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Lipid Lowering Effect of Antioxidant Alpha-Lipoic Acid in Experimental Atherosclerosis

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Summary **Accumulating data demonstrated that hypercholesterolemia and oxidative stress play an important role in the development of atherosclerosis. In the present study, a protective activity of alpha-lipoic acid; a metabolic antioxidant in hypercholesterolemic-induced animals was investigated. Eighteen adult male New Zealand White (NZW) rabbit were segregated into three groups labelled as group N, HCD and ALA ($n = 6$). Group N (normal control) was fed with normal chow, the rest (HCD and ALA) were fed with 100 g/head/day of 1% cholesterol rich diet to induce hypercholesterolemia. Four point two mg/body weight of alpha lipoic acid was concomintantly supplemented to the ALA group. Drinking water was given ad-libitum. The study was designed for 10 weeks. Blood sampling was taken from the ear lobe vein at the beginning, week 5 and week 10. Plasma was prepared for lipid profile estimation and microsomal lipid peroxidation index indicated with malondialdehyde (MDA) formation. At the end of the experiment, the animals were sacrificed and the aorta were excised for intimal lesion analysis. The plasma total cholesterol (TC) and low density lipoprotein (LDL) levels were found to be significantly low in ALA group compared to that of the HCD group ($p<0.05$). Similarly, low level of MDA ($p<0.05$) in ALA group was observed compared to that of the HCD group showing a significant reduction of lipid peroxidation activity. Histomorphometric intimal lesion analysis of the aorta showing less of atheromatous plaque formation in alpha lipoic acid supplemented group ($p<0.05$) compared to HCD group. These findings suggested that alpha lipoic acid posses a dual lipid lowering and anti-atherosclerotic properties indicated with low plasma TC and LDL levels and reduction of athero-lesion formation in hypercholesterolemic-induced rabbits.**

Key Words**: alpha lipoic acid, antioxidant, atherosclerosis, lipid peroxidation, intimal lesion**

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Introduction

The relationship between hypercholesterolemia with the prevalence of cardiovascular diseases (CVD) has been well documented. Increased in plasma low density lipoprotein (LDL) concentration has been associated with the susceptibility of developing atherosclerosis [*1–2*]. Although precise mechanism of atherogenesis still need further investigation, oxidative modification of LDL is considered to be an essential process in the activation of the inflammatory pathway leading to formation of atheromatous plaque in the intimal layer of the artery [*3–5*]. In contrast to the adverse effects of an elevation of LDL, the concentration of high density lipoprotein (HDL) correlates inversely with atherosclerosis development [*2*, *6*]. Increased oxidative stress, resulting from both increased reactive oxygen species (ROS) production appears to play an important role in the chronic inflammatory responses to hypercholesterolemia and atherosclerosis [*1*, *6–9*]. Therefore, apart from hypocholesterolemic drug intervention in reducing blood cholesterol level, the prophylaxis of atherosclerosis using antioxidant therapy has been extensively evaluated in animal experiments [*7*, *9*, *10*].

Alpha lipoic acid (ALA), also known as 1,2-dithiolane-3-pentanoic acid, 1,2-dithiolane-3-valeric acid or 6,8-thioctic acid has generated considerable clinical interest as a cellular thiol-replenishing and redox-modulating agent [*11*]. Biologically, alpha lipoic acid functions as a cofactor of oxidative decarboxylation reactions in glucose metabolism to yield energy [*12*]. It has been used for a long time in the western world to treat complications associated with diabetes [*13*]. To carry out this function, the disulfide group of the lipoic acid dithiolane ring is reduced to its dithiol form, dihydrolipoic acid, DHLA. Current findings suggest that alpha lipoic acid not only acts as a cofactor in glucose metabolism, but may also acts as an antioxidant *in vitro* and *in vivo* [*14*]. *In vitro* experiments have shown that both ALA and DHLA are potent scavengers of reactive oxygen species. Alpha lipoic acid quenches singlet oxygen, hydroxyl radical and hypochlorus acid [*15*] while DHLA scavenges hydroxyl radicals, hypochlorus acid, superoxide anions radicals and peroxyl radicals [*16*].

