CHAPTER IV: DISCUSSION

The present study was consistent with a study of Cu^{2+}-induced LDL oxidation *in vitro* assays in ten healthy with normolipidic and ten diabetics with hyperlipidemia volunteers. The plasma levels of biochemical parameters which may affect on LDL oxidation process in experiment were shown in Table 9. In this study, the levels of serum total cholesterol, triglycerides and LDL-cholesterol in diabetics were significantly higher than those in healthy group, while HDL-cholesterol levels were significantly lower. Previous report, the higher levels of LDL-cholesterol, triglycerides and lower level of HDL-cholesterol in diabetics indicate higher risks of atherosclerosis (Julier K, *et al.*, 1999). The HDL-cholesterol could protect LDL against oxidation *in vivo* because the lipids in HDL are preferentially oxidized before those in LDL. The levels of HDL-cholesterol significantly correlated with the levels of conjugated dienes in the plasma of healthy subjects and patients with coronary artery disease (Shimonov M. *et al.*, 1999). The present study, all volunteers had no significant difference in age or sex ratio between-groups, but were not observed for dietary habits, body weight, and smoking in both groups. It was also reported, that age, dietary habits and smoking may independently enhance LDL susceptibility to oxidation in diabetic patients (Liguori A, *et al.*, 2001).

The LDL was isolated by discontinuous gradient density centrifugation between range of 1.006 and 1.21 g/mL recovered the LDL by 18 hours at 60,000 rpm. the LDL isolation has high purity and was not contaminated from small molecular weight protein, the disadvantage is that it took very long time for centrifugation (Himber J, *et al.*, 1995). The method modified by BH Chung, *et al.*, (1980) was applied using the single vertical spin discontinuous density gradient ultracentrifugation at 50,000 rpm for 2.5 hours. Three major lipoprotein fractions were separated, VLDL at the upper layer, LDL at the middle layer and HDL at the bottom layer.

Our study used Cu^{2+} to induce oxidation of LDL, that is unlikely to occur *in vivo*. It was reported that increased serum copper ions concentrations associate with accelerated progression of atherosclerosis and detectable levels of redox active transition metals, including copper and iron, in human atherosclerotic lesions support a role for these metals *in vivo* (Kuzuya M. *et al.*, 1992).

In this study, LDL oxidation was initiated by addition of freshly prepared 20 μM $CuSO_4$ in the final concentration and then incubated at 37° C for 2 hours. The Cu^{2+}-induced oxidation of LDL was characterized by increasing of electrophoretic motility, conjugated diene and MDA formation compared to native LDL. The copper binds to apo B forming a complex with LDL subtracting an electron from a thiol group of the apo B or from a fatty acid giving lipid hydroperoxide radicals (Esterbauer H, *et al.*, 1993). Oxidation with Cu^{2+} involves a mechanism associated to specific sites of LDL, and probably is also present *in vivo*. Copper and iron as prooxidants were reported to be present in human atheromatous plaques (Smith C, *et al.*, 1992). Oxidation with Cu^{2+}, involves a mechanism associated to specific sites of LDL, and probably is also present *in vivo*. Copper and iron as prooxidants were reported to be present in human atheromatous plaques (Smith C, *et al.*, 1992).

$$Cu^{2+} + LOOH \longrightarrow Cu^{+} + LOO^{\cdot} + H^{+}$$

Cellulose acetate electrophoresis was used to validate the purity of the LDL fractions. ox-LDL compared to plasma native LDL. The lipoproteins in plasma control were separated into 3 bands as shown in Figure 5, LDL. VLDL and HDL bands. respectively moved from catodic to anodic poles. All three isolations were slightly moved more anodic due to increased negative charge from separating process compared with plasma control. Agarose gel or polyacrylamide gel electrophoresis should be used instead of cellulose acetate electrophoresis for better cleared separation (Gros M, *et al.*, 1973).

