Effect of alpha lipoic acid on oxidative stress and vascular wall of diabetic rats

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Abstract

Premises and objectives: Antioxidant plays an important role in preventing the progression of diabetes mellitus (DM) complications. The aim of the present study was to investigate the effect of alpha lipoic acid (ALA) supplementation on plasma lipid, oxidative stress and vascular changes in diabetic rats.

Material and methods: Diabetes was induced by a single intravenous injection of streptozotocin (STZ) (50 mg/kg). The diabetic rats were divided into two groups: (i) supplemented group with ALA (100 mg/kg/day) and (ii) non-supplemented group without ALA. Non-diabetic rats (NDM) formed the control group, which received saline injection.

Results: Following eight weeks of supplementation, fasting blood glucose (FBG) and glycosylated hemoglobin (HBA1c) in ALA-supplemented rats was found to be significantly lower than the non-supplemented group. ALA-supplementation also improved dyslipidemia that occurred in diabetic rats. ALA-supplementation also significantly increased plasma superoxide dismutase (SOD) activity and vitamin C level as compared to the No Suppl group. The increase in plasma and aorta malondealdehyde + 4-hydroxynonenal (MDA + 4-HNE) levels were also inhibited and the levels of oxidative DNA damage of peripheral lymphocytes were significantly reduced. Electron microscopic examination of thoracic aorta revealed that normal tissue organization was disrupted in STZ-diabetic rats with ALA-supplementation reducing the changes in the vascular morphology.

Conclusions: It is concluded that ALA has the potential in preventing the alteration of vascular morphology in diabetic rats probably through the improvement of glycemic status and dyslipidemia as well as its antioxidant activities.

Keywords: antioxidant, diabetes mellitus, alpha lipoic acid, atherosclerosis.

Introduction

The incidence of diabetes mellitus (DM) is prevalent over all parts of the world. It is also associated with reduced quality of life and increased mortality and morbidity factors. DM is a complex, progressive disease, which is accompanied by multiple complications. Control of blood lipid level is an important step to decrease the incidence of long-term complications of the disease [1].

Data collected from clinical studies indicate that majority of the diabetic patients die due to cardiovascular diseases and atherosclerosis, which accounts for about 8 to 10% of all diabetic deaths [2]. The established relationship between DM and atherosclerosis has fuelled suggestion to search for antidiabetic drugs with beneficial effects in reducing the atherosclerotic process in diabetic patients [3].

Hyperglycemia is attributed to the development of diabetic complications. Hyperglycemia leads to increase in oxidative stress due to the overproduction of free radicals and decreased efficiency of antioxidant defense system. It occurs particularly in patients with poor glycemic control [4].

Oxidation of lipids, proteins and other macromolecules (such as DNA) occurs during the development of diabetes and its complication. To control the influx of free radicals, aerobic cells have developed an antioxidant defense system, which directly
or indirectly scavenges free radicals or prevents their conversion to toxic products [5].

The stability and capacity of antioxidant status during chronic diabetes seriously influences the outcome of the long-term complications caused by oxidative stress [6].

ALA is a cofactor of α-ketoacid dehydrogenase complexes and it plays a fundamental role in the metabolism. ALA has been found to affect cellular metabolic processes, alter redox status of cells, interact with thiols and other antioxidants [7]. ALA is an unique antioxidant because it has beneficial effects on energy production and also an essential cofactor of mitochondrial respiratory enzymes, including the pyruvate dehydrogenase (PDH) complex [7].

Dietary antioxidants have become a popular in the treatment of the prevention of the DM and its complications. Regimes that counter hyperglycemia such as oral anti-diabetic drugs and insulin do not completely prevent the risk of developing diabetic complications [8]. Recently, the uses of antioxidants have proved to be helpful in the reduction of oxidative stress and slow the progress of diabetic vascular complications [4].

As the incident of diabetes and its complications increase rapidly, there is an urgent need to search a new source with antidiabetic properties. Therefore, the main aim of the present study was to examine if ALA-supplementation had the protective effect on the plasma lipid, oxidative stress and atherosclerotic changes in the aorta in STZ-induced diabetic rats.

