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Alpha-lipoic acid and coenzyme Q10 combination ameliorates experimental diabetic neuropathy by modulating oxidative stress and apoptosis

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ABSTRACT

Aims: Diabetic neuropathy (DN) is the most common complication of diabetes. Neuroprotective effects of alpha lipoic acid (ALA) and coenzyme Q10 (CoQ10) has been previously shown in DN, but underlying mechanisms involved not been exactly found. The present study explored the neuroprotective effects of ALA and Q10 combination in experimental DN by ameliorating oxidative stress and apoptosis.

Main methods: We investigated the effects of CoQ10 (10 mg/kg, orally, five weeks) and/or ALA (100 mg/kg, orally, five weeks) in STZ (45 mg/kg, i.p.)- induced DN in rats. After treatments motor function, oxidative stress biomarkers, ATP levels, expression of caspase 3 and UCP2 proteins were assessed by open-field, biochemical and ELISA methods and Western blot analysis. Dorsal root ganglion (DRG) neurons were histologically examined using H&E staining method.

Key findings: ALA and/or CoQ10 treatment significantly ($p < 0.05$) attenuated DN – induced motor function deficiency by modulating distance moved and velocity. ALA and/or CoQ10 treatment dramatically suppressed DN – induced oxidative stress which was associated with decrease in LPO and ROS and increase in GSH and TAC in DRG neurons. ALA and/or CoQ10 was proved to prevent apoptosis and degeneration of DRG neurons, which appears to be mediated by regulating the expression of caspase 3 and UCP2 proteins, inducing ATP and improving DN–induced changes in DRG neurons. We found maximum effectiveness with ALA and CoQ10 combination on mentioned factors.

Significance: These results provide a possible basis of the underlying mechanism for application of ALA and CoQ10 combination in treatment of DN.

1. Introduction

Diabetic neuropathy (DN) is a microvascular complication of diabetes which can leads to an extensive damage to all components of peripheral nervous system such as dorsal root ganglia (DRG) neurons; the Schwann cells [1]. DN is the most common disorder of diabetes with high morbidity, premature mortality, diminished quality of life and it occurs in approximately 50% of diabetic patients [2]. In fact, against worldwide prevalence estimates of diabetes of 592 million by the year 2035, DN may affect 296 million persons around the world [3]. Given the growing documents for increased oxidative stress, mitochondrial dysfunction, and apoptosis pathways activated in DN and the lack of

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effective therapy to prevent or treat of DN [4,5], it is important to find tools by which one can reduce effects related to these pathways. Hence therapeutic paradigms can shift to target these pathways through the use of agents such as alpha lipoic acid (ALA) and coenzyme Q10 (CoQ10).

DRG neurons identified as the target tissue in diabetic peripheral neuropathy and hyperglycemia in diabetes promote generation of reactive oxygen species (ROS), mitochondria dysfunction and subsequent apoptosis in DRG neurons [6,7]. DRG neurons are much susceptible to oxidative injury for having large mitochondria with high oxidative metabolism [8]. Moreover DRG neurons entrain higher levels of local blood flow which indicating greater metabolic and oxygen demand. It was also seen the effects of lipid peroxidation are greater in DRG neurons because impaired neurotrophic support and a more leaky blood-ganglia barrier [9]. Therefore DRG neurons can be the crucial site for continuing investigations on DN and also for examining the prevention or treatment of neuropathic disease in diabetes.

ALA is a natural compound that acts as a cofactor for a number of mitochondrial enzymes [10]. It is also as a co-enzyme in the Krebs cycle. ALA is unique among antioxidant for has both hydrophilic and hydrophobic properties. Unlike other neutral antioxidants, both the oxidized and reduced forms of ALA are potent antioxidants whose functions include: 1) scavenging of ROS, (2) regeneration of endogenous and exogenous antioxidants such as glutathione (GSH), and vitamins C and E, 3) renovation of oxidized proteins, 4) chelation of metal ions, 5) inhibition of nuclear factor kapp B, 6) regulation of gene transcription [11]. Many basic and clinical studies have shown the advantageous effect of ALA on models of disease marked by an increase in oxidative stress such as neurodegenerative diseases, diabetes and its complications [11]. It is reported that ALA can prevent and reduce micro- and macrovascular complications of diabetes in animal models [12] and also ALA administration significantly ameliorates polyneuropathies in patients with diabetes [11,13,14] and improves the symptoms of DN [10,15].

CoQ10 or ubiquinone is an endogenous synthesized lipid and a vitamin-like substance present in almost all living cells, primarily in the mitochondria [16]. CoQ10 is an electron transport in the mitochondrial respiratory chain and contributes in aerobic cellular respiration, increases energy generation in the form ATP, and enhances mitochondrial antioxidant activity [16,17]. In addition to its unique role in the mitochondria, CoQ10 serves as a potent lipophilic antioxidant, powerful scavenging free radicals and preventing lipid peroxidation [17,18]. CoQ10 capable of regenerating and recycling other antioxidants like the vitamin E and ascorbate [19] and it also stimulates cell growth, and prevents cell death [19]. Considering the mentioned properties of CoQ10, strategies to suppress oxidative stress and enhance mitochondrial function by this substance may involve to the development of novel therapies for DN. In fact the beneficial effects of CoQ10 on DN in basic and clinical studies have been reported [20–22].

Although many documents showed ALA and CoQ10 have the ability to improve DN, but molecular mechanisms involved in the effectiveness of these agents have not been exactly found. The present study was designed to evaluate the neuroprotective effects of CoQ10 and/or ALA on experimental model of DN and further explored the potential mechanisms involved in the usefulness of these compounds in the aspect of oxidative stress, apoptosis and DRG neurons degeneration.

2. Material and method

2.1. Experimental animals

Seventy male Wistar rats weighing 200–250 g and aged 6–8 weeks were purchased from the animal house of Iran University of Medical Sciences. The adult rats were maintained under 12 h light/dark cycle, 45–55% humidity, with access to standard diet and water. The experiments were approved by the Ethical Committee of Iran University of Medical Sciences.

2.2. Study design

Diabetes was induced by a single intraperitoneal injection of streptozotocin (45 mg/kg in citrate buffer). The control rats received citrate buffer in the equal volume. 72 h after STZ administration, fasting blood glucose levels were measured using the glucometer. Rats with blood glucose over 200 mg/dL were considered as diabetic rats.

