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ORIGINAL PAPER

Effects of alpha-lipoic acid on retinal ganglion cells, retinal thicknesses, and VEGF production in an experimental model of diabetes

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Abstract

Purpose The purpose of the present study was to investigate the effect of alpha-lipoic acid (ALA) on the thicknesses of various retinal layers and on the numbers of retinal ganglion cells and vascular endothelial growth factor levels in experimental diabetic mouse retinas.

Methods Twenty-one male BALB/C mice were made diabetic by the intraperitoneal administration of streptozotocin (200 mg/kg). One week after the induction of diabetes, the mice were divided randomly into three groups: control group (non-diabetic mice treated with alpha-lipoic acid, $n = 7$), diabetic group (diabetic mice without treatment, $n = 7$), and alpha-lipoic acid treatment group (diabetic mice with alpha-lipoic acid treatment, $n = 7$). At the end of the 8th week, the thicknesses of the inner nuclear layer (INL), outer nuclear layer (ONL), and full-length retina were measured; also retinal ganglion cells and VEGF expressions were counted on the histological sections of the mouse retinas and compared with each other.

Results The thicknesses of the full-length retina, ONL, and INL were significantly reduced in the diabetic group compared to the control and ALA treatment groups ($p = 0.001$), whereas the thicknesses of these layers did not show a significant difference between ALA treatment and control groups. The number of ganglion cells in the diabetic group was significantly lower than those in the control and ALA treatment groups ($p = 0.001$). The VEGF expression was significantly higher in the diabetic group and mostly observed in the ganglion cell and inner nuclear layers compared to the control and ALA treatment groups ($p = 0.001$). Therefore, the number of ganglion cells and VEGF levels did not show significant differences between the ALA treatment and control groups ($p = 0.7$).

Conclusions Our results show that alpha-lipoic acid treatment may have an impact on reducing VEGF levels, protecting ganglion cells, and preserving the thicknesses of the inner and outer layers in diabetic mouse retinas.

Keywords Diabetic retinopathy · Alpha-lipoic acid · Neurodegeneration · Antioxidants · Vascular endothelial growth factor

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Introduction

Diabetes mellitus (DM) is a good model of chronic oxidative damage and it is a particularly suitable disease for antioxidant supplementation. Oxidative stress caused by the formation of free radicals and reactive oxygen species (ROS) leads to retinal vasculature damage by stimulating the release of proinflammatory cytokines such as interleukin 1β (IL-1β) and tumor necrosis factor (TNF-α) and hence it plays an important role in the pathogenesis of DR [1]. Reactive oxygen species (ROS) products have been shown to stimulate protein kinase C (PKC) which is responsible for the upregulation of retinal vascular endothelial growth factor (VEGF) proteins [2]. Vascular endothelial growth factor, a key angiogenic growth factor, plays an important role in the angiogenic process and pathogenesis of diabetic retinopathy [3]. Since VEGF production is increased by hypoxia [4] and oxidative stress, its upregulation has been documented in the early course of diabetes in rat and mouse models [5, 6].

A significant correlation between increased blood sugar levels and depletion of antioxidants is demonstrated, which eventually leads to pathological and biochemical changes in DR [7]. One of the best characterized antioxidants is alpha-lipoic acid (ALA), a disulfide derivative of octanoic acid, which can alter the redox status of cells and interact with thiols and other antioxidants such as glutathione (GSH), vitamin C, and vitamin E, for reducing or slowing down ROS damage. It reduces oxidative stress by scavenging a number of free radicals in both membrane and aqueous domains, by chelating transition metals in biological systems and by preventing membrane lipid peroxidation [8]. ALA is contained in all cells rich in mitochondria where the energy demand is high, thus leading to the production of oxidant activity. A mitochondrial dysfunction, observed during DR, inevitably results in the apoptosis of cells with a high energy demand, such as the retinal ganglion cells and photoreceptors.

Administration of ALA to diabetic rats has been shown to inhibit the parameters of oxidative stress in various organs of diabetic rats, including kidney, nerve, and retina [9, 10].

In the present study, we aimed to evaluate the impact of ALA on the levels of VEGF and retinal ganglion cells (RGCs) and on the thicknesses of the inner and outer neuronal layers in an experimental diabetic mice model.