The observations of ALA as an antioxidant *in vitro* and *in vivo* were previously based on hyperglycaemic ambience [*13*, *17*, *18*]. However, until now, no experimental design has addressed the question as to whether ALA could work in atherosclerotic atmosphere *in vivo* thus preventing the proliferation and propagation of this degenerative disease. Lipid peroxidation, the oxidative deterioration of the polyunsaturated fatty acids (PUFA), leads to the formation of hydroperoxides, short-chain aldehydes, ketones and other oxygenated compounds. This process is considered responsible for the development of various diseases like atherosclerosis [*13*], diabetes [*19*], cancer [*20*] and may be one of the main contributing factor towards aging [*21*]. In the present study, we sought to determine whether ALA is able to exert its antioxidative effect and alter blood lipid levels in atherosclerosis-induced animals.

Materials and Methods

Experimental animals

Eighteen three month-old male New Zealand White (NZW) rabbits with an average body weight of 2.2–2.8 kg were procured from the central animal house of the Faculty Medicine and Health Science, UPM. The animals were placed in individual cages and acclimatized under room temperature and humidity (28 ± 2°C; relative humidity 60–70%) with regular light/dark cycle and free access to food and water for one week before use. Following acclimatization, the animals were segregated into three groups and fed with of the following: 1. Normal control group (N) was given 100 g/head/day of normal rabbit chow (Golden Hope, Malaysia), 2. Hypercholesterolemic-induced group (HCD) was given 100 g/head/day of 1% cholesterol-rich diet (ICN Biomedical, California) and 3. Alpha-lipoic acid-treated group (ALA) was given 100 g/head/day of 1% cholesterol-rich diet concomitantly with 4.2 g/kg/day of alpha-lipoic acid (Sigma Chemicals). Alpha-lipoic acid was supplemented by oral gavage daily. The dose of alpha-lipoic acid used in this study was based from previous work (data not shown). Drinking water was given *ad libitum*. The study was designed for 10 weeks. Blood sampling were taken prior to treatment (w = 0), at week five (w = 5) and week 10 (w = 10). Twenty five ml of ear lobe venous blood samples were drawn into EDTA coated tubes, placed in a transport ice bucket and were centrifuged at 3000 rpm in a refrigerated bench top centrifuge for 10 min at 4°C. Plasma samples were prepared and analysed for total cholesterol (TC), low density lipoprotein (LDL), high density lipoprotein (HDL), triglycerides (TG) and microsomal lipid peroxidation indicated by malondialdehyde estimation. In week ten, the animals were sacrificed by exsanguination after withdrawing the same volume of blood samples from ear vein as previously described and the plasma sample obtained were aliquoted and kept in −70°C for a maximum of 7 days before analyses. Following exsanguination, the whole aorta were excised for histomormetric intimal lesion analyses. All experimental procedures on the animals were performed in accordance with protocols approved by the Animal Care & Use Committee, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia.

Biochemical analyses

Lipid profiles estimation. Lipid profiles observed are the total cholesterol (TC), triglycerides (TG), high-density

Table 1. Blood lipids (mmol/l) and the ratio of TCHOL and HDL of animals prior to the experiment, week 5 and at week 10 (mean ± standard deviation, N = control, high cholesterol diet = 1% high cholesterol diet, ALA = 1% high cholesterol diet + 4.2 mg/kg alpha lipoic acid)