The total antioxidant capacity of curcuminoids were measured based on radical scavenging of the $ABTS^{\cdot}$ cation in an artificial, aqueous test system using the hydrophilic tocopherol analogue Trolox as reference compound compared with of α-tocopherol and ascorbic acid. Ascorbic acid exerted the highest total antioxidant capacity. The total antioxidant capacity of α-tocopherol was not significant different from curcuminoids. This comparison demonstrated that the higher total antioxidant capacity of ascorbic acid may affect on the test system, but it could not strongly protect Cu^{2+}-induced LDL oxidation *in vitro* due to its water soluble property. This study, α-tocopherol showed a stronger antioxidant capacity on inhibiting Cu^{2+}-induced LDL oxidation than curcuminoids and ascorbic acid which corresponded to previous report that α-

tocopherol had higher protection on Cu^{2+}-induced LDL oxidation than ascorbic acid (Carr AC, *et al.*, 2000).

We found that 1-30 μg/mL curcuminoids and ascorbic acid and 1-100 μg/mL , α-tocopherol had no cytotoxicity to U937 cells. The cell viability was up to 90% after incubation. The previous studies reported that 1- 30 μM commercial grade curcuminoids and 1- 25 μg/mL crude curcuminoids were not toxic to KB-V1 cells (Anuchapreeda S, *et al.*, 2002). Another report showed that different concentrations of ascorbic acid (vitamin C) and α-tocopherol (vitamin E) caused a cytotoxic effect on embryonic human fibroblasts (CLV102), human melanoma cells (ME18) and adriamycin-resistant subline cells (ME18/R), coupled with trypan blue exclusion test. Statistically significant survival decrease was observed, if the concentration of vitamin E exceeded 500 μg/mL and vitamin C exceeded 40 μg/mL in the culture media (Wozniak G and Anuszewska EL, 2002).

Our results found that the oxidative susceptibility of LDL in diabetics group was higher than in healthy group which observed by lower U937 cellular LDL uptake, longer lag time of conjugated diene formation and higher MDA concentration. The initial plasma levels of antioxidants and α-tocopherol content in LDL had not been compared in both subject groups. Diabetic patients were reported to have lower antioxidants in their plasma levels or lower vitamin E content in LDL than normal. As mentioned, vitamin E is the major antioxidant content in LDL, while the other antioxidants play minor roles in LDL. Vitamin E is a lipophilic chain-breaking antioxidant that scavenges lipid peroxyl radicals both in lipoproteins and in cellular membranes (Palanduz S, *et al.*, 2001). Vitamin E in LDL is the result of exogenous intake, it can transfer from LDL to cellular membranes or other lipoproteins, but its metabolic redox reactions occurs in LDL. The plasma level of vitamin E is strongly correlated with the level of the vitamin E content in LDL (Jain SK, *et al.*,1996). The mechanism of oxidative susceptibility of LDL were proposed by Figure 17.

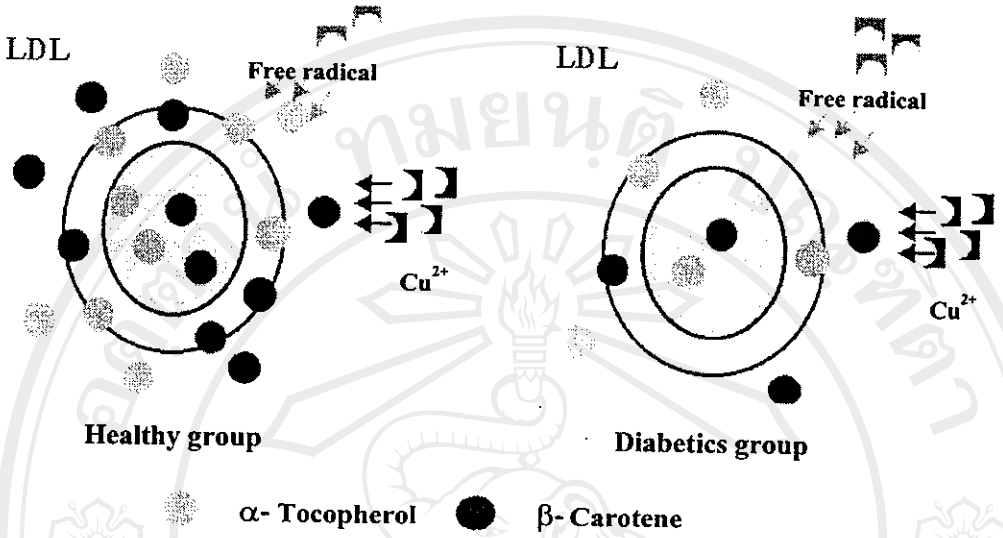

Figure 17. The oxidative susceptibility of LDL in healthy and diabetics groups. The diabetics have lower antioxidants levels in their plasma or lower antioxidants content in LDL than normal healthy group.