Materials and methods

Animals and induction of diabetes

All experiments were performed in accordance with the Guidelines for Animal Experiments in Karadeniz and Technical University adopted by the Committee on the Care and Use of Laboratory animals of the University and tenets of the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. Twenty-one male BALB/C mice aged 8 weeks were maintained in an animal room with controlled temperature (21–25 °C) and humidity (45–65%) under a 12-h light/dark cycle (light on 06:00 to 18:00). Mice had free access to standard food and water. After 7 days of acclimation, the mice were made diabetic by the intraperitoneal administration of streptozotocin (STZ) (200 mg/kg) (Streptozotocin, Sigma, Deisenhofen, Germany) dissolved in 0.1 mol/l citrate buffer (pH 4.5). One week after STZ injection, the blood samples were collected from the tail vein and all animals with plasma glucose level > 300 mg/dl were considered as diabetic and included in the study. Plasma glucose levels were determined with a commercially available Medisense Optium Xceed kit (Abbott, USA).

We evaluated the initial and final body weights and blood glucose levels among groups. The final body weights and blood concentrations are shown in Table 1.

Grouping and experimental procedure

One week after the induction of diabetes, the mice were divided randomly into 3 groups: control group (non-diabetic mice treated with ALA, $n = 7$), diabetic group (diabetic mice without treatment, $n = 7$), and alpha-lipoic acid treatment group (diabetic mice with ALA treatment, $n = 7$). Three weeks after the induction of diabetes, ALA (Sigma-Aldrich, St. Louis, MO, USA) (100 mg/kg) was dissolved in ethanol and applied intraperitoneally weekly for 5 weeks. At the end of the 8th week, all animals were euthanatized by an overdose of carbon dioxide asphyxiation followed by cervical dislocation. After cervical dislocation,

Table 1 Final body weight and blood glucose concentrations in control and diabetic mice and diabetic mice treated with ALA (n:7)

	Control	Diabetic	Diabetic + ALA
Final body weight (g)	31.4 ± 2.9	25.4 ± 2.5*	26.1 ± 1.4*
Blood glucose concentration (mg/dl)	118 ± 26.7	327 ± 20.3*	336 ± 22.8*

ALA alpha-lipoic acid

* Significantly different from controls ($p < 0.05$)

fourteen eyeballs were collected from each experimental group and fixed in 10% neutral buffered formalin solution for 24 h.

Histological analysis

The samples were embedded in paraffin blocks. Then 4-µm-thick sections from each block were cut tangentially through the pupil and optic nerve, passing through the temporal ora serrata, the optic nerve, and the nasal ora serrata, which included a full length of retina. Cryostat sections were stained with hematoxylin and eosin (H&E) for ganglion cell counting and histopathology. Ganglion cells were counted in central and peripheral retina (both peripheral sides, nasal and temporal), and the average ganglion cell number was counted per 100 mm length of the retina from each group. The thicknesses of the full-length retina and the outer and inner nuclear layers were measured in the central and peripheral retina (both peripheral sides, nasal and temporal). The respective measurements were then averaged to obtain the values. All measurements were performed under a light microscope (Olympus-BX53F, Tokyo/Japan) with an attached digital camera (Digital Camera; DP72 Olympus, Tokyo/Japan, Software; DP2-BSW Ver.2.1, Olympus Corporation, Tokyo/Japan). Immunohistochemical staining was performed by the automated immunohistochemical device (Ventana BenchMark XT, Inc. Tucson, Arizona/USA) using an anti-VEGF antibody (ABCAM Inc. USA, ab46154). VEGF expression was counted in central and peripheral retina (both peripheral sides, nasal and temporal), and the average VEGF number was counted per 100 mm length of the retina from each group. We utilized ImageJ (imagej.nih.gov/ij) program to count both VEGF and RGCs. All of the histopathological and immunohistochemical examinations were conducted by a single blind pathologist.

Statistical analysis

Statistical analyses were undertaken using SPSS (version 13.0; SPSS Inc., Chicago, IL). The results were expressed as median (min–max) and mean ± SD. Kruskal–Wallis variance analysis was used to compare the groups. Then post hoc Mann–Whitney u test was used to determine which of the two groups leads to these significant differences. A post hoc p value less than 0.016 was considered statistically significant (Bonferroni corrections = 0.05/number of comparisons).

