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Efficacy of Alpha-Lipoic Acid against Diabetes-Induced Deterioration of Blood Antioxidants and Diabetic Retinopathy in Experimental Animals

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Abstract: The present study aimed to investigate the effect of alpha-lipoic acid (ALA) administration on diabetes-induced alterations of blood antioxidants and retinal histopathological changes. Forty albino Wistar rats of both sexes were used and divided into four groups; two groups (I and II) served as controls. In group III, diabetes was induced by a single intraperitoneal injection of alloxan 100 mg/kg. In group IV, diabetes was induced and rats received a daily oral dose of ALA 60 mg/kg/day for four months. Serum glucose, serum vitamin C and erythrocyte reduced glutathione levels were measured after 2 and 4 months. Then, animals were sacrificed and subjected to light microscopic examination of the retina. Untreated diabetic animals exhibited significant deterioration of the measured biochemical parameters in addition to marked histopathological retinal changes. Treatment with ALA improved significantly the diabetes-induced deterioration of vitamin C and glutathione in blood. In addition, the retina of these animals appeared normal with no significant deviation from the control retina suggesting its protective role against diabetic retinopathy.

Key words: Alpha-lipoic acid, experimental diabetes mellitus, antioxidant, diabetic retinopathy, vitamin C, glutathione.

INTRODUCTION

Diabetic neuropathy represents a major health problem as it is responsible for substantial morbidity, increased mortality and impaired quality of life. Near normoglycemia is now generally accepted as the primary approach for prevention of diabetic neuropathy, but it is not achievable in a considerable number of patients (Ziegler, 2004). In the past two decades several medical treatments that exerted their effects despite hyperglycemia have been derived from the experimental pathogenic concept of diabetic neuropathy. Such compounds have been designed to improve or slow down the progression of the neuropathic process and are being evaluated in clinical trials to achieve the above benefits by many researchers (Negrisanu *et al.*, 1999 and Bregovskii *et al.*, 2005).

Alpha-lipoic acid (ALA) and its reduced form dihydro-lipoic acid are present in all prokaryotic and eukaryotic cells. Lipoic acid was once considered a vitamin, but now it is commonly accepted that it can be synthesized de novo in human cells. It has long been known as a coenzyme of multi-enzymatic complexes catalyzing the decarboxylation of alpha keto acids. In addition, ALA is involved in the regulation of carbohydrate and lipid metabolism (Malinska and Winiarska, 2005). Moreover, ALA was found to be a potent free radical scavenger and metal chelator. It also plays an important role in the regeneration of the active form of other cellular antioxidants including vitamins C and E (Biewenga *et al.*, 1997). Being easily absorbed from the gastrointestinal tract and able to cross the blood brain barrier without exhibiting any serious side effects along with the above mentioned features, ALA is considered a very promising drug (Malinska and Winiarska, 2005).

Alpha-lipoic acid was used successfully to ameliorate diabetic polyneuropathies in clinical trials (Ziegler, 2004). Negrisanu *et al.* (1999) have proved its value in improving the nerve conduction velocity and clinical manifestations of diabetic peripheral neuropathy. In addition, Bregovskii *et al.* (2005) observed an improvement in diabetic sensory deficiency after treatment with ALA. Moreover, Tankova *et al.*,(2004) reported its

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effectiveness in autonomic diabetic neuropathy. It was also reported to possess protective effects on the retina of diabetic mice (Johnsen-Soriano *et al.*, 2008).

In the present study, ALA is evaluated for the treatment or amelioration of diabetic retinopathy and its relation to its antioxidant properties in experimentally induced diabetes mellitus.

MATERIALS AND METHODS

Induction of the Animal Model of Diabetes Mellitus:

Wister rats were injected intraperitoneally with a single dose of 100 mg/kg alloxan (Sigma, Germany). The blood glucose level was measured after 48 hours then weekly. Diabetes was defined as a fasting blood glucose level exceeding 120 mg/dl.

Animal groups:

Forty Albino Wistar rats of both sexes weighing 140-150 grams were used. They were housed individually in separate cages under veterinary supervision. They were used in accordance with institutional guidelines and with the statement for use of animals in ophthalmic and vision research. They were fed with the standard diet and water for four months and kept in 12 hours dark/light cycles under controlled temperature and humidity. Animals were divided into four groups each consisting of ten rats.