It is well established that diabetes is one of major risk factors for atherosclerosis with about two to five-folds higher than normal. The common occurrence of risk factors in diabetic patients, such as hypertension, obesity, and hypercholesterolemia, in addition to age and smoking, did not completely explain the increased level of mortality and morbidity from atherosclerosis in diabetic patients (Steiner G, *et al.*, 1985). With regard to plasma lipids and lipoproteins, it is also cleared that plasma triglyceride (TG) usually increased in patients with diabetes, and is consistently one risk factor for atherosclerosis. Strong correlation between plasma TG levels and increasing density and decreasing size of the predominant LDL. Furthermore, diabetes is marked by characteristic alterations in lipoprotein levels, including an elevation of TG and LDL and a decreased HDL concentration. In addition, it appears that small, dense LDL subclass patterns are more common in diabetic patients. Another report demonstrated that small dense LDL is more susceptible to oxidative modification (Kondo A, *et al.*, 2001). Therefore, it is important to evaluate LDL particle size and oxidative susceptibility of LDL in examining the risk of coronary

heart disease in diabetic patients. Higher lipid levels, glucose levels and smaller LDL particles are major factors on the increased LDL oxidizability in patients with diabetes.

The effect of diabetes on decreasing lag time of total antioxidant capacity and increasing MDA concentration of LDL oxidation appears to be independent of higher plasma lipid and glucose levels (Vlassara H, 1996). It was reported that serum levels of thiobarbituric acid–reactive substances (TBARs) were significantly increased in patients with diabetes mellitus. Plasma lipid and glucose levels were significantly increased in subjects with diabetes. Oxidation of LDL begins with the abstraction of hydrogen from polyunsaturated fatty acids; LDL-fatty acid composition contributes to the process of LDL oxidation (Laker MF, *et al.*, 1991). Diabetic patient had greater amounts of LDL polyunsaturated fatty acids than in normal, the maximum oxidation level in diabetes subjects could be increased. Glycosylation (glycation) of lipoproteins has been shown to be occurred in diabetes, and it was reported that glycated LDL is more susceptible to oxidation. They also indicated that HbAlc, which is regarded as an index of degree in protein glycation, is not an independent factor affecting LDL oxidizability in subjects with diabetic (Vlassara H, *et al.*, 1996).

In diabetes patients, where an imbalance of glucose metabolism occurs, a glucose-dependent mechanism may effectively contribute to LDL oxidation *in vivo* (Bowie A, *et al.*, 1993). They reported the reduction of the length of lag time and increased MDA concentration of LDL from diabetes patients which had more susceptibility to Cu^{2-}-induced LDL oxidation than healthy group. The role of glucose in the promotion of LDL oxidation has been examined and found that glucose autoxidation resulted in the formation of hydroxyl radicals that supported LDL oxidation. This pro-oxidant effect of glucose was stimulated by copper and inhibited by metal ion chelation. Glucose stimulated Cu^{2-}-induced LDL oxidation and this effect was also inhibited by the chelation of metal ions. The results were agreed with prior report, as glucose at pathophysiologically relevant concentrations stimulated both Cu^{2+} and Fe^{2-}-induced LDL oxidation (Knott HM, *et al.*, 2003).

Quinones-Galvan A, *et al.*, (1999) found that *in vivo* insulin administration increased the susceptibility of Cu^{2-}-induced LDL oxidation and insulin administration enhanced LDL oxidizability. The effects were similar in insulin-sensitive and insulin-resistant individuals, suggested that dyslipidemia, especially hypertriglyceridemia, is associated both with insulin

resistance of glucose metabolism and with increased partially oxidized LDL particles (Quinones-Galvan A, *et al.*, 1999).