Material and Methods

Diabetes induction

Male Spraque Dawley rats weighing 260–290 g were obtained from the Animal Unit of Universiti Kebangsaan, Malaysia. Two animals were housed per cage. All animals were maintained on a balanced diet and water ad libitum without restricted. Universiti Kebangsaan (Malaysia) Animal Ethics Committee (UKMAEC) approved the study and guidelines for using live animals were followed.

Diabetes was induced in a group of rats after an overnight fast by single intravenous injection (via tail vein) of STZ (Sigma, St. Louis, MO, USA), which was freshly dissolved in normal saline. The dose of administration was 50 mg/kg body weight. Another group of rats, which only received saline formed the non-DM group (NDM) (n = 7).

Three days later, blood was collected via tail vein and glucose concentration measured by a strip-operated blood glucose sensor (Companion 2, Medisense Ltd., Birmingham, UK). Rats with blood glucose levels >15.0 mmol/L were included in the study and divided into two groups: supplemented with ALA (n = 8) and non-supplemented with ALA (n = 8). ALA (Sigma, St. Louis, MO, USA) was administered orally at a dose of 100 mg/kg body weight/day throughout the feeding period of eight weeks and the supplementation was begun on the same day. The treatment dose of ALA was based on a previous study [9].

Non-supplemented and NDM rats were left untreated.

Biochemical analysis

Following eight weeks of supplementation, the rats were fasted overnight and blood was collected by cardiac puncture under deep anesthesia with diethyl ether. Blood was collected in tubes containing sodium fluoride tube (fasting blood glucose (FBG) analysis) and EDTA.

Thoracic aorta was quickly excised, the upper third was cut for transmission electron microscopy evaluation and the rest was used for biochemical analysis. Aortic homogenates were prepared essentially as described by Upston JM et al. [10].

Blood was kept on ice and centrifuged at 3000 rpm for 20 minutes at 4°C and the obtained plasma was stored at -40°C until analysis.

Plasma glucose levels were analyzed using enzymatic glucose-oxidase kits (Trace Scientific, Melbourne, Australia, catalogue no. TR 15104). Blood HbA1c was determined using method described by Eross J et al. [11] and express as percent of total hemoglobin.

Determination of total cholesterol (TC), triglyceride and high-density lipoprotein (HDL–C) were carried using kits (Teco Diagnostics, 1286N. Lakeview Ave., Anaheim) and low-density lipoprotein (LDL–C) determination was calculated using method by Friedewald WT et al. [12].

Protein concentrations were measured by the method of Bradford MM [13] and MDA + HNE levels were assayed by lipid peroxidation using a kit (Calbiochem’s Lipid Peroxidation Assay, catalogue no. 437634). SOD activities were determined according to Beyer WF Jr and Fridovich I [14]. In this method, SOD activities were measured by the photochemical nitro blue tetrazolium (NBT) reduction (one unit of SOD is the amount which causes a 50% decrease of the SOD-inhibitable NBT reduction).

Plasma vitamin C was measured according to a previous study [15]. Briefly, 100-µL plasma was mixed with 100 µL of 10% perchloric acid containing 1% of meta-phosphoric acid. All were mixed in 1.5 Eppendorf tubes, wrapped in aluminum foil to ensure the vitamin C was not be degraded by light and kept at -40°C.

Vitamin C analysis was performed within one week of storage using high-performance liquid chromatography (HPLC) at room temperature. The HPLC system consisted of a Gilson 307 pump, Rheodyne manual injector, 20-µL sample loop and a Gilson 151 UV/VIS set at 245 nm. Column for separation was a Metaphase Crestpak C18S (4.6 mm I.D. × 150 mm L).

Mobile phase consisted of 0.015% metaphosphoric acid in 20 mM ammonium dihydrogen phosphate (pH 2.95). Flow rate was set at 1 mL/min. with a run time of 10 minutes.

Eluted peak of vitamin C was detected using UV/VIS Gilson set at 245 nm and results were analyzed based on the area under the peak.