	TCHOL			HDL			TG			LDL			TC:HDL ratio		
	N	*HCD*	*ALA*	*N*	*HCD*	*ALA*	*N*	*HCD*	*ALA*	*N*	*HCD*	*ALA*	*N*	*HCD*	*ALA*
Week zero (w0)	4.60 ± 0.22	3.83 ± 0.57	4.31 ± 0.32	1.23 ± 0.09	1.68 ± 0.18	1.66 ± 0.11	4.31 ± 0.22	4.57 ± 0.43	4.80 ± 0.35	1.38 ± 0.06	1.76 ± 1.03	1.35 ± 0.03	3.74 ± 0.11	2.28 ± 0.24	2.61 ± 0.22
Week 5 (w5)	4.55 ± 0.34	17.29 ± 4.05	22.22 ± 2.30	1.40 ± 0.17	1.39 ± 0.19	1.70 ± 0.09	4.12 ± 0.12	4.77 ± 0.36	4.40 ± 0.22	1.13 ± 0.06	14.54 ± 4.06	17.39 ± 0.96	3.27 ± 0.16	12.79 ± 3.78	3.27 ± 2.08
Week 10 (w10)	6.65 ± 0.10	33.16 ± 4.03	21.52 ± 2.80	1.33 ± 0.08	1.32 ± 0.12	1.63 ± 0.11	4.14 ± 0.09	4.86 ± 0.32	4.65 ± 0.28	2.44 ± 0.08	30.43 ± 3.98	15.72 ± 0.74	4.26 ± 0.20	25.65 ± 4.45	13.23 ± 0.92

lipoprotein (HDL) and low-density lipoprotein (LDL). Samples were processed following protocols provided by the manufacturer of the commercial kit (Sigma Chemicals) and were analysed spectrophotometrically on Hitachi Chemistry Analyzer.

Microsomal lipid peroxidation and protein estimation. Plasma samples obtained were used to study lipid peroxidation *in vivo*. Malondialdehyde (MDA) as thiobarbituric acid reactive substance was measured at 532 nm spectrophotometrically (Bolkent *et al.*, 2005) whereas the protein concentration was determined by Biuret's method as previously described [*22*].

Quantification of aortic atheroslerosis. The full length of the aorta from the ascending to the common iliac were isolated immediately after exsanguination and washed with iced-cold normal saline to remove debris and blood clot. The aorta was stripped from excess adventitial and fat tissues. A longitudinal section was made on the aorta using a scissor to exposed the lumen. The tissue was pinned on a wooden board with the luminal surface is exposed above following immersion in 10% formalin for 24 h. After the incubation period, the tissue was rinsed with 70% ethanol followed by immersion in Sudan IV staining (Sigma Chemicals) for 15 min to identify lipid containing atheromatous plaque. The tissue was rinsed with tap water to remove excess staining and the luminal surface was photographed using a single lens reflex digital camera (Canon EOS 300D, Japan). The intimal lesion area was measured further using a computerised image analyser system and area ratio was calculated as area of lesion/area of intimal surface. The image analysis system consisted of a Macintosh lix computer (Apple) equipped with a Frame Grabber Card (Quick Capture, Data Translation), a Sony high-resolution video camera, and Trinitron Super Mac 21 inch color monitor.

Statistical analysis. Sample were analysed in triplicate. All data were expressed as mean ± standard deviation. Statistical analysis was done by one-way ANOVA using the SIGMAStat version 2.01 computer software. Tukey post-tests were performed for multiple group comparison. In all cases, statistical significance was set at $p<0.05$.

Results

Plasma lipids analysis

The results of the plasma lipid profiles are shown in Table 1. All lipid fractions at w = 0 demonstrated a low basal level in all groups.

Total cholesterol (TC). When fed with 1% high cholesterol diet, rabbits in both HCD and ALA groups developed marked hypercholesterolemia although there was no significant different in plasma HCD and ALA at w = 5. Both of these groups (HCD and ALA) exhibit hypercholesterolemia at week 5 (both $p<0.05$) with 3.8 and 4.8 times higher than that of the N group in the same time period. The level of TC in HCD group further increased significantly ($p<0.05$) at w = 10 with 1.9 times higher compared at w = 5 and 4.98 times higher than N group at w = 10. Supplementation of 4.2 mg/kg of alpha lipoic acid daily was found to be able to sustain the plasma TC level in ALA group with no significant different at w = 5 and w = 10 respectively. Infact, TC level in ALA group was shown to be at low level significantly ($p<0.05$) compared to HCD group at w = 10 with 35% reduction.