HDL-cholesterol and apo A-I levels in subjects with diabetes were reported to be lower than those in normal. It may be that the decreased levels of HDL and apo A-I, regarded as a protector against LDL oxidation, augment the oxidative susceptibility of LDL in patients with diabetes were positively correlated with lag time of LDL oxidation only in healthy subjects. The earliest stages of HDL oxidation are accompanied by the oxidation of specific methionine residues in apolipoprotein A-I and apolipoprotein A-II. HDL also has antioxidant activity, which is probably induced by the esterase against oxidative damage to lipids and lipoprotein particles in human (Sanguinetti SM, *et al.*, 2001).

Diabetes patients have increased incidence of vascular disease when compared with healthy group. Oxidative stress can be increased in diabetes because of a prolonged exposure to hyperglycemia, hyperlipidemia, low vitamin E content in LDL and low levels of HDL. Accelerated, non-enzymatic glycosylation has been linked with glucose autoxidation, and glycosylated lipoproteins are an important source of free radicals. Further studies should be investigated for the susceptibility of LDL oxidation in person with hyperlipidemia or hypertension or in smokers compared to diabetic and all of study plays an important risk in the pathology of atheroslcerosis.

This study aims to demonstrate the antioxidant properties of herbal preparation widely used in food ingredients complementary and alternative medicine from a tropical plant native to south and southeast tropical Asia: *Curcuma Longa* Linn. Turmeric extracts were prepared in the same manner as they are consumed as folk remedies. Crude curcuminoids donated from Dr. Songyot Anuchapreeda, consisted of three major component mixtures; curcumin (48.4-48.8 %), demethoxycurcumin (20.6-20.8 %) and bisdemethoxycurcumin (30.7-31.0 %) (Anuchapreeda S, *et al.*, 2002). The results showed that curcuminoids could inhibit Cu^{2+}-induced oxidation of LDL in both healthy and diabetic groups as shown by increase of cellular LDL uptake, reduced dienes production and MDA concentration after curcuminoids treatment. It may be due to antioxidant composition changing of LDL or free radical scavenging enhanced removal from the Cu^{2+}, and the susceptible of LDL oxidation reducing. The presence of 30 μg/mL of curcuminoids retarded the LDL oxidation compared to the absence of curcuminoids in healthy and diabetic