Prior to injection of the sample into the system, 200-µL HPLC mobile phase was added to sample,
vortex and centrifuged at 20 000 g for 1 minute. A total of 10-µL supernatant was then injected into the HPLC for vitamin C analysis.

Standard curve to extrapolate true concentration of vitamin C in sample was performed daily by diluting 10 mg L-(-)-ascorbic acid (BDH, Poole, UK) in 10 mL fresh mobile phase. The mixed solution was then diluted further with 1% metaphosphoric acid and 10% perchloric acid to produce a stock solution with a concentration of 100 µg/mL.

External control of analysis was done by injecting a vitamin C standard for every 10 injections. An external control concentration deviation of 5% would result in the creation a new standard curve.

Alkaline Comet Assay

The Alkaline Comet Assay procedure was performed as per previous study [16].

Electrophoresis slides observation were made using Leitz Laborlux fluorescence microscope (Nikon) equipped with an epifluorescence mercury lamp source (excitation filter 515, barrier filter 590 nm) and X40 fluorescence objective (numerical aperture 0.85) and X40 equipped with an epifluorescence mercury lamp source (Leitz Laborlux fluorescence microscope (Nikon)).

The Alkaline Comet Assay procedure was performed as per previous study [16]. Briefly, after dissection, the thoracic aorta was cut into small pieces, fixed at 4°C for 2 hours in 2.5% buffered glutaraldehyde, and post-fixed for 1.5 hour with osmium tetroxide.

Tissues were dehydrated in ethyl alcohol followed by propylene oxide and embeded in araldite. Semi thin sections of plastic embedded samples were stained with toluidine blue.

Ultrathin circumferential sections were stained with uranyl acetate and lead citrate and subsequently examined by the Tecnai G2 Transmission Electron Microscope (FEI, USA).

Results

FBG, HbA1c, TC, LDL–C and HDL–C and triglycerides levels for all groups are shown in Table 1.

FBG and HbA1c levels were found to be higher in the non-supplemented and ALA groups than in the NDM group. However, a significantly reduction in FBG and HbA1c levels were seen in the ALA group as compared to the non-supplemented group. Plasma TC and LDL–C and triglyceride concentrations were markedly increased in non-supplemented diabetic rats as compared to NDM rats.

ALA-supplementation prevented the increase of TC, LDL–C and triglyceride and these levels were significantly lower compared with the non-supplemented rats. On the other hand, the ALA-supplementation group also has significantly higher levels of HDL–C as compared with non-supplemented group.

The oxidative stress parameters in plasma and aortic homogenates are shown in Table 2.

Table 1 – Plasma glucose, HbA1c and lipid profile in NDM, No Suppl and ALA group following eight weeks of study

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Groups</th>
<th>NDM</th>
<th>No Suppl</th>
<th>ALA</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBG (nmol/L)</td>
<td>6.90 ± 0.22</td>
<td>31.22 ± 0.15*</td>
<td>27.73 ± 1.10**</td>
<td></td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>4.57 ± 0.28</td>
<td>10.95 ± 0.93*</td>
<td>9.01 ± 0.52**</td>
<td></td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>45.59 ± 2.44</td>
<td>72.11 ± 3.50*</td>
<td>48.04 ± 2.94**</td>
<td></td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>37.64 ± 1.08</td>
<td>149.35 ± 11.19*</td>
<td>105.73 ± 4.84**</td>
<td></td>
</tr>
<tr>
<td>LDL–C (mg/dL)</td>
<td>18.93 ± 1.01</td>
<td>38.94 ± 3.87*</td>
<td>10.42 ± 1.00*</td>
<td></td>
</tr>
<tr>
<td>HDL–C (mg/dL)</td>
<td>20.84 ± 1.59</td>
<td>4.16 ± 0.58*</td>
<td>16.31 ± 0.74*</td>
<td></td>
</tr>
</tbody>
</table>

Data are the mean ± SEM. *p<0.05 vs. NDM group; **p<0.01 vs. No Suppl group.