Results

The thickness of the full-length retina was 79 µm (60–95), 105 µm (99–135), and 117 µm (106–171) in diabetic, ALA treatment, and control mouse retinas, respectively. The thickness of ONL was 19 µm (14–21), 27 µm (22–35), and 31 µm (23–45) in the diabetic, ALA treatment, and control mouse retinas, respectively. The thickness of INL was 10 µm (7–12), 14 µm (7–12), and 19 µm (17–25) in the diabetic, ALA treatment, and control mouse retinas, respectively. Diabetic mouse retinas showed a significant decrease in the thickness of the full-length retina, ONL, and INL compared to the control and ALA-treated mouse retinas ($p = 0.001$). The thicknesses of each layer among groups are shown in Table 2 per mouse. However, the thicknesses of these layers did not show a significant difference between the ALA treatment and control mouse retinas ($p = 0.07$) (Table 3). The numbers of ganglion cells were counted as 94 (80–100), 180 (160–192), and 185 (120–204) in the diabetic, ALA treatment, and control mouse retinas, respectively. H&E stained retinal sections showed a significant loss of ganglion cells in diabetic mouse retinas compared to the control and

Table 2 The thickness of each layer among groups were shown per mouse

	Total retina (μm)	ONL (μm)	INL (μm)
Control (n:14)	123	35	25
	106	32	19
	107	28	18
	115	28	19
	171	45	24
	117	23	17
	118	31	18
	121	33	23
	109	34	21
	119	31	20
	119	26	17
	116	26	22
	106	28	19
	117	31	19
Diabetic (n:14)	75	18	9
	87	19	10
	95	21	12
	79	21	11
	63	19	7
	79	18	10
	60	14	7
	78	20	11
	84	19	8
	93	20	10
	81	19	8
	66	15	9
	74	19	10
	79	19	10
Diabetic + ALA (n:14)	103	28	17
	105	25	12
	135	22	12
	110	28	14
	104	35	22
	99	25	15
	115	27	12
	107	27	15
	108	27	14
	105	24	13
	108	26	13
	101	28	16
	102	24	13
	105	27	14

ONL outer nuclear layer, *INL* inner nuclear layer, *ALA* alpha-lipoic acid

ALA-treated mouse retinas ($p = 0.001$). The number of ganglion cells did not show a significant difference between the control and ALA-treated mouse retinas ($p = 0.7$) (Fig. 1). The numbers of VEGF expressions were 40 (33–48), 22 (16–29), and 20 (18–26) in the diabetic, ALA treatment, and control mouse retinas

Table 3 The thicknesses of full length retina, inner and outer segment layers among groups

Thickness (min–max)	Control (n:14)	Diabetic (n:14)	Diabetic+ALA (n:14)
Total retina	117 μm (106–171)*	79 μm (60–95)	105 μm (99–135)*
ONL	31 μm (23–45)*	19 μm (14–21)	27 μm (22–35)*
INL	19 μm (17–25)*	10 μm (7–12)	14 μm (7–12)*

n number, *ONL* outer nuclear layer, *INL* inner nuclear layer, *ALA* alpha-lipoic acid

