±1.8	3.0±0.7	3.6±1.9	2.6±1.1	3.0±2.1
18:0	9.8±2.1	3.0±0.4	3.2±0.4	7.6±2.1	6.3±2.0	7.3±1.8	8.1±3.4
18:1	19.8±5.0	52.7±2.1	55.2±1.7	38.3±5.6	42.2±8.4	53.3±7.8	41.3±14.8
18:2	13.6±3.8	8.7±1.0	9.6±0.9	16.9±3.3	12.3±8.6	11.2±4.6	10.4±4.8
18:3	1.2±1.0	0.2±0.1	0.3±0.1	0.4±0.2	0.2±0.8	1.0±0.9	0.5±0.2
20:4	0.2±0.1	0.1±0.04	0.1±0.06	0.2±0.2	0.2±0.2	0.5±0.8	0.1±0.1
Vitamin E	3.3±0.9	5.4±2.2	27.6±4.2	21.2±4.5	27.6±14.3	3.6±2.2	11.5±3.6

* Rabbits were fed palm oil diets without supplementary vitamin E (denoted as PO–E), enhanced with 240 ppm *dl*-α-tocopheryl acetate (PO+α-T), enhanced with 209 ppm γ-tocotrienol (PO+γ-T_3), enhanced with 965 ppm δ-tocopherol (PO+δ-T), enhanced with 1000 ppm vitamin C but without supplementary vitamin E (PO+C), or enhanced with 1000 ppm vitamin C and 210 ppm *dl*-α-tocopheryl acetate (PO+CE). Values are means ± standard deviations, n = number of rabbits. Baseline values are for all rabbits before dietary treatment.

indicate that different types of antioxidants did not exert large changes on the fatty acid profiles except that rabbits fed γ-T_3, δ-T and vitamin C plus α-T have lower plasma levels of oleic acid, but the data are not significant.

Effect on oxidative susceptibility of LDL

The antioxidant effects of various vitamin E components and vitamin C were also evaluated by the copper(II)-catalyzed oxidation of LDL samples obtained from rabbits fed palm oil diets with or without supplementary antioxidants, and the results are diagrammatically shown in Fig. 3.3. It is obvious that LDL from the rabbits fed on diets without supplementary vitamin E (PO-E group) is the most susceptible to the oxidation. Supplementation of various vitamin E to rabbits resulted in increases in their plasma vitamin E contents as shown in Table 3.8. Rabbits fed with α-T and δ-T have a higher vitamin E content (about 28 μg/mL) than for the rabbits fed γ-T_3 (i.e. 21 μg/mL). The LDL derived from rabbits fed α-T and δ-T were found to be more resistant to oxidation with average lag times of 111 and 113 min, respectively; the LDL of rabbits fed γ-T_3 was more susceptible to the oxidation apparently due to its lower content of vitamin E. There could be other explanations, tocotrienols are perhaps excreted more rapidly.

Average lag times for the oxidation of LDL derived from the rabbits fed with a combination of α-T and vitamin C supplementation (PO+CE group) are the highest for the series (Fig. 3.3), the data are significantly ($p < 0.01$) better than those without vitamin E supplementation (PO-E group), and also better (data not significant) than supplementation singly with α-T (PO+α-T group) or vitamin C (PO+C group). Plasma vitamin E levels of PO-E rabbits without vitamin E supplementation are relatively low (i.e. 5.4 μg/mL); supplementation of α-T to the PO+α-T rabbits remarkably elevated the plasma vitamin E levels to 27.6 μg/mL and these resulted in LDL being more resistant to the oxidation. It is noted that inhibition of the oxidation of LDL can be achieved by supplementation of dietary vitamin E. The antioxidant effect of vitamin E has been enhanced by the dietary vitamin C as shown in the resistance of the LDL against oxidation. Vitamin E levels as