A- Control Groups:

Group I. The animals received an equivalent volume of distilled water once daily by means of a stomach tube (negative control).

Group II. The animals received a daily oral dose of 60 mg/kg/day ALA (Thiotacid from EVA Pharma, Egypt) dissolved in distilled water by means of a stomach tube for four months (positive control).

B- Diabetic Model Groups:

Group III. Diabetic model was induced and the animals were left untreated.

Group IV. Diabetic model was induced and the animals received a daily oral dose of ALA 60 mg/kg/day dissolved in distilled water using a stomach tube for four months starting from the third day of induction of diabetes.

Biochemical Analysis:

Serum glucose was enzymatically determined using biomerieux kit according to the method described by Siest *et al.* (1981). Serum vitamin C and erythrocyte reduced glutathione were measured by colorimetric methods described by Jogata and Dani (1982) and Beutler *et al.* (1963) respectively.

Histopathological Examination:

Histopathological examination was carried out according to Drury and Wallington (1980). The eyes were enucleated, dissected and immediately double fixed in 4% glutraldehyde buffer, then 1.3% osmium tetraoxide in phosphate buffer (pH 7.3). Retinal specimens were processed and embedded in araldite Cy 212. Semi-thin sections were stained with toluidine blue (TB) and examined by light microscopy.

Statistical Analysis:

Values of serum glucose, serum vitamin C and erythrocyte reduced glutathione were expressed as mean±SD. Analysis of variance (ANOVA) and student t test were performed to compare the values between groups. A post-hoc test was used to isolate significant differences if ($P < 0.05$).

RESULTS AND DISCUSSION

Biochemical Analysis:

The mean glucose, vitamin C serum levels and erythrocytes reduced glutathione (at the 2nd and 4th months) are shown in Tables (1, 2 and 3). Control rats (groups I and II) showed normal values of the estimated parameters which were not significantly changed all through the duration of the experiment.

Regarding induction of diabetes model in group III, rats exhibited a noticeable deterioration in the tested parameters. The blood glucose level increased significantly to 143.83±11.23 and 153.00±23.66 mg/dl at the 2nd and 4th months respectively (Table 1). While vitamin C serum level dropped markedly to 0.65±0.30 and 0.56±0.25 mg/dl (Table 2) and the glutathione level decreased markedly to 42.00±6.81 and 40.66±8.33 mg/dl at the 2nd and 4th months respectively (Table 3). All these values were significantly different from the control values. After treatment of diabetic rats with ALA (group IV), it was observed that the serum glucose levels were slightly improved to 125.17±8.47and128.33±15.46 mg/dl at the 2nd and 4th months respectively. However, they were still significantly higher than control group values. Comparing serum glucose levels of group IV with untreated animals of group III, it was found to be significantly lower than diabetic model values after 2 months but insignificantly lower after 4 months (Table 1). It is also noticed that vitamin C level was improved in animals treated with ALA. It reached 1.00±0.58 and 1.36±0.38 mg/dl at the 2nd and 4th months respectively. Although vitamin C level was insignificantly higher than its level in the untreated diabetic model after 2 months, it was more improved after 4 months of ALA treatment to be significantly higher than that of the untreated animals of group III. In addition, it was improved to be insignificantly different from the control group values (Table 2). Moreover, group IV showed markedly improved glutathione levels reaching values that were significantly higher than the untreated diabetic model values and insignificantly lower than control group values. These values were 60.66±15.95 and 67.50±21.51 mg/dl at the 2nd and 4th months respectively (Table 3).

Table 1: Mean levels (±SD) of serum glucose at the 2nd and 4th months of the experiment in group I (negative controls), group II (positive controls receiving 60 mg/kg/day ALA orally for 4 months), group III (untreated diabetic model) and group IV (diabetic model treated with 60 mg/kg/day ALA orally for 4 months).

Groups	Group I	Group II	Group III	Group IV
Mean ± SD after 2 months	69.17 ± 8.70	71.54 ± 10.25	143.83 ± 11.23	125.17 ± 8.47
P1		0.769	0.000*	0.000*
P2				0.031*
Mean ± SD after 4 months	69.33 ± 9.00	71.66 ± 7.92	153.00 ± 23.66	128.33 ± 15.46
P1		0.503	0.000*	0.000*
P2				0.147

Data are expressed as mean ± SD; n = 10; ALA: Alpha lipoic acid; * Significant difference at $p < 0.05$.
P1 compared to group I; P2 compared to group III.