High density lipoprotein (HDL). The level of HDL in both of N and ALA groups (inter and intra group) do not changed significantly throughout the experimental period. The level of HDL in N group at the beginning of the experiment was found to be lower compared to the other groups. However, it is presumed to be normal with no treatment given and do not affect the overall HDL analysis. The HDL level in HCD group was found to be lower significantly ($p<0.05$) at w = 5 and w = 10 with 1.2 and 1.3 times reduction compared to that of at w = 0. The level of HDL in alpha lipoic acid supplemented group however remained constantly high in w = 10 (1.63 mmol/l) resembling the level of which at w = 0 (1.66 mmol/l) and the level is significantly higher ($p<0.05$) compared to that of HCD and N group respectively in the same time period.

Low density lipoprotein (LDL). The plasma LDL level was initially similar in all groups at the beginning of the experiment (w = 0) (mean of basal plasma LDL of three groups was 1.51 ± 0.23 mmol/l), but were significantly higher at w = 5 in HCD and ALA groups, compared to the

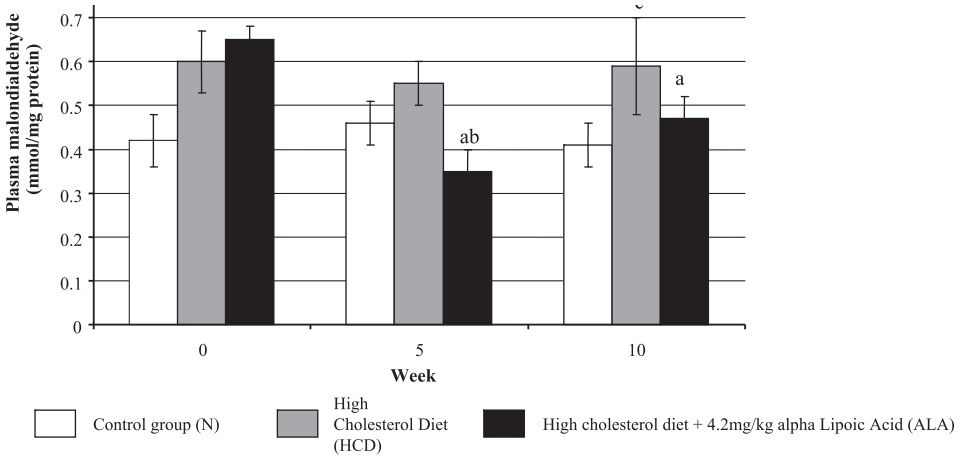

Fig. 1. The microsomal lipid peroxidation indicated by malondialdehyde (MDA) level in plasma of normal control group (N), high cholesterol diet (HCD) and high cholesterol diet + alpha lipoic acid group (ALA) at the respective week of treatment. Data are expressed as mean ± SD, $n = 6$. a $p<0.05$ compare to ALA at week zero (ALAw = 0), b $p<0.05$ compare to HCDw = 5, c $p<0.05$ compare to Nw = 10.

control group which remained at low level. The level of plasma LDL in HCD group was found to increased further significantly at w = 10 with 2 times increment from w = 5 level ($p<0.05$). The level of LDL in ALA group however was found to be not significantly difference at w = 10 compared to its level at w = 5. Infact, the level of LDL in alpha lipoic supplemented group at w = 10 was significantly low with almost two times lower than that of HCD group in the same experimental period.

Triglycerides (TG). No significant difference of plasma TG level was observed in all groups throughout the experimental period. However, there is a pattern of increased TG level in HCD group and reduced TG level in ALA group both at w = 5 and w = 10.