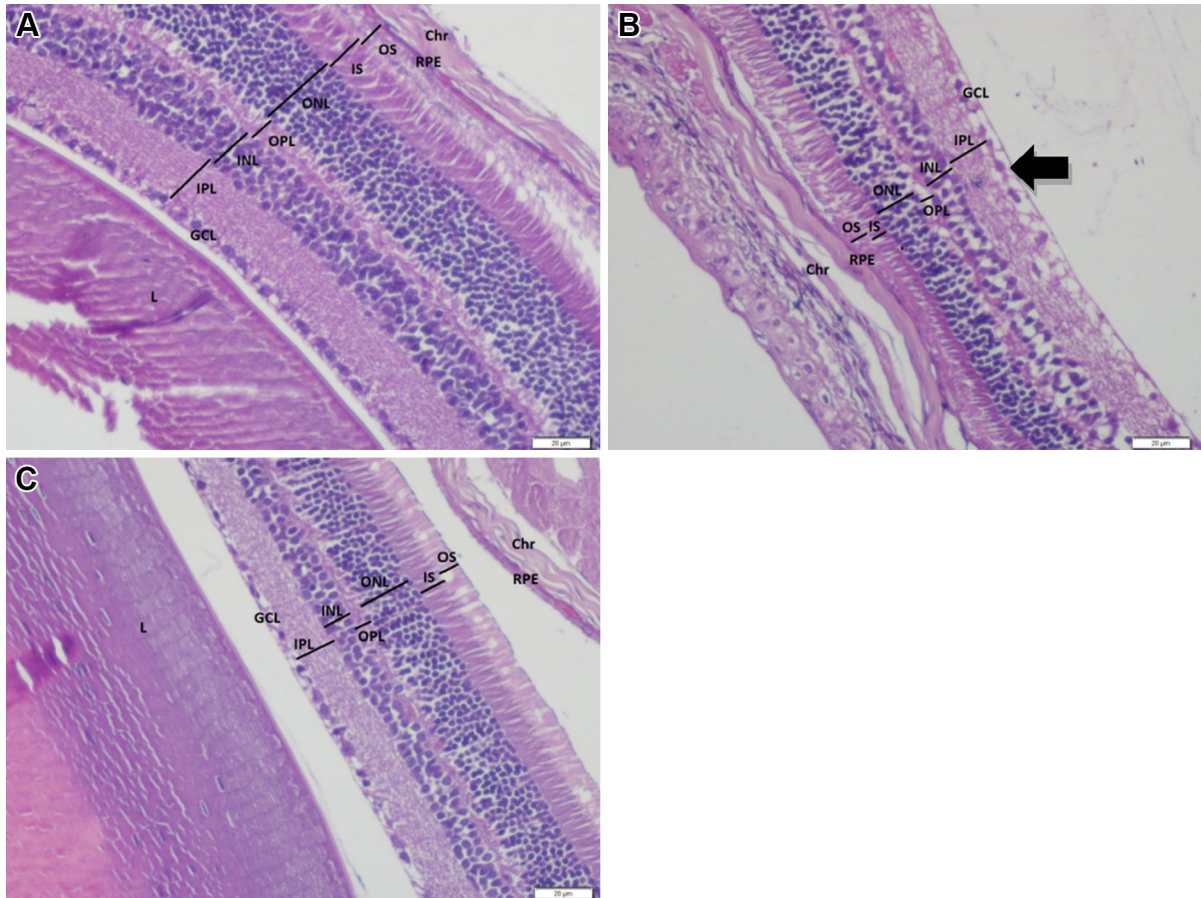* Significantly different from the diabetic group mice ($p = 0.001$)

Fig. 1 An illustration of the retinal sections of mouse retinas using H&E staining among the groups: **a** control mouse retina, **b** diabetic mouse retina, and **c** alpha-lipoic acid-treated mouse retina. Magnification ×400. *GCL* ganglion cell layer, *IPL* inner plexiform layer, *INL* inner nuclear layer, *OPL* outer plexiform layer, *ONL* outer nuclear layer, *IS* inner segment, *OS* outer segment, *RPE* retinal pigment epithelium, *Chr* choroid, *L* lens, *arrow* RGC

respectively. VEGF expression was highest in the diabetic group compared to the other groups ($p = 0.001$) and the expression was mostly observed in the ganglion cell and inner nuclear layers. VEGF expression did not show a significant difference between the ALA-treated and control mouse retinas

($p = 0.09$) (Table 4). The VEGF staining in groups is illustrated in Fig. 2.

Mice with STZ-induced diabetes and ALA treatment showed a significant decrease in body weight and a significant increase in blood glucose compared with non-diabetic controls ($p < 0.05$) (Table 1). No

Table 4 RGCs and VEGF counts among groups

	Control (n:14)	Diabetic group (n:14)	Diabetic+ALA (n:14)
RGC (Min–max)	185 (120–204)*	94 (80–100)	180 (160–192)*
VEGF (Min–max)	20 (18–26) *	40 (33–48)	22 (16–29)