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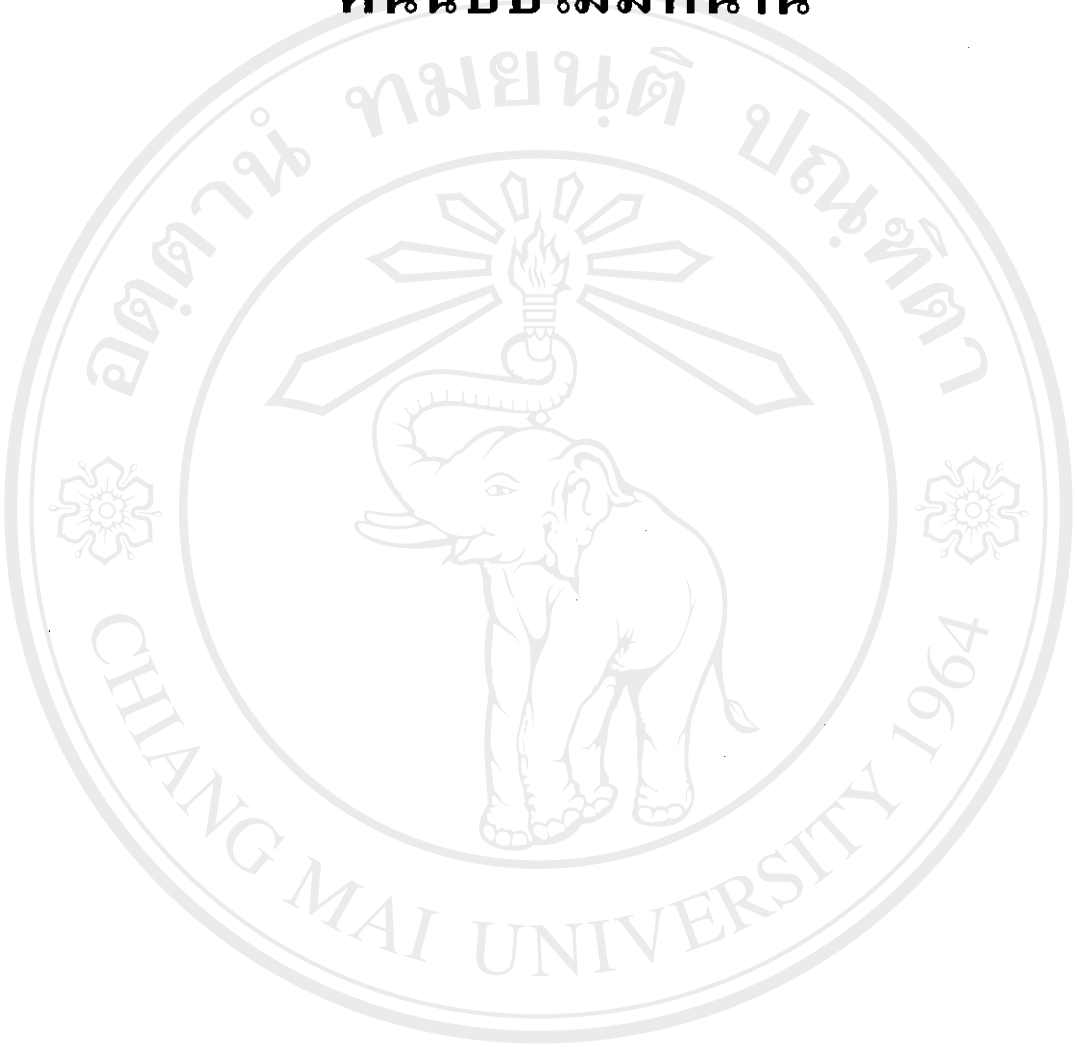

healthy and diabetic groups were approximately 83%, 88%, 84% and 77%, 82%, 78%, respectively. The effect of α-tocopherol on Cu^{2+}-induced oxidation of LDL was significantly greater than curcuminoids and ascorbic acid while the effect of curcuminoids was not different from ascorbic acid in both groups. The mechanism of curcuminoids, α-tocopherol and ascorbic acid on Cu^{2+}-induced oxidation of LDL were compared and proposed as in Figure 19. Previously, several mechanisms were proposed by which the antioxidants, ascorbate (vitamin C) and α-tocopherol (vitamin E), may inhibit both LDL oxidation and leukocyte adhesion to the endothelium and vascular endothelial dysfunction. The α-tocopherol appears to be more effective than ascorbate in an antioxidant or a pro-oxidant to inhibit lipid peroxidation in LDL (Carr AC, *et al.*, 2000).