Table 2: Mean levels (±SD) of serum vitamin C at the 2nd and 4th months of the experiment in group I (negative controls), group II (positive controls receiving 60 mg/kg/day ALA orally for 4 months), group III (untreated diabetic model) and group IV (diabetic model treated with 60 mg/kg/day ALA orally for 4 months).

Groups	Group I	Group II	Group III	Group IV
Mean ± SD after 2 months	1.33 ± 0.39	1.40 ± 0.35	0.65 ± 0.30	1.00 ± 0.58
P1		0.709	0.010*	0.217
P2				0.248
Mean ± SD after 4 months	1.36 ± 0.36	1.50 ± 0.51	0.56 ± 0.25	1.36 ± 0.38
P1		0.421	0.019*	1.00
P2				0.004*

Data are expressed as mean ± SD; n = 10; ALA: Alpha lipoic acid; * Significant difference at $p < 0.05$.
P1 compared to group I; P2 compared to group III.

Table 3: Mean levels (±SD) of erythrocyte reduced glutathione at the 2nd and 4th months of the experiment in group I (negative controls), group II (positive controls receiving 60 mg/kg/day ALA orally for 4 months), group III (untreated diabetic model) and group IV (diabetic model treated with 60 mg/kg/day ALA orally for 4 months).

Groups	Group I	Group II	Group III	Group IV
Mean ± SD after 2 months	79.00 ± 6.89	74.50 ± 12.66	42.00 ± 6.81	60.66 ± 15.95
P 1		0.570	0.000*	0.063
P 2				0.025*
Mean ± SD after 4 months	77.83 ± 6.21	80.33 ± 8.52	40.66 ± 8.33	67.50 ± 21.51
P 1		0.381	0.000*	0.232
P 2				0.036*

Data are expressed as mean ± SD; n = 10; ALA: Alpha lipoic acid; *Significant difference at $p < 0.05$.
P1 compared to group I; P2 compared to group III.

Histopathological Examination:

The histological examination of the retinas of rats in the negative controls (group I) showed the normal layers of the retina (Fig. 1). In addition, the retinas of rats of group II treated with ALA (positive control) appeared fairly normal (Fig. 2).

Light microscopic examination of the retinas of diabetic rats (group III) revealed edema of all layers and accumulation of dark stained granules at the periphery of pigmented cells. In addition, the nuclei of the outer nuclear layer appeared dense stained and clear spaces between the rows of nuclei were observed. Some nuclei of the inner nuclear layer showed condensation of chromatin. Small blood capillaries with thickened basement membrane were also seen in this layer (Fig. 3). The ganglion cell layer showed lucent cytoplasm and the nerve fiber layer displayed dilated capillaries with thickened membrane. The lumens of these dilated capillaries were filled with fragmented deformed adherent blood elements (Fig. 3).

Diabetic animals treated with ALA (group IV) exhibited well protection of the retina against the above diabetic changes. Light microscopic examination of the retina of these rats showed no significant deviation from the control retina (Fig. 4).

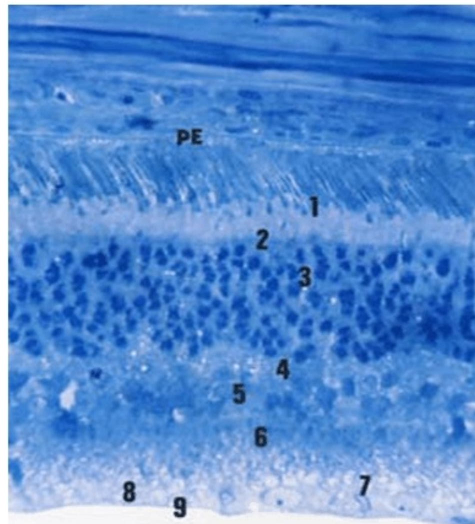

Fig. 1: Light micrograph of a semi-thin section of control rat retina (group I) showing the supporting retinal pigment epithelium (PE) and the normal nine retinal layers: 1- photoreceptors, 2- outer limiting membrane, 3- outer nuclear layer, 4- outer plexiform layer, 5- inner nuclear layer, 6- inner plexiform layer, 7- ganglion cell layer, 8- nerve fiber layer and 9- inner limiting membrane (TB X500).