The rats were randomly divided into ten groups, each group comprised 7 animals (n = 7): (1) Co (normal control group that received distilled water orally); (2) Olive oil (the group that received olive oil "CoQ10 solvent" orally for five weeks); (3) CoQ10 (the group that received 10 mg/kg CoQ10, five weeks, orally); (4) ALA (the group that received 100 mg/kg alpha lipoic acid, five weeks, orally); (5) CoQ10 + ALA (the group that received 10 mg/kg CoQ10 + 100 mg/kg alpha lipoic acid, five weeks, orally); (6) DN (diabetic neuropathy group, diabetic rats that received distilled water orally for five weeks); (7) olive oil + DN (diabetic rats received olive oil orally for five weeks); (8) CoQ10 + DN (diabetic rats that received 10 mg/kg CoQ10, five weeks, orally); (9) ALA + DN (diabetic rats that received 100 mg/kg alpha lipoic acid, five weeks, orally); (10) CoQ10 + ALA + DN (diabetic rats that received 10 mg/kg CoQ10 + 100 mg/kg alpha lipoic acid, five weeks, orally). It should be noted that oral treatments have been performed by gavage. After the end of treatment period, rats were anesthetized with ketamine and xylazine injection. Then DRGs from the second cervical (C2) to second lumbar (L2) spine region on both sides were removed and immediately transferred to liquid nitrogen for using next experiments. A number of other DRG neurons were also fixed by 10% formalin for the morphological study.

2.3. Processing of DRG neurons for further analysis

DRG neurons were placed in lysis buffer (10 mM Tris-base, 150 mM NaCl, 1 mM EDTA, 1% NP40 in double distill H_2O) containing suitable enzyme inhibitors on ice. Then the tissues were homogenized by a mechanical grinding pestle for 30 s on ice and then were centrifuged for 15 min at 16,000 $\times g$ at 4 °C and finally the supernatant was used as sample for further analysis [23].

2.4. Generation of reactive oxygen species (ROS)

ROS generation in all groups was assayed using a fluorescent dye 2′, 7-dichlorofluoresceindiacetate (DCFH-DA). Briefly, samples were incubated with DCFH-DA for 30 min at 37 °C. The fluorescence intensity was measured at 485 and 528 nm as excitation and emission wavelengths respectively, using a Microplate Reader (BioTek Instruments, USA).

2.5. Measurement of lipid peroxidation (LPO)

Lipid peroxidation was determined by the thiobarbituric acid reactive (TBARS) method. The samples were mixed with thiobarbituric acid (TBA) and were heated for 30 min in the boiling water. The assay was based on the conjugation ability of MDA with TBA to form a red product that its absorbance was taken at 532 nm by microplate reader (BioTek Instruments, USA).

2.6. Measurement of total antioxidant capacity (TAC)

TAC were evaluated by FRAP method as described previously [24]. FRAP is based on the reduction of Fe^{3+} to Fe^{2+} by the samples. The complex between Fe^{2+} and 2, 4, 6-tris (2-pyridyl)-1, 3, 5-triazine (TPTZ) gives a blue color with absorbance of 593 nm that was measured by spectrophotometer.

2.7. Assessment of glutathione (GSH)

GSH concentration (μmol/L) was determined by a colorimetric GSH assay kit (Zell Bio GmbH Ulm, Germany) in accordance with the protocols.

2.8. Measurement of adenosine diphosphate (ADP) and ATP

In order to measure the level of ADP and ATP, DRG neurons was homogenized in 1 mL of ice-cold 6% TCA and was centrifuged at 12,000 *g* for 10 min at 4 °C. The supernatant was neutralized with 4 M KOH to a pH of 6.5 and was used to determine the concentrations of ATP and ADP (μg/mL per mg of tissue) using reverse-phase HPLC. Energy changes were expressed as an ADP/ATP ratio [25].

2.9. Western blot analysis

The expression of UCP2 and caspase-3 in dissected DRGs (supernatant) was performed by Western blotting. Total protein was extracted by the Bradford assay. The proteins were separated by SDS-PAGE. Then proteins transferred to nitrocellulose membrane. After blocking with non-fat dry milk, membranes were probed by primary antibodies: rabbit polyclonal anti-caspase 3 1:1000, and rabbit monoclonal anti-UCP2 1:1000 (Cell Signaling Technology, Danvers, MA, USA) overnight at 4 °C. Then the membrane incubated with secondary antibody: anti-rabbit antibody conjugated with horse-radish peroxidase 1:2000 (Santa Cruz Biotechnology, USA) at room temperature for 1 h. β-Actin was used as internal control. The protein bands were visualized with Enhanced Chemiluminescence (ECL) (Amersham Pharmacia Biotech, Buckinghamshire, UK) and their densitometry was done using Image J software (Total lab software, Wales, UK).

2.10. Histological preparation and morphometry of DRG neurons

Dissected DRGs were fixed in the paraformaldehyde and embedded in paraffin. The DRGs in paraffin blocks were cut into 40 μm sections. These sections were stained with hematoxylin and eosin (H&E) and were used for microscopic analysis. Olympus microscope (LX71, Japan) was used to determine the number and diameter of A and B cells in all groups. Stereological studies were performed by image evaluation program (Optika, Italy) [25].

2.11. Measurement of motor function

Motor function was evaluated by open-field activity tests. The rats were put in an open-field box where distance moved (cm) and velocity (cm/sec.) were recorded by a camera on the top of the box for 15 min. The EthoVision tracking system (Noldus Information Technology, Wageningen, the Netherlands) was used to measure motor function by determining speed and distance of animal movement.

2.12. Statistical analysis

All results obtained from different treatments were analyzed using one-way ANOVA and Tukey's post hoc tests and presented as the mean ± SEM. A $p < 0.05$ was considered as statistically significant.

3. Results

3.1. The effect of CoQ10 and/or ALA on oxidative stress biomarkers

3.1.1. The effect of CoQ10 and/or ALA on ROS generation

Table 1 illustrates the effects of five weeks administration of CoQ10 and ALA on ROS generation. Generally, ROS level was significantly ($p < 0.001$) increased in DN group compared with the control group. Treatment of rats with CoQ10 and ALA significantly ($p < 0.05$ and $p < 0.001$, respectively) inhibited the DN-induced ROS production. Notably, coadministration of CoQ10 and ALA more effectively decreased the ROS generation that it was significance ($p < 0.05$) compared with the CoQ10 group.

3.1.2. The effect of CoQ10 and/or ALA on LPO and TAC levels

The level of MDA in DN group was significantly ($p < 0.001$) higher compared to control group. The MDA level of both CoQ10 and ALA groups was significantly ($p < 0.001$) lower compared to DN group. However, the MDA level in diabetic rats exposed to CoQ10 and ALA combination was lower compared to diabetic rats exposed to CoQ10 and ALA alone, but there was no significant difference (Table 1).

As shown in Table 1, the TAC level was significantly ($p < 0.001$) lower in DN group when compared to control group. TAC level was observed to be increased in diabetic rats received the CoQ10 or ALA, but combination treatment of CoQ10 and ALA significantly ($p < 0.01$) increased the TAC level compared to DN group and also this increase had significant difference ($p < 0.05$) compared with the CoQ10 group.

3.1.3. The effect of CoQ10 and/or ALA on glutathione level

In this study a significant ($p < 0.001$) reduction of GSH level was observed in DN group compared with control group. These alterations were improved in diabetic rats treated with CoQ10 and significantly ($p < 0.01$) with ALA. Coadministration of CoQ10 and ALA also significantly ($p < 0.01$) increased the glutathione level compared to DN group, however, there was no different between this group and CoQ10 and ALA groups statistically (Table 1).