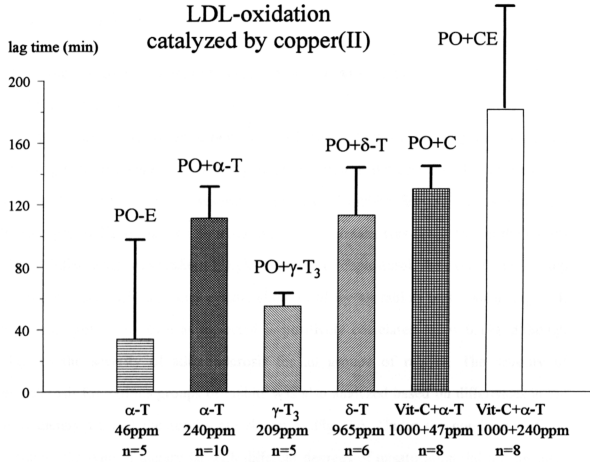


Fig. 3.3 The effects of vitamin E and vitamin C supplementation in protecting oxidation of LDL.

present in LDL are probably insufficient to effectively protect against oxidation of all the PUFA [159]. Recent work [291] has indicated that without vitamin C, vitamin E is not only insufficient to protect LDL against oxidation but may even be a prooxidant. The present results on dietary vitamin E and C confirm their synergistic effects in protecting the LDL from oxidation.

3.3.3 Effects of Dietary Fats and Antioxidants on Atherosclerosis

Effect of dietary fats

Atheroma on the aortas of rabbits fed atherogenic diets containing various dietary fats were grossly examined. A relatively large variation in the severity of atherosclerosis was generally observed in the respective groups of rabbits fed with cholesterol and different types of dietary fat and no atheroma or fatty streaks was detectable in the rabbits fed POWC-diet which was without supplementation of cholesterol. An overall relationship between atherosclerosis and total plasma cholesterol for all rabbits is shown in Fig. 3.4. The plasma cholesterol level as expected is positively correlated ($r^2 = 0.19$), although weakly, to the severity of atherosclerosis for all groups of rabbits. The severity of atherosclerosis for various groups of rabbits was also analyzed based on differences in the type of dietary fat and the results are shown in Fig. 3.5. Under the conditions of the experiment, the type of dietary fat with different degrees of unsaturation did not produce remarkable differences in the severity of atherosclerosis.

The combined action of cholesterol and fatty acids on atherosclerosis may be implicated by evidence that in the absence or presence of very high level of cholesterol, no significant difference was observed between the saturated and unsaturated fatty acids. The elevation of cholesterol and oleic acid in atherosclerotic rabbits implies that atherosclerosis is probably promoted by cholesterol oleate which has been demonstrated to be accumulated predominantly in fatty streak [203,292,450,588].

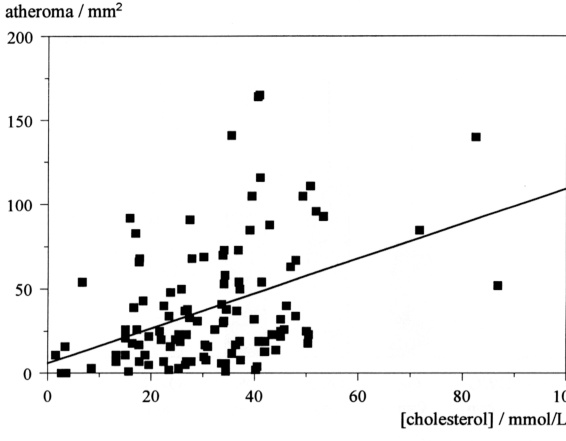
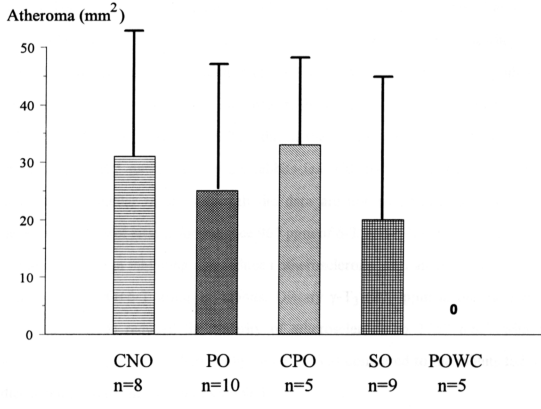


Fig. 3.4 Correlation between the atheroma area and the total plasma cholesterol level.