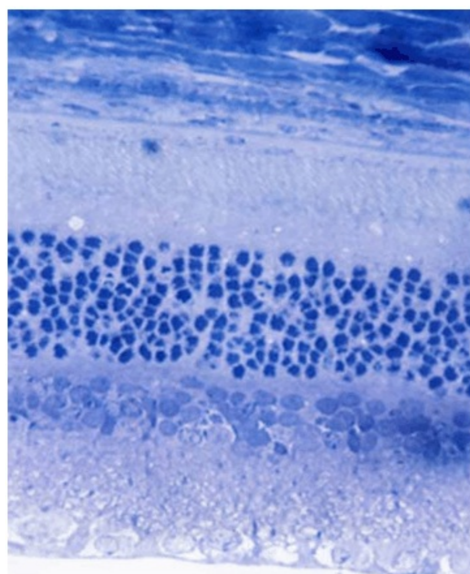

Fig. 2: Light micrograph of a semi-thin section of rat retina of group II (positive controls) appearing fairly normal (TB X500).

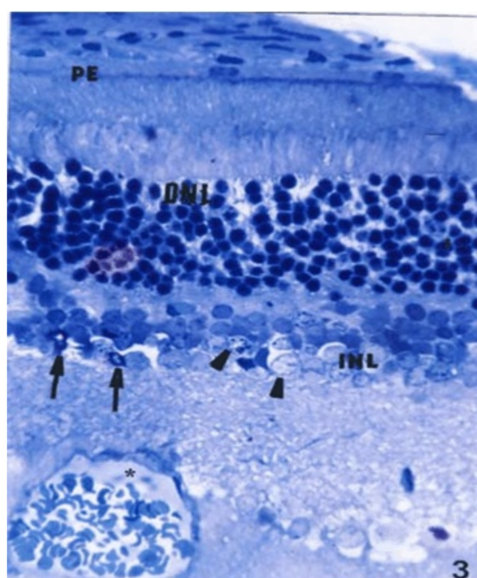

Fig. 3: Light micrograph of a semi-thin section of rat retina of group III (untreated diabetics) showing edema of all retinal layers. Dark stained granules are present at the periphery of pigment epithelial cells (PE). Most nuclei of the outer nuclear layer (ONL) appear dense stained with clear spaces between some of the rows of nuclei. The inner nuclear layer (INL) shows condensation of chromatin in some nuclei (arrow heads). Small blood capillaries with thickened basement membrane are present in this layer (arrows). The lumen of a dilated retinal capillary is filled with deformed adherent blood elements (*) (TB X500).

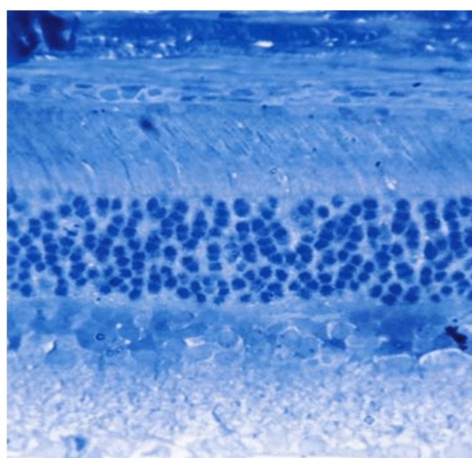

Fig. 4: Light micrograph of a semi-thin section of rat retina of group IV (diabetic rats treated with 60 mg/kg/day alpha-lipoic acid for four months) showing no significant deviation from the control retina (TB X500).

Discussion:

Alpha-lipoic acid, also referred to as thioctic acid, has a therapeutic potential in diabetic complications. It favorably influences the vascular abnormalities of diabetes such as impaired microcirculation, increased indices of oxidative stress and vascular dysfunction (Ziegler, 2004).

In the present work, a significant impairment of the biochemical parameters was observed in the untreated diabetic rats (group III) as indicated by the significant increase in the serum glucose level and the significant decrease in glutathione and vitamin C levels after 2 and 4 months of diabetes induction. These findings are in agreement with those mentioned by previous studies (Noh *et al.*, 2002; Stadler *et al.*, 2004 and Stoppa *et al.*, 2006).