3.2. The effect of CoQ10 and/or ALA on ADP/ATP ratio

The result showed ADP/ATP ratio in DRG neurons significantly ($p < 0.001$) increased in the DN group in comparison with the control group. CoQ10 and ALA significantly decreased ($p < 0.001$) this ratio through enhancing ATP level. Compared with each of CoQ10 and ALA groups alone, the coadministration of both CoQ10 and ALA significantly ($p < 0.001$) decreased the ADP/ATP ratio (Fig. 1).

3.3. The effect of CoQ10 and/or ALA on caspase 3 and UCP-2 proteins expression

Western blot analysis showed an increase of caspase 3 protein expression in DRG neurons of rats with DN compared to control. In addition, a decrease in expression of UCP-2 protein was observed in the same conditions. Treatment with CoQ10 and/or ALA demonstrated an improvement in referred proteins expression in DRG neurons compared to rats with DN (Fig. 2a). Therefore, caspase 3 protein expression was found to be significantly up regulated in DRG neurons of rats with DN compared to control ($p < 0.05$) after densitometric analysis. Treatment of diabetic rats with CoQ10 and/or ALA significantly ($p < 0.05$) inhibited expression of caspase 3 protein (Fig. 2b). In other hands, we found a significant down regulate ($p < 001$) in expression UCP-2 protein in DRG neurons of DN rats compared to control and treatment with CoQ10 and/or ALA did not a notable improvement in the expression of this protein (Fig. 2c).

3.4. Qualitative effects of CoQ10 and/or ALA on DRG neurons observations

We observed a prominent vacuolation in HE-stained DRG neurons in rats with DN. There found also an overrepresentation of small, more basophilic neurons (type B) and an underrepresentation of the largest diameter clear neurons (type A) in DN rats compared with control rats. After CoQ10 or ALA administration, we observed noticable improvement changes in DRG neurons compared with the DN group, but we found considerable further improvement in the treatment with combination of CoQ10 and ALA in comparison with CoQ10 or ALA alone (Fig. 3).

Table 1
CoQ10 and/or ALA modulate DN-induced oxidative stress biomarkers changes in DRG neurons. Diabetic rats were treated with CoQ10 (10 mg/kg) and/or ALA (100 mg/kg) for five weeks and then ROS, LPO, TAC, glutathione was measured in DRG neurons. Results are mean ± SEM, n = 7. Difference between control and other groups is significant at $p < 0.001$ (^{aaa}). Difference between DN and other groups is significant at $p < 0.001$ (^{bbb}), $p < 0.01$ (^{bb}) and $p < 0.05$ (^{b}). Difference between CoQ10 + DN and other groups is significant at $p < 0.05$ (^{c}). Co: control; CoQ10: coenzyme Q10; ALA: alpha lipoic acid; CoQ10 + ALA: coenzyme Q10 + alpha lipoic acid; DN: diabetic neuropathy; Olive oil + DN: olive oil + diabetic neuropathy; CoQ10 + DN: coenzyme Q10 + diabetic neuropathy; ALA + DN: alpha lipoic acid + diabetic neuropathy; CoQ10 + ALA + DN: coenzyme Q10 + alpha lipoic acid + diabetic neuropathy.

Groups	ROS (μ/mg protein)	LPO (μmol/mg protein)	TAC (mM)	Glutathione (mmol/mg protein)
Co	1.00 ± 0.01	109.0 ± 4.9	215.8 ± 10.6	2.15 ± 0.08
Olive oil	0.98 ± 0.07	112.1 ± 0.5	209.9 ± 8.6	2.16 ± 0.12
CoQ10	1.01 ± 0.01	106.1 ± 2.2	208.5 ± 4	2.32 ± 0.28
ALA	0.85 ± 0.07	102.9 ± 2.3	223.7 ± 11.2	2.43 ± 0.19
CoQ10 + ALA	0.73 ± 0.08	99.3 ± 2.4	233.7 ± 11.2	2.49 ± 0.20
DN	2.26 ± 0.12^{aaa}	157.7 ± 4.1^{aaa}	131.8 ± 2.9^{aaa}	0.87 ± 0.10^{aaa}
Olive oil + DN	2.29 ± 0.09	157.8 ± 4.1	133.2 ± 2	0.88 ± 0.03
CoQ10 + DN	1.72 ± 0.07^{b}	130.5 ± 2.1^{bbb}	142.3 ± 2.1	1.410.09
ALA + DN	1.47 ± 0.18^{bbb}	126.7 ± 6.7^{bbb}	164.2 ± 1.6	1.65 ± 0.09^{bb}
CoQ10 + ALA + DN	1.21 ± 0.1^{bbbc}	120.8 ± 1.8^{bbb}	178.9 ± 3.6^{bbc}	1.76 ± 0.09^{bb}

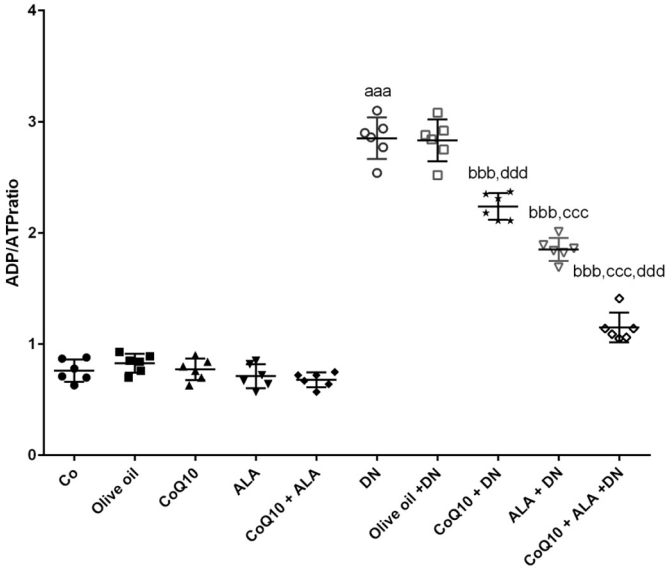

Fig. 1. CoQ10 and/or ALA reduce DN-induced ADP/ATP ratio in DRG neurons. Diabetic rats were treated with CoQ10 (10 mg/kg) and/or ALA (100 mg/kg) for five weeks and then ADP/ATP ratio was measured in DRG neurons by HPLC. Results are mean ± SEM, n = 7. Difference between control and other groups is significant at $p < 0.001$ (^{aaa}). Difference between DN and other groups is significant at $p < 0.001$ (^{bbb}). Difference between CoQ10 + DN and other groups is significant at $p < 0.001$ (^{ccc}). Difference between ALA + DN and other groups is significant at $p < 0.001$ (^{ddd}).Co: control; CoQ10: coenzyme Q10; ALA: alpha lipoic acid; CoQ10 + ALA: coenzyme Q10 + alpha lipoic acid; DN: diabetic neuropathy; Olive oil + DN: olive oil + diabetic neuropathy; CoQ10 + DN: coenzyme Q10 + diabetic neuropathy; ALA + DN: alpha lipoic acid + diabetic neuropathy; CoQ10 + ALA + DN: coenzyme Q10 + alpha lipoic acid + diabetic neuropathy.