n number, *RGC* Retinal ganglion cell, *VEGF* Vascular endothelial growth factor

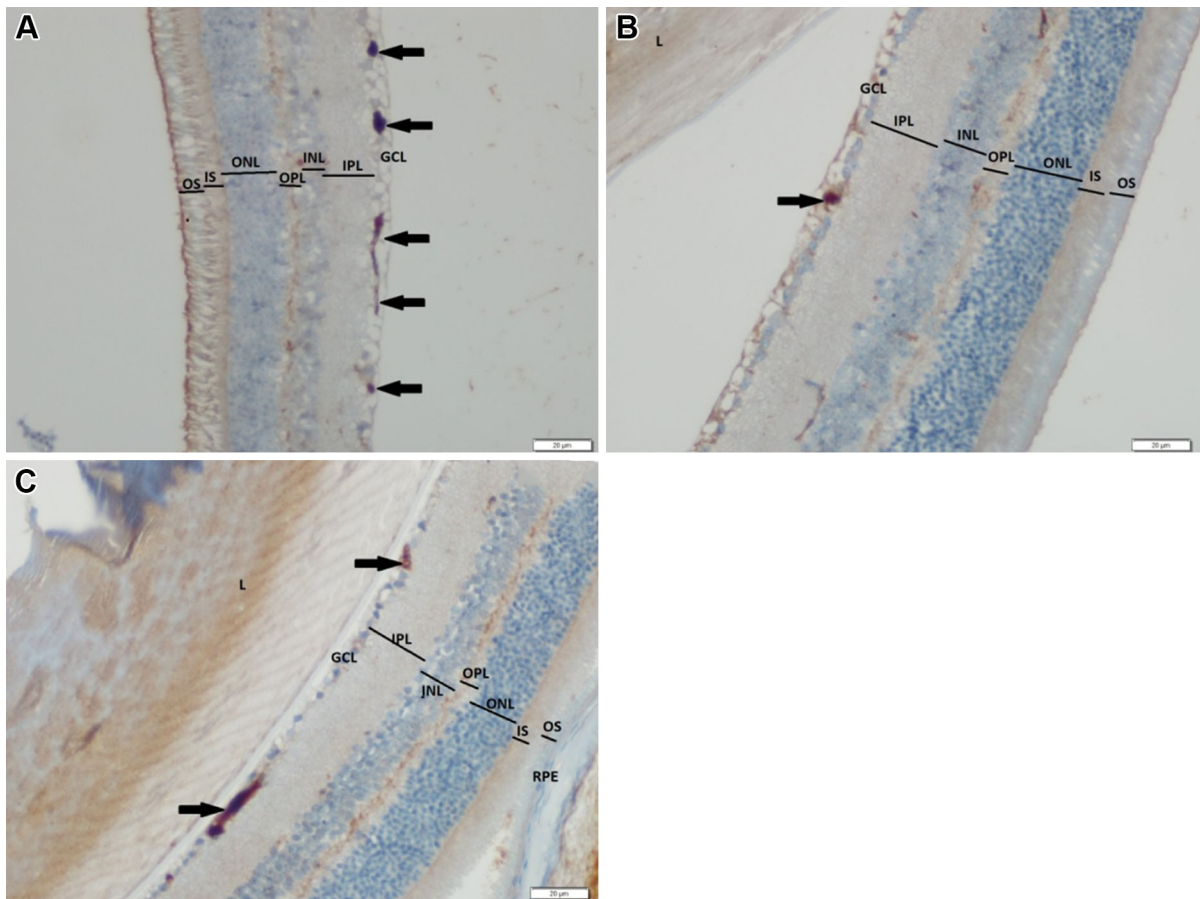* Significantly different from diabetic group ($p = 0.001$)

Fig. 2 An illustration of immunohistochemical analysis of the retinas using anti-VEGF antibody among diabetic groups: **a** diabetic mouse retina, **b** control mouse retina, and **c** alpha-lipoic acid-treated mouse retina. Magnification ×400. *GCL* ganglion cell layer, *IPL* inner plexiform layer, *INL* inner nuclear layer, *OPL* outer plexiform layer, *ONL* outer nuclear layer, *IS* inner segment, *OS* outer segment, *arrow* VEGF staining in the GCL

statistically significant difference was found between the final body weights and blood glucose concentrations in diabetic mice and diabetic mice treated with ALA. Treatment with ALA did not significantly change these metabolic variables in the diabetic mice ($p > 0.05$).

Discussion

In the present study, we immunohistochemically analyzed the retinas of diabetic, control, and ALA-treated mice by measuring the thicknesses of various retinal layers and by counting the RGCs in ganglion

cell layer. Besides, we counted the VEGF expressions in the groups and compared with each other. Retinal ganglion cell counts were significantly reduced in the retinas of diabetic group compared to those of the control and ALA treatment groups. We also found statistically higher levels of VEGF in diabetic mouse retinas compared to the ALA-treated and control mouse retinas.