Table 2 – MDA + HNE and SOD plasma and aorta, plasma vitamin C levels, and DNA damage in peripheral lymphocyte in NDM, No Suppl and ALA group following eight weeks of study

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Groups</th>
<th>NDM</th>
<th>No Suppl</th>
<th>ALA</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA + HNE plasma (nmol/g protein)</td>
<td>14.51 ± 1.44</td>
<td>25.00 ± 1.08*</td>
<td>17.26 ± 0.07*</td>
<td></td>
</tr>
<tr>
<td>MDA + HNE aorta (µmol/g protein)</td>
<td>10.71 ± 1.15</td>
<td>14.79 ± 1.46*</td>
<td>9.72 ± 1.25*</td>
<td></td>
</tr>
<tr>
<td>SOD plasma (u.e/g protein)</td>
<td>3.33 ± 0.14</td>
<td>1.27 ± 0.11*</td>
<td>1.97 ± 0.12*</td>
<td></td>
</tr>
<tr>
<td>SOD aorta (u.e/g protein)</td>
<td>3.19 ± 0.06 x10⁷</td>
<td>1.50 ± 0.19 x10⁸*</td>
<td>1.74 ± 0.18 x10⁸*</td>
<td></td>
</tr>
<tr>
<td>Vitamin C plasma (µg/mL)</td>
<td>1.17 ± 0.08</td>
<td>0.66 ± 0.08*</td>
<td>1.29 ± 0.07*</td>
<td></td>
</tr>
<tr>
<td>DNA Comet tail of lymphocyte (%)</td>
<td>4.00 ± 2.46</td>
<td>7.11 ± 0.35*</td>
<td>5.09 ± 0.47*</td>
<td></td>
</tr>
<tr>
<td>Tail moment of lymphocyte</td>
<td>0.46 ± 0.04</td>
<td>5.76 ± 0.38*</td>
<td>0.27 ± 0.04*</td>
<td></td>
</tr>
</tbody>
</table>

Data are the mean ± SEM. *p<0.05 compared with NDM group; **p<0.01 compared with No Suppl group.
STZ-induced diabetic resulted in a significant decrease in plasma and aortic homogenates SOD activities as well as plasma vitamin C levels. However, plasma SOD activities in ALA group were significantly higher as compared to non-supplemented group.

ALA-supplementation also inhibited the reduction of plasma vitamin C in diabetic rats. MDA + HNE levels of plasma and aortic homogenates also significantly increased in non-supplemented diabetic groups as compared with NDM group.

Treating diabetic rats with ALA also normalized MDA + HNE levels of plasma and aortic homogenates. The levels of endogenous DNA damage, measured by the % tail DNA of the lymphocytes were significantly higher in non-supplemented rats compared to NDM rats and ALA-supplementation managed to normalize the % tail DNA in ALA diabetic group.

Tail moment, which is defined as the product of the tail length and the fraction of DNA in the tail was also used as a parameter to describe the extent of DNA damage. Diabetic rats showed significantly higher levels of tail moment compared to NDM rats. ALA-supplementation was also able to normalize the tail moment value.

**Morphological observation of the aorta**

By electron microscopic examination, the aorta from control rats was found to compose mainly the smooth muscle cells (M), elastic lamina (EL), and a small amount of extracellular matrix (EM) between the elastic lamina and smooth muscle cells (Figure 1A).

The aortic media also showed a regular disposition of smooth muscle cells between the elastic lamina within a homogenous interstitial matrix. Endothelial cells in aorta from control rats were in closed contact with the underlying vessel wall and were smooth and uniform (Figure 1B).

STZ-diabetes caused severe alteration of vascular structure in non-supplemented diabetic rats as shown in Figures 2 and 3.

Intimal surface of aorta were irregular and the endothelial cells showed irregular distribution, become atrophic and lost their squamous characteristics. Subendothelial region was patchily thickened with the present of infiltrating mononuclear cells.

Aortic media showed irregular disposition of smooth muscle cells between partly fragmented elastic lamina within degenerative interstitial matrix.

Internal elastic lamina also appeared fragmented and reduplicated. Irregularly fragmented and atrophic smooth muscle cells were frequent observed together with increasing in extracellular matrix, predominantly consisting electron-dense amorphous material.