3.5. The effect of CoQ10 and/or ALA on the number and diameter of A and B cells in DRG neurons

The number of B cells considerably exceeded that of A cells in DN as compared to controls ($p < 0.001$). There was a significant increase in the number of large cells in Q10 or ALA-treated groups ($p < 0.01$ and $p < 0.001$, respectively) especially with combination of ALA and Q10 ($p < 0.001$) in comparison with DN rats. ALA or Q10 also counteracted ($p < 0.001$) the DN-induced changes in B cell and we found more improvement with ALA + Q10 in comparison with ALA or Q10 alone. Diameter of large and small cells was significantly reduced in DN compared with control animals ($p < 0.001$). The application of combination of ALA and Q10 were able to prevent these DN-mediated changes ($p < 0.01$) in large cells. Administration of ALA and also combination of ALA and Q10 significantly ($p < 0.05$) increased DN-reduced small cells diameter (Table 2).

3.6. The effects of CoQ10 and/or ALA on motor function deficiency

A notable deficiency in motor function was found in DN rats. The distance moved (cm) (Fig. 4a) and velocity (cm/s) (Fig. 4b) were significantly ($p < 0.01$) lower in DN than in control rats. Although administration of CoQ10 or ALA improved motor function but it was no significant. Surprisingly, combination of CoQ10 and ALA reversed distance moved (cm) and velocity significantly ($p < 0.05$) in comparison with DN rats.

3.7. Correlation between results

Correlation analysis between ROS as an oxidative marker, Caspase-3 and UCP-2 protein expression as apoptosis markers, ADP/ATP ratio, and other factors which are related to number and diameter of A and B cells in DRG neurons and motor function deficiency are shown in Table 3. As it is observed, only caspase-3 expression doesn't have correlation with the other factors; and all of the others have significant correlation with each other.

Statistically significant positive correlation is observed between ROS level by ADP/ATP ratio, number and diameter of B cells and distance moved ($p < 0.01$). In the other hand, ROS has significant negative correlation by UCP-2 expression, number and diameter of A cells, and velocity ($p < 0.01$). Distance moved represents positive correlation by level of ATP, and velocity shows negative correlation by that ($p < 0.01$). Also, there are correlations between number and diameter of A and B cells by other factors (Table 3).

4. Discussion

DN is one of the most usual chronic disorders of diabetes which induces disability of lifetime and unfortunately there is no suitable therapy to patients suffering. Therapies for DN can be divided into treatments that target pathologic mechanisms and those that aim to relieve symptoms [2]. Due to the main roles played by oxidative stress in mediating DN it is not surprising the use of antioxidants in the search for an efficient and efficacious treatment for this disease. Generally, antioxidants work with two strategies for improving DN conditions: 1-strategies targeted directly against ROS such as ALA. 2-strategies targeted at mitochondria such as CoQ10 [10]. Although many documents showed ALA and CoQ10 have the ability to improve DN, but molecular mechanisms involved in the effectiveness of these compounds not fully understand yet. Moreover at present, no antioxidant treatment for DN