The light microscopic examination of the diabetic retinas revealed the microvascular abnormalities of diabetic retinopathy. Most of the dilated retinal capillaries were filled with deformed, fragmented and adherent blood elements. Another microvascular retinal change was the decreased membrane pericytes that support the walls of retinal blood capillaries. These changes of diabetic retinopathy are believed to indicate threatening ischemic and proliferative retinopathy when critical numbers of retinal capillaries become nonperfused (Mizutani *et al.*, 1996 and Friedman, 1999).

In the present investigation, it was observed that the serum glucose level was decreased after treatment of diabetic rats with ALA. However, these levels were significantly lower than the untreated diabetic rats only after 2 months but not after 4 months of ALA treatment. Similarly, it was previously reported that ALA could decrease blood glucose level in experimentally induced diabetes mellitus (Gavrovskaia *et al.*, 2008 and Winiarska *et al.*, 2008). This effect can be explained by the ability of ALA to increase cellular uptake of glucose by recruiting glucose transporter-4 to the cell membrane which is evidenced in cell culture experiments (Henriksen, 2006 and Packer *et al.*, 2001).

Treating the diabetic rats with ALA for 4 months resulted in a marked improvement of the antioxidant tested parameters. The serum vitamin C and erythrocyte reduced glutathione levels reached values that were significantly higher than the untreated diabetic model values and insignificantly different from the control group values. These results are in agreement with previous studies which reported the antioxidant effects of ALA. Alpha-lipoic acid was reported to increase significantly the total serum antioxidant capacity in patients with autonomic diabetic neuropathy (Tankova *et al.*, 2004). It was also effective in improving diabetes-induced decrease in blood glutathione redox state in alloxan diabetic rabbits (Winiarska *et al.*, 2008).

On histological examination of the ALA treated diabetic animals (group IV), it was found that the retinas are free from the histopathological changes observed in those of untreated diabetic rats (group III). This finding confirms the protective effect of ALA on the retina and is in agreement with Johnsen-Soriano *et al.* (2008) who studied the effect of ALA on the retina of diabetic mice. They found that early administration of ALA to diabetic mice prevented significantly the decrease of glutathione content and glutathione peroxidase activity in addition to normalization of malonedialdehyde concentration in the retina. They also reported that ALA could restore the electroretinogram b-wave amplitude changes of diabetic animals to normal values.

Kowluru *et al.* (2006) explained that oxidative stress is linked to accelerated apoptosis of retinal capillary cells which is an early event in the pathogenesis of diabetic retinopathy. Mitochondria are the major endogenous source of superoxide which is the causal link between hyperglycemia and biochemical pathways involved in development of vascular complications of diabetes mellitus. Therefore, protection from oxidative damage may be a broadly applicable treatment strategy in diabetic retinopathy. This was also suggested by Komeima *et al.* (2007) who reported that a mixture of antioxidants including ALA could reduce the retinal cellular death induced by oxidative stress. It was also supported by Lin *et al.* (2006) who reported that hyperglycemia induce the mitochondria to overproduce reactive oxygen species with marked deterioration of the antioxidant parameters in retinal specimens. The same authors found occluded acellular capillaries together with decreased number of membrane pericytes that support the walls of retinal blood capillaries. They proved that ALA treatment prevented the microvascular damage through normalizing the overproduction of mitochondrial reactive oxygen species and preserving the pericyte coverage of retinal capillaries (Lin *et al.*, 2006).

To explain the role of ALA in oxidative stress conditions affecting the retina, Stoyanovsky *et al.* (1995) had documented that vitamin E (α tocopherol) is considered the major lipid soluble antioxidant in retinal cell membranes. It scavenges peroxyl radicals forming peroxy-α tocopherol radical. Vitamin C in retinal cells is able to reduce the oxidized vitamin E radical to its reduced state and protect it from oxidation induced by ultraviolet radiation. Alpha-lipoic acid was found to be able to enhance the protective effect of vitamin C by regenerating it from dehydroascorbate. This effect of ALA explains the increased vitamin C and glutathione levels observed in the present study and can also explain the beneficial role of ALA as an antioxidant aiming to protect the retina from oxidative stress caused by different stressful conditions (Akpinar *et al.*, 2007; Berkowitz *et al.*, 2007; Komeima *et al.*, 2007 and Derin *et al.*, 2009).

In conclusion, ALA was effective in restoring diabetes-induced deterioration of blood antioxidants; namely vitamin C and glutathione. Retinal histopathological changes observed in diabetic animals were ameliorated by administration of alpha-lipoic acid which suggests its protective role against diabetic retinopathy.

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