The migration of smooth muscles cells from the media into the intima with the breaking through the inner elastic lamina.

ALA-treatment of diabetic rats reduced the smooth muscle cell proliferation the formation of electron-dense amorphous material in the aortic media.

Elastic lamina lamina was more regular. Endothelial cells of aorta from ALA-treated rats were having squamous characteristic with the intima surface appeared smoother, with fewer defect.

![Figure 1](image-url)
Effect of alpha lipoic acid on oxidative stress and vascular wall of diabetic rats

Figure 2 – The ultrastructure of the aorta in diabetic rats following eight weeks of study. (A) Proliferation and fragmentation of medial smooth muscle cells (SMC) in both intima and media layer. The extracellular matrix also consisting predominantly amorphous material (AM) was apparent in the media of diabetic rats. Endothelial cell (EC) lost its squamous characteristic and become atrophic (original magnification ×4400). (B) A smooth muscle cell (SMC) is migrating from the media into the intima, breaking through the elastic lamina (EL) (original magnification ×4400). (C) The present of mononuclear cell (MC) and fragmented smooth muscle cell (SMC) at the subendothelial layer of the intima. There was fragmentation and reduplication of elastic lamina (original magnification ×6500).

Figure 3 – The ultrastructure of the aorta in ALA group following eight weeks of study. The elastic lamina (EL) appeared more regular and smooth muscle cells (SMC) does not actively proliferate. Although the amorphous materials (AM) in the extracellular matrix still present, but the amount is less. The endothelial cell (EC) still having squamous characteristic (original magnification ×3200).
Discussion

In the present study, blood glucose level in STZ diabetic rats was significantly higher as compared to the control rats. Administration of STZ caused destruction of the β-cell of pancreas and lead to reduction of insulin secretion, thereby leading to increase in plasma glucose levels. Oral administration of ALA had shown hypoglycemic effects against STZ-induced diabetes in rats and these observations are consistent with previous findings reported [18, 19].

ALA is a potent antioxidant that has effects on fuel metabolism and also acts as an essential cofactor of mitochondrial respiratory enzymes, including the pyruvate dehydrogenase (PDH) complex [20]. Quercetin, which contains antioxidant properties, had the beneficial effects in decreasing blood glucose concentration, promoting regeneration of the pancreatic islets and increasing insulin release in STZ-induced diabetic rats [20]. Luostarinen R et al. [21] had reported that supplementation of vitamin E for four weeks was capable to prevent hyperglycemia condition following fish oil intake in healthy volunteers through the decrease in insulin production and insulin/glucose ratio. They had postulated that the antioxidant effects of vitamin E could have the protective effect to β-cell destruction, which occurred due to the lipid peroxidation process following the fish oil intake.

Elevation of plasma lipid is one of several risk factors present in most diabetic patients that accelerated the long-term development of cardiovascular complication [22]. The present study had shown that STZ-diabetes leads to dyslipidemia condition and this is in agreement with the results of previous investigation [17]. The increase in TC, LDL-C, and triglycerides as well as decrease HDL-C concentrations may be as a result of reduced lipoprotein lipase activity secondary to reduce in plasma insulin levels [23]. Cholesteryl ester transfer protein, which is important in regulating lipoprotein lipid composition was increased in DM condition, which may have contributed to the dyslipidemia [23].

ALA-supplementation corrected the dyslipidemia occurred and the mechanism action of ALA is believed through the controlling the activity of enzyme that involved in lipid metabolism. ALA was found to increase HMG-CoA reductase activity and increase lipoprotein lipase and lecithin cholesterol acyl transferase (LCAT) [24].

In the present study, the Alkaline Comet Assay measured the basal levels of DNA damage in freshly isolated lymphocyte. There were significant increases in the levels of DNA damage in the lymphocyte from non-supplemented diabetic rats. Our results also indicated that MDA + HNE levels were significantly higher in non-supplemented diabetic rats. These findings suggest that DNA damage is associated with the production of free radicals. Free radicals including hydroxyl radicals, singlet oxygen, peroxy radicals and peroxynitrite are able to produce other modification to the DNA bases as well as strand breakage and various DNA-damage [25].