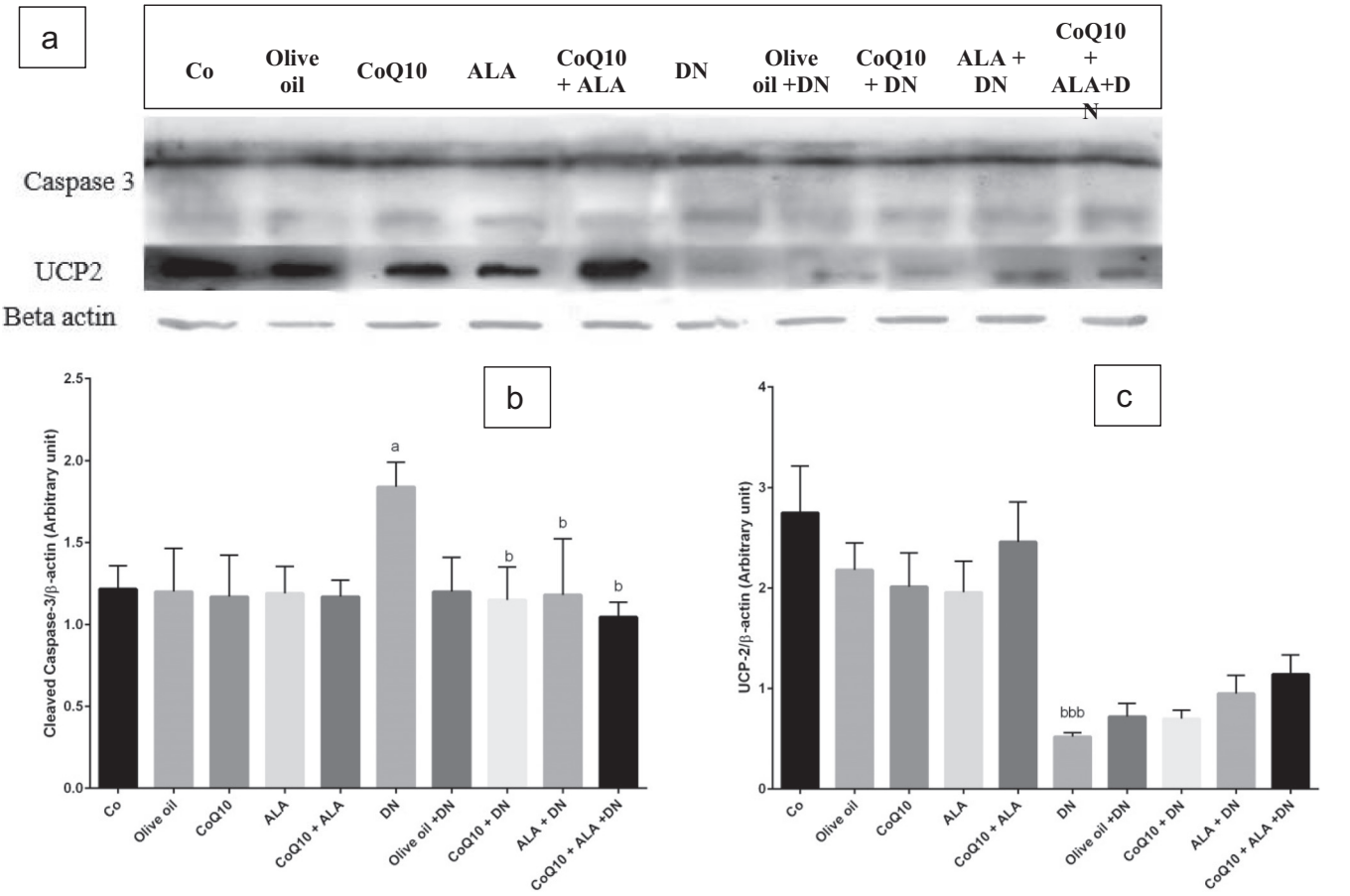

Fig. 2. CoQ10 and/or ALA modulate DN effects on caspase 3 and UCP-2 proteins expression in DRG neurons. Diabetic rats were treated with CoQ10 (10 mg/kg) and/or ALA (100 mg/kg) for five weeks and then Western blot analysis was performed. a) β-Actin was used as an internal control. Co (1), Olive oil (2), CoQ10 (3), ALA (4), CoQ10 + ALA (5), DN (6), Olive oil + DN (7), CoQ10 + DN (8), ALA + DN (9), CoQ10 + ALA + DN (10). After that, Western blotting densitometry was performed. Results are mean ± SEM, n = 7. b) CoQ10 and/or ALA effect on caspase 3 protein expression. c) CoQ10 and/or ALA effect on UCP-2 protein expression. Difference between control and other groups is significant at $p < 0.05$ (^{a}). Difference between DN and other groups is significant at $p < 0.001$ (^{bbb}), $p < 0.05$ (^{b}). Co: control; CoQ10: coenzyme Q10; ALA: alpha lipoic acid; CoQ10 + ALA: coenzyme Q10 + alpha lipoic acid; DN: diabetic neuropathy; Olive oil + DN: olive oil + diabetic neuropathy; CoQ10 + DN: coenzyme Q10 + diabetic neuropathy; ALA + DN: alpha lipoic acid + diabetic neuropathy; CoQ10 + ALA + DN: coenzyme Q10 + alpha lipoic acid + diabetic neuropathy.

has been approved by the United States Food and Drug Administration. Therefore this study was designed to evaluate the neuroprotective effects of ALA and CoQ10 on experimental model of DN to determine the possible mechanisms involved in the usefulness of these agents. In this study we focus on pathways of oxidative stress and mitochondria dysfunction and consequently DRG neurons death induced by DN and examines protective effects of ALA and CoQ10 against these pathways and will also have a comparison between the two compounds in these conditions.

It has been proved that hyperglycemia in diabetes promote generation of intracellular ROS, mitochondria dysfunction, and cell apoptosis in DRG neurons [26]. DRG neurons have been identified as primary target in diabetic peripheral neuropathy [27]. Recent studies show that all of the pathways involved in DN leading to a unique result: enhanced cellular oxidative stress and ROS level [28–31]. Additional evidence demonstrates that the nervous system is very sensitized to ROS damage for high levels of polyunsaturated lipids [32], and also it has been shown oxidative stress is more severe at the DRG neurons [33]). The hyperglycemia in diabetes induces two pathways: 1) autooxidative glycosylation which is the major cause of increased ROS generation, 2) glycation of antioxidant enzymes [34] that lead to decreased activity or availability of antioxidant enzymes, both of which are involved in DN [10]. Our results in support of previous findings [33,35–37] revealed that, ROS level significantly increased in DRG neurons in rats with DN. In addition to, our result showed that total antioxidant capacity and glutathione level significantly decreased in the same conditions.

GSH is an essential antioxidant for detoxification of toxic substances and is also a main regulator of intracellular redox potential [38]. Many studies in support of my results showed a reduction of glutathione content in DN [35,39–41]. Therefore, deficiency of antioxidant capacity in DN leads to free radicals attack to cells membranes and start lipid peroxidation [2]. In the present study MDA level was more eminent in DRG neurons in rats with DN that confirm previous studies [22,28,35,40,42]. On the other hands, our data indicated that treatment of diabetic rats with CoQ10 or ALA inhibited the DN – induced ROS generation and lipid peroxidation as well as, increased DN-reduced GSH and TAC levels in DRG neurons. We observed a little more improvement with ALA compared to CoQ10 in these factors. In support of our results previous studies found that CoQ10 decrease oxidative stress in peripheral nervous system by acting as an anti-oxidant and free-radical scavenger [43]. CoQ10 protects cell membranes and mitochondrial DNA against lipid peroxidation [35,44] by functions as intracellular antioxidant and also by increasing the expression of endogenous antioxidants [16] and thus exhibiting neuroprotective activity in DN.

Many studies revealed that ALA is a powerful free radical scavenger in peripheral nerves both in vivo and in vitro [45,46]. ALA has potent