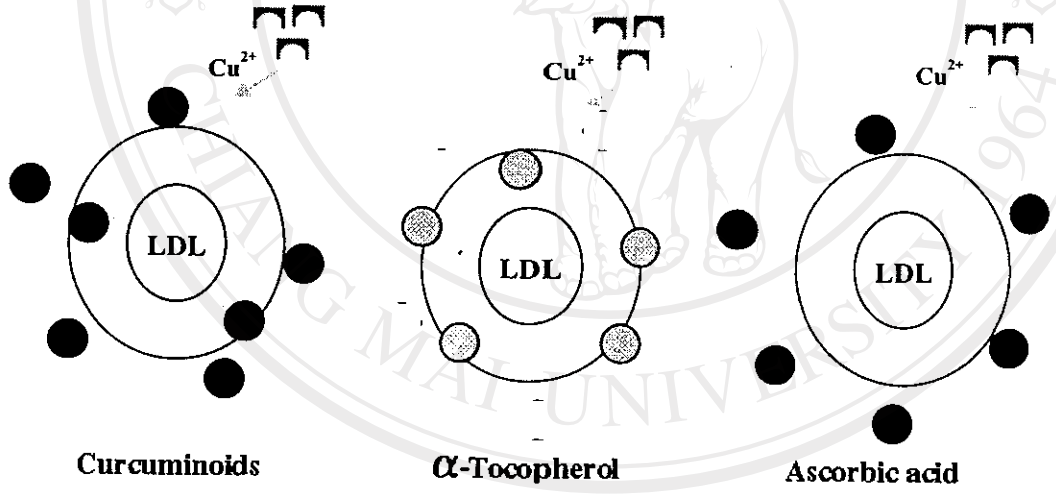

Figure 19. Effect of curcuminoids, α-tocopherol and ascorbic acid on Cu^{2+}-induced oxidation of LDL. Alpha-tocopherol is a lipid soluble and major antioxidants content in LDL. The inhibition of Cu^{2+}-induced LDL oxidation was greater than curcuminoids and ascorbic acid.

There was a report suggested that lipophilic antioxidants, such as vitamin E and beta-carotene, are carried in LDL and may protect polyunsaturated fatty acids against oxidation by competing with them for free radicals. Once antioxidants are depleted from LDL, the propagating

chain reaction of lipid peroxidation, so an increase in the amount of antioxidants in LDL would be expected to delay the onset of this process. Changing in lag time and MDA concentration are likely the most appropriate measure of the effect of antioxidant on LDL oxidation because it reflects altered susceptibility of the LDL particle to oxidation (Belcher JD, *et al.*, 1993).

A water solubility of ascorbic acid is known to serve as a source of reducing equivalents for the redox cycling of transition metal ions and as a result of this activity has been shown to potentiate lipid peroxidation in some oxidizing systems. The protective effect of ascorbic acid in Cu^{2+}-exposed LDL observed in previous studies indicate that any such potentiation, if it occurs, is overcome by the antioxidant effects of this agent (Retsky KL, *et al.*, 1993). The balance between the pro-oxidant and antioxidant activities of ascorbic acid could be different in LDL, leading to subfraction differences in its net inhibitory effect. Protection by ascorbic acid may also occur independently of its hydrogen-donating activities. The inhibition of Cu^{2+}-catalyzed LDL oxidation may involve covalent modification by ascorbic acid oxidation products, which in turn may inhibit LDL-Cu^{2+} binding.

Antioxidants may inhibit atherogenesis and improve vascular function by two different mechanisms. First, lipid-soluble antioxidants presented in LDL, including α-tocopherol, and water-soluble antioxidants present in the extracellular fluid of the arterial wall, including ascorbic acid (vitamin C), inhibit LDL oxidation through an LDL-specific antioxidant action. Second, antioxidants present in the cells of the vascular wall decrease cellular production and release of reactive oxygen species (ROS), inhibit endothelial activation, and improve the biologic activity of endothelium-derived nitric oxide (EDNO) through a cell- or tissue-specific antioxidant action (Frei B, 1999).

Combined supplementation with ascorbate, β-carotene, and α-tocopherol is not superior to high-dose α-tocopherol alone in inhibiting LDL oxidation. Hence, α-tocopherol therapy should be favored in future coronary prevention trials involving antioxidants (Jialal I and Grundy SM, 1993). The effects of α-tocopherol and β-carotene on LDL oxidation is that α-tocopherol and β-carotene inhibit the oxidative modification of LDL in the system by decrease in lipid peroxide (thiobarbituric acid reactive substances, TBARs, activity). The effect of α-tocopherol on the generation of TBARs was stronger than that of β-carotene (Cheng X, *et al.*, 1993).