ALA which been used as a nutritional supplementation, has several potential benefits including therapeutic potential and capable of scavenging free radicals [7]. In the present study, ALA-supplementation managed to prevent the increase in MDA + HNE levels and inhibit DNA damage in diabetic rats. The ability of ALA to decrease lipid peroxidation process as demonstrated in the present study was to confirm the previous finding [26, 27]. This finding indicates that ALA has the potential in reducing oxidative damage. The percentage of comet tail in ALA group is significantly low when compared to untreated diabetic rats, but markedly higher when compared to the NDM group. However, the levels of DNA damage as measured by tail moment, showed no significant difference between NDM and ALA groups. This indicated that the DNA-damage that occurred in ALA-supplementation rats had been repaired.

This finding is an agreement with the previous study, which found ALA has the capability in preventing DNA-damage induced by palmitic acid [28]. Supplementation of ALA had caused a substantial increase in nucleic acid content and protein in aged rats. Previous study had showed that antioxidants such as fluvastatin [29, 30] and vitamin E [31] exhibited protective effect against oxidative DNA-damage.

Reduction in plasma and aorta SOD activities in the present study may be due to the elevated of free radicals and the analogous findings were observed in experimental diabetes [32]. Vitamin C is an excellent hydrophilic antioxidant in plasma, because it disappears faster than other antioxidant when plasma is exposed to reactive oxygen species [33]. The elevated levels of vitamin C and SOD activities in ALA-supplemented group play a protective role against oxidative stress in diabetic condition. ALA replenishes vitamin C, glutathione and vitamin E through the reduction of their radicals via the redox cycle [7]. This observation showed that ALA could improve the antioxidant defense mechanism, decreased in free radicals levels and thereby reduce the oxidative damage that occurred in diabetic condition.

The morphological findings of this study have shown that that ultrastructural organization of aorta is disturbed in STZ-induced diabetic rats. In the current study, various morphological changes in endothelial cells indicative of endothelial injury. Infiltration of macrophages in the subendothelial space and migration of medial smooth muscle cells to the intima and proliferation of medial smooth muscle cells in the intima layer were observed in diabetic rats. These findings were considered to be early events in the development of the atherosclerotic lesion [34].

The increased in MDA + HNE levels and decreased in SOD activities of thoracic aorta reflects the increased in oxidative stress had occurred. Kamalakkannan N and Prince PS [35] reported that the elevation in MDA + HNE levels in aorta are consequences of increased production and liberation of lipid peroxides due to the pathological changes occurred.

This study is consistent with previously study, which demonstrated that hyperglycemia, hyperlipidemia and
high oxidative stress might cause morphological alteration in the aorta and could be considered to be important factor in the initiation of atherosclerotic lesions development in STZ-diabetic rats [17].

This study-demonstrated supplementation of ALA at eight weeks to the diabetic rats reduced the alteration in vascular morphology of aorta. The mechanism by which ALA-supplementation exerts the vascular morphology is probably through its antioxidant properties whereby supplementation with ALA to the diabetic rats effectively prevents the increase in oxidative stress in plasma and the thoracic aorta.

According to Packer L et al. [7], ALA is capable to scavenge reactive oxygen species generated during the lipid peroxidation and protects the cell structure against damage. ALA is functionally efficient in helping cells to recover from oxidative damage [9]. Besides that, ALA-supplementation also effectively inhibits dyslipidemia and improved hyperglycemia condition. Therefore, the mechanism by which ALA-supplementation exerts the vascular morphology is probably through its antioxidant properties and the effects on carbohydrate and lipid metabolism.

Conclusions

The present findings show that hyperglycemia, dyslipidemia and high oxidative stress play an important role in vascular morphological changes in diabetic rats. Hypoglycemic, hypolipidemic, and antioxidative stress effects of ALA associates with the prevention of development of abnormalities in the structure of aorta in STZ-diabetic rats. Therefore, ALA is a potent antioxidant for the protection of the vascular wall against oxidative injury associate with diabetes mellitus.

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