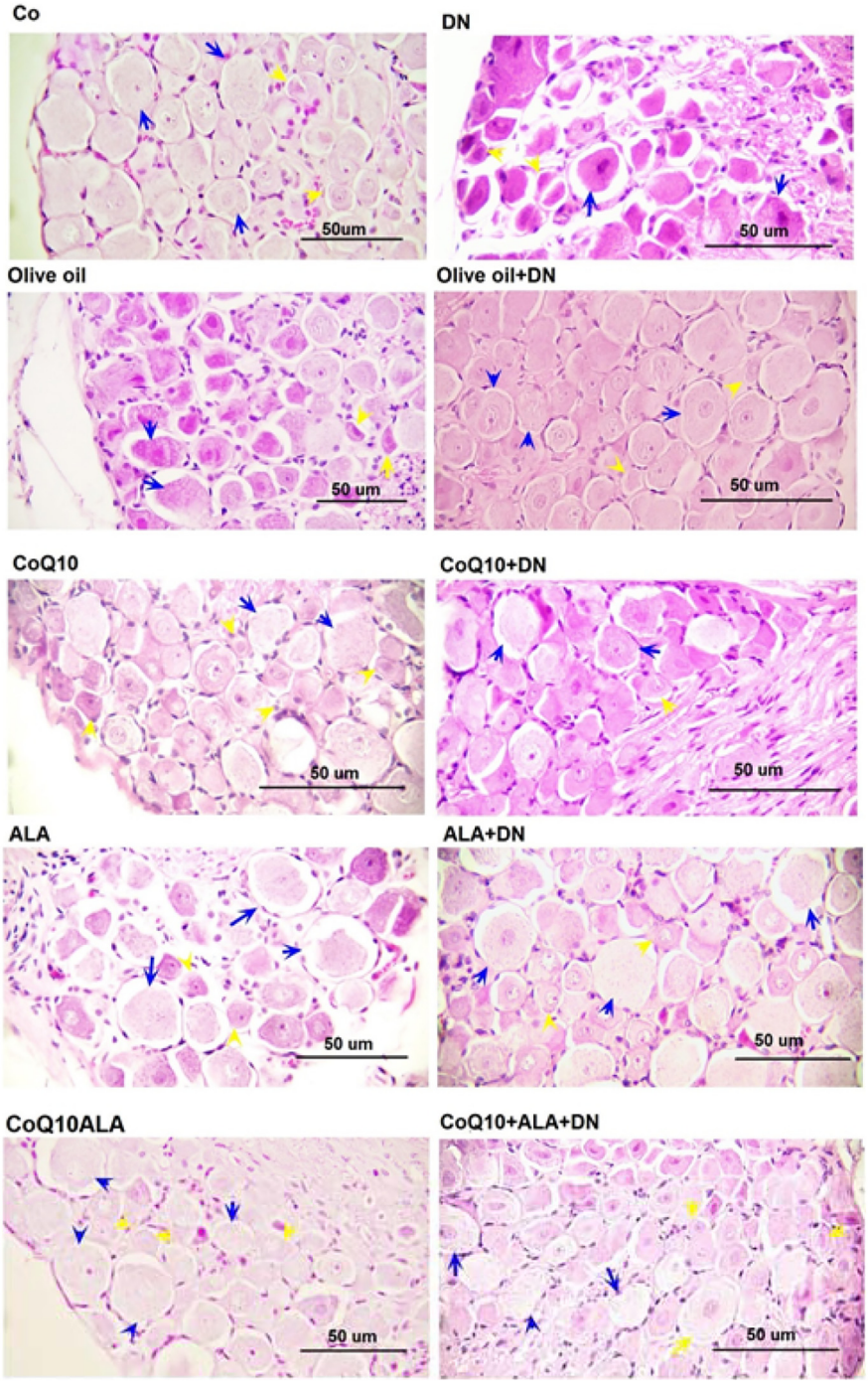

Fig. 3. HE-stained DRG neurons. Co: control; CoQ10: coenzyme Q10; ALA: alpha lipoic acid; CoQ10 + ALA: coenzyme Q10 + alpha lipoic acid; DN: diabetic neuropathy; Olive oil + DN: olive oil + diabetic neuropathy; CoQ10 + DN: coenzyme Q10 + diabetic neuropathy; ALA + DN: alpha lipoic acid + diabetic neuropathy; CoQ10 + ALA + DN: coenzyme Q10 + alpha lipoic acid + diabetic neuropathy. DN neurons tend to be small and expose a stronger basophilic staining attitude and have more and larger vacuoles. Also, we found a decrease in A cells size and conversion of A cells into B cells in DRG neurons of rats with DN and administration of ALA and/or Q10 abolished these effects. Blue arrows: A cells; Yellow arrows: B cells. Scale bar: 50 μm (400×). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

ability to scavenge ROS and reducing oxidative stress, so it is beneficial in diabetic complications in animal models [10,47]. ALA was able to improve the levels of endogenous antioxidants and decreased biochemical abnormalities such as the lipid per oxidation [10,48]. These findings could explain the protective effect of ALA on neurons in experimental models [16]. ALA is capable to reducing the oxidized forms of other antioxidants such as GSH [11]. Previous studies showed that treatment with ALA increases reduced glutathione (GSH) in vitro and in vivo [45]. On the other hands, CoQ10 has ability for regenerating and recycling other antioxidants such as GSH [44]. In addition to, it has been seen that CoQ10 and ALA have beneficial effects on total antioxidant capacity (TAC) [4,49]. It is surprising in this study ALA and CoQ10 cotreatment was more potent than the single treatment with ALA and CoQ10 in improve of oxidative stress markers. In support of our results previous studies demonstrated ALA and CoQ10 combination protected against DN- induced oxidative stress by increasing endogenous antioxidants such as cellular glutathione, restoring TAC levels, preventing lipid peroxidation and scavenging ROS [16,50–53]. Explanation this phenomenon may be the fact that, ALA is able to reduce CoQ10 to ubiquinol “a main component of the electron transport chain of mitochondrial” which can additionally reduce free radicals [11]. In other hands, combination of two antioxidants can be more effective than a single antioxidant because they can complement each other [54].

DRG neuronal apoptosis is a key mechanism involved in the pathogenesis of diabetic sensory neuropathy [27,56]. Moreover, in vivo and in vitro documents suggested that hyperglycemia affected the survival or death of DRG neurons [57–61]. Hyperglycemia activates the

Table 2
CoQ10 and/or ALA improve number and diameter changes of large and small cells in DN rats. Diabetic rats were treated with CoQ10 (10 mg/kg) and/or ALA (100 mg/kg) for five weeks and then number and diameter of A and B cells was measured in DRG neurons. Results are mean ± SEM, n = 7. Difference between control and other groups is significant at $p < 0.001$ (^{aaa}). Difference between DN and other groups is significant at $p < 0.001$ (^{bbb}), $p < 0.01$(^{bb}) and $p < 0.05$ (^{b}). Co: control; CoQ10: coenzyme Q10; ALA: alpha lipoic acid; CoQ10 + ALA: coenzyme Q10 + alpha lipoic acid; DN: diabetic neuropathy; Olive oil + DN: olive oil + diabetic neuropathy; CoQ10 + DN: coenzyme Q10 + diabetic neuropathy; ALA + DN: alpha lipoic acid + diabetic neuropathy; CoQ10 + ALA + DN: coenzyme Q10 + alpha lipoic acid + diabetic neuropathy.

	Number		Diameter (μm)	
Groups	A cells	B cells	A cells	B cells
Co	13.4 ± 0.68	11.4 ± 0.75	23.7 ± 0.59	13.45 ± 0.54
Olive oil	13.6 ± 0.51	11.4 ± 1.12	23.7 ± 0.73	13.38 ± 0.67
CoQ10	13.4 ± 0.6	11.4 ± 1.12	23.8 ± 0.49	13.30 ± 0.54
ALA	13.6 ± 0.51	11.2 ± 0.58	23.9 ± 0.57	14.01 ± 0.5
CoQ10ALA	13.8 ± 0.37	11.4 ± 1.12	24.0 ± 0.68	14.09 ± 0.40
DN	5.8 ± 0.66^{aaa}	23.2 ± 0.74^{aaa}	19.0 ± 0.23^{aaa}	9.46 ± 0.64^{aaa}
Olive oil + DN	6.2 ± 0.86	23.4 ± 0.68	18.5 ± 0.66	10.22 ± 0.67
CoQ10 + DN	9.8 ± 0.37^{bb}	17.2 ± 0.86^{bbb}	21.2 ± 0.27	10.85 ± 0.49
ALA + DN	11 ± 0.63^{bbb}	17.2 ± 0.86^{bbb}	21.3 ± 0.44	12.09 ± 0.44^{b}
CoQ10 + ALA + DN	11.8 ± 0.66^{bbb}	14.6 ± 0.51^{bbb}	22.4 ± 0.25^{bb}	12.38 ± 0.59^{b}

caspase cascade in neurons through mitochondria dysfunction. Activity of mitochondrial electron transport chain alters in DRG neurons in DN [60,62]. Actually, extensive generation of ROS attack the mitochondrial membrane lipids and lead to loss of mitochondrial transmembrane potential which cause an increase in the ADP/ATP ratio and an absolute decrease in ATP levels. This in turn is coupled with release of cytochrome *c* from mitochondria into the cytosol, activation of caspase-3, and resulting in DRG apoptosis [63]. ATP and function of mitochondrial membrane in hyperglycemia condition are regulated by uncoupling proteins (UCPs). UCPs are a family of proton carriers which are available in inner mitochondrial membrane and can disperse the mitochondrial proton gradient, bypassing the ATP production via oxidative phosphorylation. Documents have shown that UCPs can prevent the production of ROS from the mitochondria and subsequently decrease oxidative stress [64]. UCP2 expression is widespread in different tissues, although we had difficulty measuring UCP2 similar to what Vincent and colleagues showed difficulty detecting UCP2 in DRG neurons [65]. According to documents, UCPs expression is rapidly downregulated by hyperglycemia in DRG neurons which can reflect them contribution to overcoming on oxidative stress and apoptosis [65,66]. UCPs can also prevent apoptosis upstream of mitochondrial membrane potential reduction, ROS formation, and activation of caspase [67]. Our result in the depletion of ATP, increase of the formation of ROS, caspase 3overexpression, and UCP2 downregulation in DRG neurons in rats with DN provide a conditioning that ultimately leads to apoptosis in DRG neurons and previous mentioned studies confirmed our data. Progressive loss of sensory fibers in peripheral nerves which is created by degeneration and loss of parent DRG neurons is a characteristic phenomenon of DN. In general, diabetic neurons tend to be smaller, expose a stronger basophilic staining attitude with more and larger