TC: HDL ratio. The value of TC:HDL ratio has been used as an indicator of lipid lowering property. In this experiment, the ALA group showing significantly lower TC: HDL ratio both at w = 5 and w = 10 ($p<0.05$) compared to that of the HCD group. No significant different was obtained in ALA group at w = 5 and w = 10 respectively. The 1% cholesterol load to NZW rabbits was found to increase TC level significantly in both HCD and ALA groups but the lower TC: HDL ratio observed in the alpha lipoic acid treatment group suggested that supplementation of alpha lipoic acid may have possibly facilitated the hepatic HDL biosynthesis *in vivo* than the overall total cholesterol concentration.

Microsomal lipid peroxidation

The concentration of tiobarbituric acid reactive substance (TBARS) as microsomal lipid peroxidation marker is shown in Fig. 1. No significant different was observed in the level of TBARS in N and HCD group at w = 5, but was significantly lower ($p<0.05$) in ALA group in the same week compared to its control at w = 0 and also to HCD at w = 5. However TBARS level in ALA group was increased at w = 10. Nevertheless, the increment was considered small and still at low pace compared to its control at w = 0 ($p<0.05$). The level of TBARS in HCD group showed to be highest throughout the experimental period, indicating that 1% cholesterol load to NZW rabbit for 10 weeks was able to cause marked hypercholesterolemia and allowing microsomal lipid peroxidation process to be overwhelming *in vivo*.

Aortic intimal lesion analysis

The histomorphometric analysis of the development of aortic intimal lesion is shown in Fig. 2A. This study demonstrated that high cholesterol diet could generate not only hypercholesterolemia, but also proceeded to the formation of atheromatous plaque in the luminal surface of the aorta. However, the group of high cholesterol diet supplemented with ALA showed a significatly lesser athero-formation in the aortic lumen compared to that of the group which was not given ALA supplementation. The percentage area of the lesion was shown to be lower in the alpha lipoic acid supplemented group ($p<0.05$), compared to that of the HCD group and no significant difference was found between ALA and the control (N) group. There is also minute atherosclerotic plaque formation was observed in the intimal layer of the aorta of the N group with less than 1% whereas the distribution of the developed lesions was found to be more at the aortic arch with less at the abdominal region in all groups (see arrow in Fig. 2B).