Further studies needed to determine the antioxidant combination activity on protection of Cu^{2+}-induced LDL oxidation such as curcuminoids combined with ascorbic acid, or curcuminoids combined with α-tocopherol. In this study, we demonstrated a reduction in the susceptibility of LDL to oxidation and the inhibition of LDL oxidation in the presence or absence of curcuminoids, α-tocopherol, and ascorbic acid in healthy and diabetic groups using U937 cellular LDL uptake model. The result presented the first evidence that native LDL isolated form healthy and diabetic groups were taken up in U937 cells more than oxidized LDL. It was interesting that native LDL, and ox-LDL were capable of cellular uptake *via* receptor on U937 cells. We found that native LDL and ox-LDL induced difference cellular uptake by U937 cells. The oxidation of LDL by 2 hours exposure to $CuSO_4$ resulted in 16-fold reducing the uptake by U937 cells compared to native LDL. Ox-LDL is a well-known ligand for scavenger receptors. U937 cells can uptake LDL particles by apo B receptor or LDL receptor more than ox-LDL particle. In this experiment, cellular LDL uptake by receptors are difficult to extrapolate to other determinations because the receptors on U937 cells may be more than one type. Ox-LDL are usually taken up by macrophage scavenger receptors. Our original hypothesis that ox-LDL were not be taken up by apo B receptors on U937 cells, but under our experimental conditions, we speculated that there are two possible routes for the accumulation of extracellular LDL in U937 cells, first, LDL oxidation process are not complete and the remain of native LDL was taken up *via* LDL receptors on U937 cells. The second, ox-LDL accumulated in the cells were taken up by another kind of receptor on U937 cells such as macrophage scavenger receptors which was formerly shown that ox-LDL can stimulate expression of scavenger receptor (CD36) in U937 cells (Han J, *et al.*, 1997).

Cu^{2+}-induced oxidation of LDL involves the peroxidation of polyunsaturated fatty acids within the LDL phospholipid monolayer, which transforms them to reactive aldehyde-derivatized fragments such as malondialdehyde and 4-hydroxynonenal. Once created, these reactive aldehydes undergo reactions with the amino group of lysine residues on the LDL apo B-100 molecule. This type of modification neutralizes the net positive charge of apo B-100, which reduces its affinity for the LDL receptor and increases its affinity for the scavenger receptor (Gieseg SP, *et al.*, 1994).

U937 cells lack the 3-ketosteroid reductase activity in the cholesterol synthesis pathway, thus they cannot synthesize cholesterol for their growth (Billheimer JT, *et al.*, 1987) and U937 is a monocytic cell line dependent on low density lipoprotein (LDL) receptor-induced uptake of cholesterol for proliferation (Frostegard J, *et al.*, 1995). The interaction of LDL with the LDL receptor is due to specific binding of its major protein, apo B-100 to negatively charged cysteine-rich motifs on the receptor protein. The binding regions of apo B-100 are believed to be clusters of positively charged amino acids, namely lysine and arginine. Despite intensive investigation the critical binding domain has not yet been identified and indeed it has been suggested that a broad region may contribute to binding. Alternatively, the cell growth rate can be influenced by the cholesterol uptake, which is induced by the binding of LDL apo B-100 to the LDL receptors. Accordingly, the growth rate of U937 cells can be used to determine the binding properties of LDL to its receptor and the delivery of exogenous cholesterol by LDL. The delivery is dependent on the effective and efficient binding of LDL apo B to the LDL receptors. The results strongly suggest that cellular LDL uptake found in U937 cells with LDL are lipid droplets enriched. It was noted that ox-LDL fractions failed to uptake *via* apo B receptor expression (Brown MS, *et al.*, 1981). Our result also found that there were droplets of LDL particles in the cells localized by oil red O staining.

The *in vitro* experiments showed that the concentration at 30 μg/mL of all antioxidants studied can increase the resistance of LDL to oxidation without cytotoxicity to U937 cells. In conclusion, we clearly showed, for the preliminary study, that curcuminoids added to LDL *in vitro* could protect LDL from Cu^{2+}-induced oxidation as shown by the determination of increased U937 cellular LDL uptake. LDL with $CuSO_4$ in the presence of curcuminoids could be uptaken by LDL receptor on U937 cells more than LDL with $CuSO_4$ in the absence of curcuminoids. Moreover, a more differentiated determination of the protective effect of curcuminoids is proposed based, for the first time, on biochemical and physical chemical results: all indicate, the protective effect of curcuminoids from Cu^{2+}-induced oxidation of LDL.