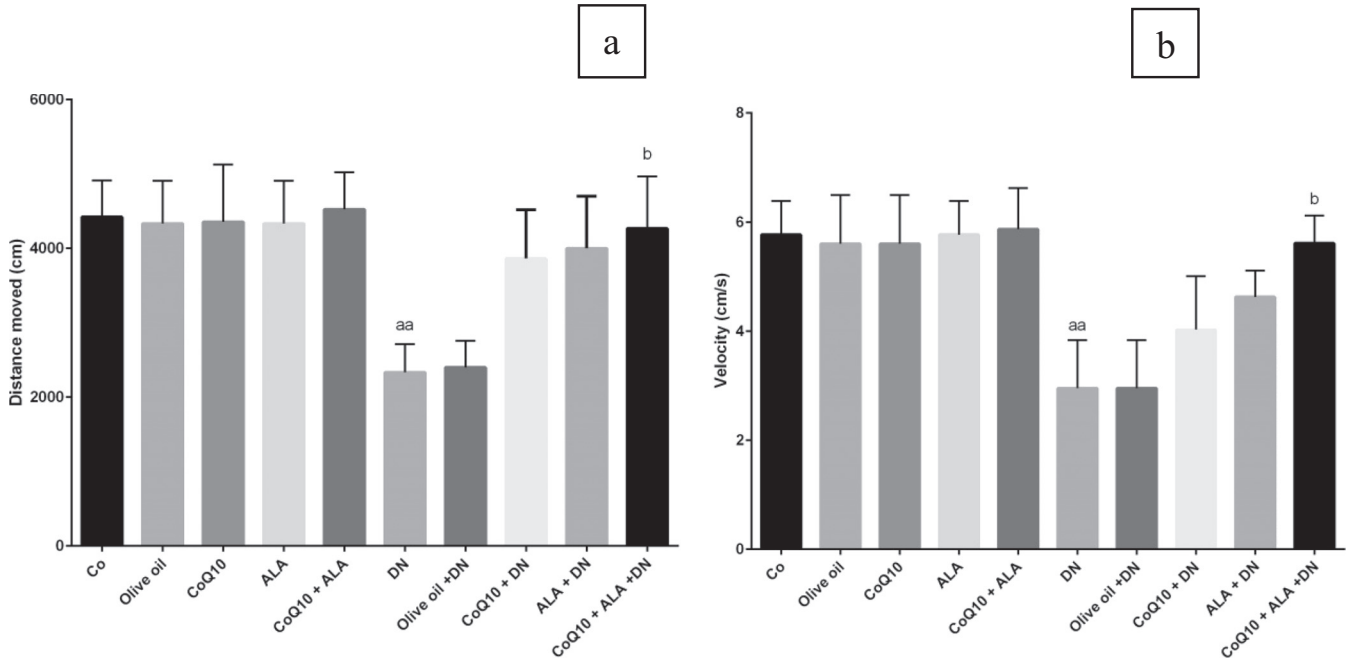

Fig. 4. CoQ10 and/or ALA improve distance moved (a) and velocity (b) changes in rats with DN. Diabetic rats were treated with CoQ10 (10 mg/kg) and/or ALA (100 mg/kg) for five weeks and then motor function was evaluated by open-field activity tests. Results are mean ± SEM, n = 7. Difference between control and other groups is significant at $p < 0.01$ (^{aa}). Difference between DN and other groups is significant at $p < 0.05$ (^{b}). Co: control; CoQ10: coenzyme Q10; ALA: alpha lipoic acid; CoQ10 + ALA: coenzyme Q10 + alpha lipoic acid; DN: diabetic neuropathy; Olive oil + DN: olive oil + diabetic neuropathy; CoQ10 + DN: coenzyme Q10 + diabetic neuropathy; ALA + DN: alpha lipoic acid + diabetic neuropathy; CoQ10 + ALA + DN: coenzyme Q10 + alpha lipoic acid + diabetic neuropathy.

Table 3
Correlation between different markers of the study.

Correlations											
		ROS level	ADP/ATP ratio	Caspase-3	UCP-2	Number of A cells	Number of B cells	Diameter of A cells	Diameter of B cells	Distance	Velocity
ADP/ATP ratio	Pearson correlation	0.987**	1	0.529	−0.896**	−0.978**	0.983**	−0.985**	−0.978**	0.856**	−0.987**
	Sig. (2-tailed)	0.000		0.116	0.000	0.000	0.000	0.000	0.000	0.002	0.000
	N	10	10	10	10	10	10	10	10	10	10
Caspase-3	Pearson correlation	0.550	0.529	1	−0.350	−0.604	0.552	−0.508	−0.575	0.512	−0.535
	Sig. (2-tailed)	0.100	0.116		0.321	0.065	0.098	0.134	0.082	0.130	0.111
	N	10	10	10	10	10	10	10	10	10	10
UCP-2	Pearson correlation	−0.867**	−0.896**	−0.350	1	0.854**	−0.877**	0.884**	0.896**	−0.719*	0.901**
	Sig. (2-tailed)	0.001	0.000	0.321		0.002	0.001	0.001	0.000	0.019	0.000
	N	10	10	10	10	10	10	10	10	10	10
Number of A cells	Pearson correlation	−0.989**	−0.978**	−0.604	0.854**	1	−0.991**	0.988**	0.972**	−0.885**	0.983**
	Sig. (2-tailed)	0.000	0.000	0.065	0.002		0.000	0.000	0.000	0.001	0.000
	N	10	10	10	10	10	10	10	10	10	10
Number of B cells	Pearson correlation	0.984**	0.983**	0.552	−0.877**	−0.991**	1	−0.998**	−0.962**	0.916**	−0.980**
	Sig. (2-tailed)	0.000	0.000	0.098	0.001	0.000		0.000	0.000	0.000	0.000
	N	10	10	10	10	10	10	10	10	10	10
Diameter of A cells	Pearson correlation	−0.988**	−0.985**	−0.508	0.884**	0.988**	−0.998**	1	0.964**	−0.903**	0.984**
	Sig. (2-tailed)	0.000	0.000	0.134	0.001	0.000	0.000		0.000	0.000	0.000
	N	10	10	10	10	10	10	10	10	10	10
Diameter of B cells	Pearson correlation	−0.983**	−0.978**	−0.575	0.896**	0.972**	−0.962**	0.964**	1	−0.776**	0.995**
	Sig. (2-tailed)	0.000	0.000	0.082	0.000	0.000	0.000	0.000		0.008	0.000
	N	10	10	10	10	10	10	10	10	10	10
Distance	Pearson correlation	0.846**	0.856**	0.512	−0.719*	−0.885**	0.916**	−0.903**	−0.776**	1	−0.818**
	Sig. (2-tailed)	0.002	0.002	0.130	0.019	0.001	0.000	0.000	0.008		0.004
	N	10	10	10	10	10	10	10	10	10	10
Velocity	Pearson correlation	−0.994**	−0.987**	−0.535	0.901**	0.983**	−0.980**	0.984**	0.995**	−0.818**	1
	Sig. (2-tailed)	0.000	0.000	0.111	0.000	0.000	0.000	0.000	0.000	0.004	
	N	10	10	10	10	10	10	10	10	10	10