Chronic hyperglycemia causes different events such as the activation of aldose reductase metabolic pathway, activation of the diacylglycerol–protein kinase C pathway, and non-enzymatic glycation of proteins with the formation of advanced glycation/lipoxidation end products (AGE/ALE) with the release of superoxide radicals [11]. These reactive radicals, located near retinal capillaries, are responsible for the apoptosis of the retinal capillary cells (pericytes and endothelial cells), leading to the loss of pericytes and the formation of microaneurysm by mitochondrial DNA modifications [12]. Asnaghi et al. examined TUNEL-positive cells in diabetic rat retinas and found a four-fold increase in the number of apoptotic neurons [13]. These characteristic changes occur before the appearance of histopathological lesions of diabetic retinopathy despite good glycemic control [14].

Because there is a strong understanding that oxidative stress may be the trigger of all other pathologies implicated in the pathogenesis of diabetic retinopathy, the use of appropriate antioxidants may have therapeutic potential effects on the metabolic and functional abnormalities found in diabetic retinopathy [15].

Alpha-lipoic acid and its reduced form, dihydrolipoic acid, reduce oxidative stress by scavenging a number of free radicals such as superoxide, hydroxyl radicals, and singlet oxygen, by preventing membrane lipid peroxidation and protein damage through the redox regeneration of other antioxidants such as vitamins C and E. Additionally, Kawabata and Suzuki found the inhibitory effects of ALA on glycation process, which is one of the major pathways involved in the pathogenesis of diabetic retinopathy by non-covalent binding to albumin [16, 17]. ALA has also been shown to exhibit a protective effect against apoptosis. The anti-apoptotic effect of ALA has been shown in a study in which ALA decreased the number of apoptotic capillary cells and acellular capillaries in the retina of diabetic rats [18].

Retinal ganglion cells serve as an ideal model to investigate cell death and improve the novel treatments for retinal degeneration, particularly in diabetes [19, 20]. Rapid decreases in RGC counts in diabetic animals have been reported in various studies. For example, Zheng et al. observed a 20% loss of cells in the ganglion cell layer 4 months after the induction of diabetes [21]. In another study, STZ-induced diabetes resulted in an extensive loss of RGCs, with 20–25% fewer cells in the GCL compared with age-matched control mice after 14 weeks of diabetes [22]. Although we evaluated RGC decrease earlier (8 weeks after the induction of diabetes), our findings are consistent with the previous observations with reduced numbers of RGCs in diabetic retinas. This result shows that the loss of RGCs may occur by 8 weeks after the onset of diabetes. Moreover, in the present study the number of RGCs was found to be statistically higher in ALA-treated mouse retinas compared to the diabetic mouse retinas, supporting the idea that ALA treatment might have prevented cell death.

The thicknesses of the layers were also evaluated in diabetic mice. Barber et al. reported significant differences in the thickness of the INL as a consequence of diabetes [23]. Park et al. reported the thinning of INL and the marked thinning of ONL by 24 weeks after the onset of diabetes [24]. In the present study, thinning in both the inner and outer nuclear layers was significantly higher in diabetic mouse retinas compared to the ALA-treated mouse retinas which might turn out the protective effect of ALA against neuronal cell loss.

VEGF, an angiogenesis inducer, plays a pivotal role in diabetic retinopathy and is implicated as the mediator and an initiator of non-proliferative and proliferative diabetic retinopathies. Local hypoxia and the increased levels of inflammatory cytokines, AGEs, and ROS that occur in diabetes can induce VEGF gene expression [25, 26]. As AGEs were shown to play a role in angiogenesis by the induction of VEGF production in diabetic retinopathy, non-enzymatic glycation inhibitors were found to suppress retinal VEGF immunoreactivity in the experimental models [27, 28]. In the present study, we evaluated the effect of ALA on VEGF expression and compared the results between groups. As shown in Fig. 2a, diabetic mouse retinas showed statistically higher levels of VEGF expression compared to the control and ALA-treated mouse retinas, which is mostly localized in the GCL

and inner retinal layers. Median VEGF levels were similar between the ALA-treated and control mouse retinas ($p = 0.09$).