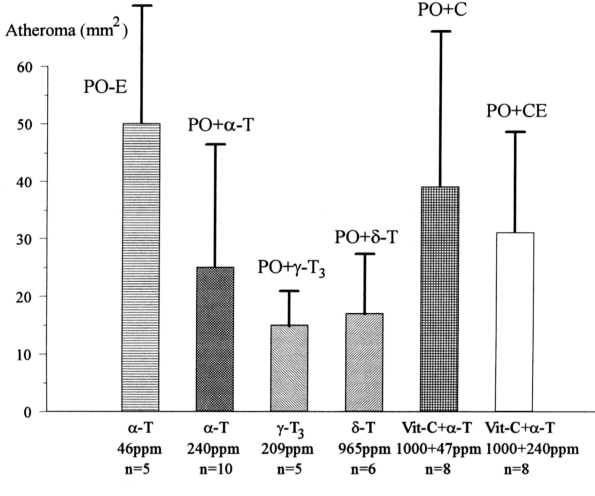
CNO = coconut oil (86.7%) and corn oil (13.3%); PO = refined-bleached-deodorized palm olein; CPO = crude palm olein; SO = soyabean oil. POWC = PO-based diet but without cholesterol supplementation. All diets except POWC were supplemented with 0.5% cholesterol.

Fig. 3.5 The effects of various dietary fats on atherosclerosis development.

Effect of antioxidants

The effects of various antioxidants on the development of atheroma were examined, and the results are shown in Fig. 3.6. It was found that PO-E rabbits fed with a palm oil diet depleted of natural vitamin E had more atheroma (50±21 mm^2) than other groups; the atheroma area for PO+C group of rabbits is 39±27 mm^2, indicating that supplementation of vitamin C (1000 ppm) alone did not produce a statistically significant inhibitory effect on the development of atherosclerosis. However, supplementation of 240 ppm α-T to the PO+α-T group of rabbits reduced the severity of atherosclerosis to an atheroma area of 25±22 mm^2, the PO+CE rabbits fed with α-T and vitamin C (31±18 mm^2) also showed similar results although the data are not statistically significant. A higher level of tocopherol intake, for instance 965 ppm of δ-T in the diet, is significantly ($p < 0.05$) more effective in inhibiting diet-induced atherosclerosis with an atheroma area of 17±11 mm^2 for the PO+δ-T group of rabbits. Dietary γ-T_3 (209 ppm in the diet) also significantly ($p < 0.05$) reduced the severity of atherosclerosis to 15±7 mm^2, with a reduction in the average area of atheroma by about 70% as compared to the rabbits fed on PO-E diet without supplementation of vitamin E.

There has been considerable confusion concerning the effect of vitamin E on plasma and lipoprotein cholesterol levels. Previously, elevated plasma tocopherol levels together with elevated LDL levels have been reported in hypercholesterolemic diseases such as cerebral thrombosis, and hypertensive cardiovascular disease [127,512,514,518]. In the present study, all atherosclerotic rabbits had concomitantly higher levels of plasma vitamin E and plasma cholesterol as compared to the POWC rabbits which were not fed cholesterol and did not exhibit atherosclerosis. The present results suggest that the increase in plasma cholesterol level resulting from dietary cholesterol has an obvious influence on the elevation of vitamin E level; this may just reflect the lipid solubility of vitamin E, however, the effect of cholesterol on hepatic metabolism of vitamin E is unclear and need to be further investigated.



All groups were fed 15% refined-bleached-deodororized palm olein and 0.5% cholesterol.

Fig. 3.6 **The effects of vitamin E and vitamin C dietary supplementation in the inhibition of atherosclerosis development.**

Although coronary heart disease and atherosclerosis have generally been linked to an excessive consumption of diets containing high levels of cholesterol and some saturated fats, it is now realized that the resistance of LDL against peroxidation is also an equally important factor for consideration in dietary recommendations. For example, epidemiological studies [208] have shown that antioxidants in the plasma are protective of cardiovascular disease. In many countries, where high fat and/or high cholesterol diets are not correlated with high mortality from cardiovascular disease, there is usually a high consumption of vegetables and fruits which apparently provides antioxidants. It is apparent that in view of the importance of maintaining oxidative stability of LDL, provision of dietary antioxidants is essential while a high monounsaturated fat diet with moderate amounts of saturates and polyunsaturates would be desirable.