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).

vacuoles. The loss of fibers in DN is selective, affecting the largest neurons. The ratio of large to small neurons is reduced in rats with DN, this is while total count of A and B cells are almost equal in this condition [57,68]. It is conceivable that vacuolar changes in DRG neurons are due to an perturbed neuronal energy metabolism and impaired sodium handling through modified Na^+/K^+-ATPase activity and/or decrease of DRG neurons blood flow in DN [69]. Our results in confirmation of previous studies showed more and larger vacuoles and a significant decrease in count of A cells and also size of A and B cells in DRG neurons in rats with DN.

It is noteworthy that, diabetic rats treated with CoQ10 or ALA showed an improvement in all listed factors associated with apoptosis and also a reduce atrophy and loss of DRG neurons. we also found that treatment with ALA had more improvement than CoQ10 in this conditions. In confirm of our study, more documents have shown that ALA inhibited the activation of apoptosis molecular pathways such as caspase-3 and the powerful regulatory effect of ALA on these pathways was mediated by preventing the production and reducing the activity of ROS [47]. ALA can helps to maintenance of mitochondrial membrane potential changes and regulation of the redox state by controlling required substrate for respiratory chain that lead to increase of ATP production. Also documents revealed that ALA is the most common first-line antioxidant therapy for DN which its antioxidative effect is through regulation of mitochondrial proteins such as UCP-2 [70]. Since anti-apoptotic and neuroprotective effects of ALA lead to use of it in diseases characterized by mitochondrial dysfunction like DN through prevention of neurons loss of DRG [4,16,71]. Previous studies revealed that CoQ10 (a mitochondrial antioxidant) prevents apoptosis induced by oxidative stress through inhibition of the mitochondria dependent caspase-3 pathway [10,22,72]. In addition to, CoQ10 facilitate electron transfer between the redox components of the electron transport chain that lead to provide a proton gradient across the inner mitochondrial membrane and increased ATP production [35]. Document has demonstrated that CoQ10 is a cofactor of mitochondrial metabolism and UCP2 activity which leading to reduced loss of DRG neurons [21,73]. Therefore, CoQ10 may play a main role in neuroprotection against DN because of prevent mitochondrial damage and subsequently prevent nerve damage [22,72]. Animal and clinical trial studies have also shown neuroprotective role of coenzyme Q10 in neurodegenerative disease [74,75]. Study in animal models of neurodegeneration showed that CoQ10 has protective effect against neuronal loss [16] and it was shown CoQ10 can attenuate neurons loss of DRG [72] and improve size and number of this neurons in DN [21]. Moreover this study demonstrated that ALA and CoQ10 cotreatment would have more improvement than the single treatment with ALA and CoQ10 against atrophy and loss of DRG neurons in diabetic rats. In confirm of our findings, previous studies showed that the combination of ALA and CoQ10 could significantly decrease apoptosis scores and consequently DN- induced neurotoxicity compared with ALA or CoQ10 alone. More effectiveness of this combination may be through an increased cellular mitochondrial density and ATP synthesis which reverse some of the neuron damages caused by the reduction of ATP. It has also been seen an increase in

complex I and II respiratory capacity following ALA and CoQ10 combination [50,51]. Moreover, this combination would suppress the hazardous effects of oxidative stress induced by the respiratory chain impairment. This synergetic effect is probably because combination treatment could decrease one or more of the final pathways of mitochondrial dysfunction that lead to improve of mitochondrial ATP production [76]. Another explanation is that two antioxidants can be more effective than a single antioxidant because they can complement each other [54]. Totally, ALA can improve the ability of CoQ10 in inhibiting oxidative stress and apoptosis resulting from mitochondrial dysfunction in DN [77].

Although motor and sensory conduction disorders are present in DN, but because motor conduction defect occurs early in the DN and progress over a period of time [68], we evaluated motor function for confirming neuropathy. Although conduction velocity has direct connection with the size of DRG neurons, but atrophy or loss of these neurons occurs much later than conduction velocity defect in DN model. It seems that slowing of conduction may be due to metabolic changes such as decreased mitochondrial generation of ATP induced by diabetes. Disorder of the bioenergetics system in DRG neurons changes cellular functions that lead to atrophy of neurons and consequently motor function deficiency. Also mitochondrial dysfunction – induced motor abnormality indirectly supports oxidative hypothesis due to accumulation of oxygen radicals [78,79]. In confirmation of previous studies this study demonstrated a significant decrease in motor function in rats with DN compare to control [57,80] and treatment with CoQ10 or ALA showed an improvement in distance moved and velocity in this condition. Of course ALA has little more improvement compared with CoQ10 in this condition. Previous studies in support of our results showed ALA and CoQ10 can improve motor nerve conduction velocity (MNCV) in rats with DN [4,21,81–83]. Our results showed that ALA and CoQ10 cotreatment had significant improvement compared to ALA or CoQ10 alone on distance moved and velocity in rats with DN. Moreover, it has been seen that ALA and CoQ10 combination has the effect of further improvement on neurotoxicity by improving nerve conduction velocity compared to ALA or CoQ10 alone [16] that support our study.

5. Conclusions

The findings of this study imply that administration of CoQ10, ALA, and especially their combination effectively attenuated oxidative stress and mitochondria dysfunction and consequently DRG neurons death in an experimental model of DN. These modulatory effects of CoQ10, ALA, and especially their combination are mediated, at least in part, through increasing TAC and GSH, preventing lipid per oxidation and ROS, modulation of expression of caspase 3 and UCP2 proteins, inducing ATP, inhibition of DRG neurons death and improving motor function. These effects could be due to CoQ10 and ALA antioxidant nature, which include anti-apoptotic properties and their neuroprotective activities. Thus, these findings would encourage future clinical trials of these agents in patients with DN to whom coadministration of neuroprotectants is required to prevent neurotoxicity.

Conflicts of interest statement

The authors declare no conflict of interest.

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