U937 cells were reported to be very active in the receptor-induced binding of human LDL. The receptor-induced catabolism of LDL consists of three stages that include the initial binding of LDL to high affinity receptors on the cell surface, internalization through endocytosis, and degradation in lysosomes (Baillie G, *et al.*, 2002). The inducing the differentiation of U937

cells into monocyte-macrophage-like cells using TPA (12-tetradecanoyl-phorbol-13-acetate), that had been induced scavenger receptors expression (Hayashi K, *et al.*, 1998).

Foam cell that accumulate lipid droplets intracellularly is a remarkable feature of atherosclerotic lesions. The mechanisms of foam cell formation have been extensively studied for more than a decade. More than 10 scavenger receptors have been identified and their pathological properties have been investigated. The presence of ox-LDL will become ligand for scavenger receptors in human circulating plasma. However, the fate of foam cells after lipid droplet formation is still poorly understood. Because atherosclerosis is considered to be a chronic condition requiring decades to develop, understanding the fate of foam cells could be of great importance in the elucidation of the precise pathophysiology of the disease. In one previous report, THP-1 cells (Human monocyte; acute monocytic leukemia) were used to study the accumulation of intracellular LDL oxidation in foam cells by macrophage scavenger receptor (Ravandi A, *et al.*, 1999). They reported that the LDL lipid glycation and LDL oxidation product, promotes LDL uptake and accumulation by THP-1 macrophages. Further study should be performed on investigation the inhibition of curcuminoids on LDL oxidation by the reduction of foam cell. Due to the specific uptaking of ox-LDL in cells *via* macrophage scavenger receptor, THP-1 cells or TPA-treated U937 cell may be included rather than U937 alone.

The last experiment, the effect of curcuminoids, α-tocopherol and ascorbic acid on ox-LDL in healthy and diabetic groups were studied. The percentage of cellular LDL uptake were not changed indicated that curcuminoids, α-tocopherol and ascorbic acid had no effect on ox-LDL. The amount of antioxidants used, 30 μg/mL, and incubation time, of the 2 hours, could not reverse ox-LDL oxidation to native LDL. Further study should be investigated for the effect of curcuminoids on ox-LDL using various concentrations and time periods of LDL oxidation.

The dried rhizome of *Curcuma longa* Linn. (turmeric), which has been used for centuries as a spice, food ingredients and a coloring agent, has been found to be a rich source of beneficial phenolic compounds known as the curcuminoids. Curcuminoids has two distinct antioxidant properties, it prevents the formation of free-radicals due to environmental stress and neutralizes those that have already formed. As mentioned, curcuminoids usefulness in promoting overall health is wide-ranging. Most people recognize turmeric as the yellow color found in curry powder; however, it has great importance as a medicinal herb due to the presence of curcuminoids

(phenolic compounds) called curcumin, dimethoxycurcumin and bis-dimethoxycurcumin. The proposed local delivery administration of curcuinoids to nutritional supplement at dosages of 500 mg 1-3 x daily can be taken supplementarily without side effects. Long term therapy may be benefit in a healthy heart or prevention of atherosclerosis, lower LDL-cholesterol oxidation and could reduce blood sugar and cholesterol levels or prevention of hyperlipidemia (Arun N and Nalini N, 2002).

In conclusion, our preliminary report showed for the first time that the oxidative susceptibility of LDL oxidation was increased in diabetes with hyperlipidemic more than healthy with normolipidemic groups. Second, the presence of curcuminoids, α-tocopherol or ascorbic acid could inhibit Cu^{2+}-induced oxidation of LDL in both groups and can inhibit Cu^{2+}-induced oxidation of LDL in healthy group more than in diabetic groups. The data presented here indicate that curcuminoids had slightly lower effect on inhibition Cu^{2+}-induced oxidation of LDL than α-tocopherol, but similar to ascorbic acid. Curcuminoids, α-tocopherol and ascorbic acid had no effect on ox-LDL or could not reverse ox-LDL to native LDL. This finding indicated that curcuminoids could protect the early phases of LDL oxidation and progression of atherosclerosis.