Discussion

In this experiment, 1% cholesterol load to the animals

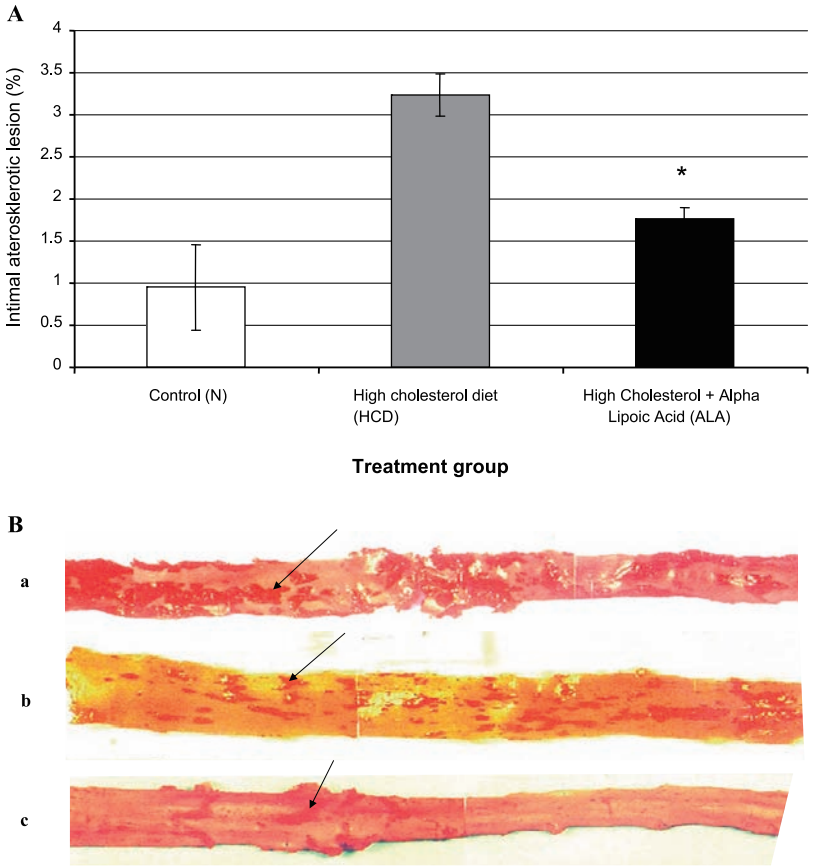

Fig. 2. A. Histomorphometric analysis of intimal lesion area in alpha lipoic acid treated group (ALA) showing a less progression of atheromatous plaque formation compared to the group with high cholesterol diet only (HCD). Data are expressed as mean ± SD, $n = 6$. *$p<0.05$ compare to atherosclerotic group.
B. The macroscopy observation of the atheromatous plaque formation on the luminal surface of the aorta stained with Sudan IV from the group of high cholesterol diet only (a), the high cholesterol diet + alpha lipoic acid (b) and the normal control group (c). Arrow indicated the presence of atheroma.

generated hypercholesterolemia in the model used. Studies both in animals and human have clearly demonstrated that prolonged high cholesterol concentration in the circulating blood have a positive correlation on developing atherosclerosis [*4*, *18*, *19*, *23*]. Results from the plasma lipid profiles of 1% high cholesterol fed animals for 10 weeks showed concentration of plasma TC and LDL respectively has increased 4.5 and 8.3 times higher in w = 5 and further increased 8.7 and 17.3 times in w = 10 compared to the level before the experiment, whereas HDL was decreased. These finding were similar to findings from previous studies [*7*, *18*]. These changes are associated with a phenomenon that excessive load of cholesterol to the liver above the acceptable level of its normal process causes the system to be unable in metabolising the lipids resulting in high cholesterol return in the circulating blood [*24–25*].

The value of TC: HDL ratio has been used as an indicator of lipid lowering property. In this experiment, the ALA group showed significantly ($p<0.5$) lower TC: HDL ratio at both w = 5 and w = 10 with 4 and 2 times reduction respectively in the values when compared to the HCD group. The 1% cholesterol load to NZW rabbits was find to increase the TC level significantly in both HCD and ALA groups but the low TC: HDL ratio observed in the ALA group indicating that supplementation of alpha-lipoic acid may have possibly facilitated the hepatic HDL biosynthesis *in vivo*.

In the present study, when Atherogenic Index in Plasma (AIP) was calculated (log TG/HDL), it was found that there were no significant differences in the value of AIP between ALA (0.41 and 0.46) and N (0.47 and 0.49) at both w = 5 and w = 10 respectively, which suggest the antiatherogenic property of alpha-lipoic acid to the animals. Interestingly, the value of AIP in ALA supplemented group also remained relatively stable throughout the study period (0.41–0.46). The use of AIP is a useful predictor of coronary risk potentials as it gives indirect measure of lipoproteins (LDL and HDL) particle size. The significantly lower AIP in both N and ALA groups relative to the HCD group (0.5–0.6 at

w = 5 and w = 10 respectively) observed in this study correspond well to the inverse correlation of the LDL size and AIP values analyzed from 35 cohorts of 1433 subjects with various risk of atherosclerosis and a cohort of 35 normal subjects [*26*].