The mechanism by which ALA may inhibit the production of VEGF was elicited in some studies. In an experimental diabetic rat model, after rats had been made diabetic, they were treated with ALA and VEGF levels were measured and compared with the untreated diabetic mouse retinas. Retinal VEGF upregulation occured as early as 6 weeks after diabetes induction and VEGF levels were found to be lower in ALA-treated mouse retinas compared to the diabetic mouse retinas [29]. Lee et al. evaluated the effect of ALA on the formation of ROS and angiogenic factors. In diabetic rats, the administration of ALA reduced the production of ROS. Treatment with ALA suppressed the expression of VEGF, angiopoietin 2, and erythropoietin induced by high glycemic levels [30]. In an another study, ALA was shown to inhibit diabetes-induced activation of two oxidants, such as nitrotyrosine and nuclear transcription factor (NF-kB), which have been postulated to play roles in the pathogenesis of VEGF secretion in diabetic rat retinas [18]. In our study, decreased levels of VEGF expression found in the ALA-treated mouse retinas might be the result of the antioxidative and inhibitory effects of ALA on the glycation process.

In the present study, since we did not observe any histopathologic changes demonstrating diabetic retinopathy, we assumed our retinas as preretinopathic diabetic retinas. Recently, the effects of ALA on preretinopathic diabetic patients were also evaluated by some authors. In a study, preretinopathic diabetic patients were randomized into two groups: one of which received oral AO treatment with ALA and the other group received a placebo treatment. The concentration of free radicals and AOs in plasma was measured by analytical techniques, and ERG was performed to measure oscillatory potentials (OPs) in patients. Plasma AO levels and ERG OP oscillatory potential values were found to be increased in the group treated with ALA compared to the control group. Results of this study suggested that ALA treatment might have a protective effect on retinal cells, in preretinopathic diabetic subjects, as detected by ERG analysis [31].

Numerous early retinal function tests in diabetic patients and molecular studies in the retinas of experimental diabetic animals suggest that neurons are vulnerable to damage shortly after the onset of diabetes. Alpha-lipoic acid has been used with the aim of preventing the initial damage in experimental diabetic models. In a study aiming to evaluate the early effects of ALA treatment, glutathione (GSH) content, malondialdehyde (MDA) concentration, and glutathione peroxidase (GPx) activity, which are the indicators of oxidative stress, were measured 3 weeks after diabetes induction in mouse retinas. Retinal function of diabetic mice was also studied by electroretinography (ERG), which was used to detect early signs of retinal damage. Early administration of lipoic acid to diabetic mice prevented the statistically significant decrease of GSH content and GPx activity and normalized MDA concentration. The b-wave amplitude was reduced, indicating the onset of early damage in retinal function caused by diabetes. The administration of ALA restored b-wave amplitude by 70% in a statistically significant manner [32].

There are various routes of administration of ALA such as oral and intraperitoneal (i.p.) use in various animal models. Although oral ALA is used, the most common approach for the administration of ALA is intraperitoneal administration in rats and mouse models. Although up to 90% of an orally administered dose is absorbed from the gut, gastrointestinal absorption of ALA may be variable [33]. That is why in the present study we preferred i.p. use of ALA instead of oral intake. In the diabetic neuropathy clinical trials, the oral dose of ALA varied among trials in the range of 100–1800 mg/day and IV dose varied from 600 to 1200 mg/day. In this study, we used a 100 mg/kg dose in mice, which can be translated to approximately 10 mg/kg in humans. This dose is considered safe in human clinical trials [34].

Our result shows that alpha-lipoic acid treatment reduces VEGF levels, protects ganglion cells, and preserves the inner and outer layer thicknesses. To our knowledge, our study is the first to analyze these effects together on mouse retinas. We think that ALA may have a potential therapeutic benefit in both neuroprotection and inhibition of early neovascularization in preretinopathic retinas. Since oxidative stress plays a major role in the pathogenesis of DR, we think that the observed beneficial effects of ALA may make it a candidate as an early adjunct therapeutic supplement to limit the initial damage and slow down or even prevent the onset of diabetic retinopathy. However, further studies are recommended to elucidate its role in diabetes mellitus.

Compliance with ethical standards

Conflict of interest The authors have no conflict of interest.

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