In the present study, we also found that supplementation of alpha lipoic acid in high cholesterol fed animals could inhibit the progression of atherosclerosis. The formation of atheromatous plaque in the alpha lipoic acid treated group was found to be significantly lower compare to that of the non-treated group (HCD). This data may provide to a new lipoate activity *in vivo*. The inhibition effect by alpha lipoic acid in atherogenesis might be attributable partly to its hypocholesterolemic property. Previous study indicated that the related metabolic functions of alpha lipoic acid is on its role in blood glucose disposal [*13*] through the glucose-metabolizing enzymes, prostaglandin dehydrogenase and alpha keto-glutarate dehydrogenase eventhough some researchers suspects a more direct role in cellular glucose uptake at the cellular membrane [*27*]. However, the involvement of this substance in cardiovasular related diseases and hypercholesterolemia have not been studied. Nevertheless, the prophylaxis of atherosclerosis using antioxidant therapy has been extensively evaluated in animal experiments.

Oxidative stress is one of the causative factors that link hypercholesterolemia with the pathogenesis of atherosclerosis [*4*, *6*, *28*]. Alpha lipoic acid through its reduced form DHLA exhibits a protective measure against free radicals activity eventhough its precise mechanism is still yet to be discovered. In our study, the TBARS level indicated by MDA concentration at w = 5 was significantly lower in the alpha lipoic acid treated group compared to that of the untreated group (HCD) indicating that apart of its hypocholesterolemic effect, alpha lipoic acid also may act as antioxidant. This findings was parallel to previous reports suggesting that alpha lipoic acid would be a potent metabolic antioxidant source to quench free radicals *in vitro* and *in vivo* [*21*, *29–30*]. Sen and coworkers [*27*] reported that alpha lipoic acid analogue have a positively charged in physiologic pH. DHLA is a potent sulfhydryl reductant; redox potential of the DHLA: ALA couple is –0.32 V (compared to –0.24 V for the glutatione:oxidized glutatione couple) [*31*]. This low redox potential may enable DHLA to easily donate its electron to electron-deprived molecules subsequently neutralized free radical activity. Studies with human Jurkat T cells have shown that when added to the culture medium, lipoate readily enters the cell where it is reduced to DHLA and rapidly efflux into the culture medium [*16*]. The concentration of MDA increased in w = 10. This phenemenon was speculated due to high retention of cholesterol, particularly the LDL in the plasma that in turn exposing them to free radical attacks. It is well known that high cholesterol diet induces the overproduction of reactive oxygen species (ROS) which could initiate lipid peroxidation (LPO) [*14*, *32*]. Supplementation of alpha lipoic acid has shown to have favourable effect for the first five week of treatment, however, excessive cholesterol level in blood due to continual cholesterol load may possibly have overshadowed the existing effect in the subsequent period. This study also reveals that prolonged intake of high cholesterol diet may cause retention of TC and LDL therefore exposing them to free radical attacks even with supplementation of exogenus antioxidant. The extra high concentration of cholesterol may accumulated in the hepato-biliary system resulting in relative high blood LDL. Reduction of TC and LDL would heavily depend on the cholesterol concentration in the diet.

This experiment suggest that alpha lipoic acid supplementation may increase lipid and lipoprotein regulation. The mechanism on how alpha lipoic acid able to reduce TC and LDL concentration is unknown, but probably via lipoprotein lipase (LPL) activity or through cholesterol metabolisme by the liver [*32*]. Chiba and co-worker reported LPL activity and HDL level is increased in cholesterol fed NZW rabbit after administration of NO-1886 [*33*]. Alpha lipoic acid probably capable to initiate LDL receptor synthesis in the liver which in turn increase the uptake of cholesterol back to the hepatic system and increase synthesis of apoprotein A component (moeity of HDL particles) for reversed cholesterol transport [*14*]. With consideration to the dose of alpha lipoic acid, progression of atherosclerotic disease probably could be reduced to some extent. Although the present data do not allow the conclusion that alpha lipoic acid supplementation could prevent atherosclerosis-free radical activity, the data are in agreement with a model in which antioxidant supplementation may contributes to a reduction of bad cholesterol in the circulatory system.

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