

Neuroprotective effects of α -Lipoic acid alone and in combination with ferulic acid in diabetic neuropathy induced rats

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Abstract

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The present pre-clinical activity was undertaken to screen the two antioxidants, mainly α -lipoic acid and ferulic acid alone, and in combination in neuropathic pain induced by diabetes in rats. The activity was confirmed by assessing various behavioral as well as biochemical and histopathological studies. The study was performed on adult albino rats. The rats were divided into different groups, and each group contained six rats. Diabetic neuropathy in rats was induced by administering a freshly prepared single dose of streptozotocin (60 mg/kg, i.p). After development of neuropathy the rats were treated with α -lipoic acid (25 mg/kg/day, p.o), ferulic acid (10 mg/kg/day, p.o) and standard drug Pregabalin (30 mg/kg/day i.p). One group received the combination of antioxidants, i.e., α -lipoic acid (12mg/kg, p.o) and ferulic acid (05 mg/kg/day, p.o) respectively, for two weeks. Neuropathic pain was assessed using mechanical allodynia, mechanical hyperalgesia, cold allodynia, and thermal allodynia. Biochemical parameters of blood glucose, nitric oxide, level of lipid peroxidase, reduced glutathione, and membrane-bound ATPases activities were also studied. Neuropathic pain induced rats showed a significant alteration in behavioral and biochemical parameters. Treatment with α -lipoic acid in combination with Ferulic acid significantly restored the altered parameters towards normal as compared to single antioxidants, thus provided proper neuroprotection. This effect might be due to the strong free radical scavenging potential of α -lipoic acid and ferulic acid.

Abbreviations: α -lipoic acid (ALA), Streptozotocin (STZ), Ferulic acid (FA), Pregabalin (PG), Intraperitoneal injection (i.p.), By mouth (p.o.), Diabetic neuropathy (DN), Neuropathic Pain (NP), Percentage of maximum possible effect (%MPE), Lipid peroxidation (LPO), Reduced glutathione (GSH), Nitric oxide level (NO)

1. Introduction

Diabetic neuropathy is considered one of the microvascular complications of long term hyperglycemia. It has been reported that people with chronic diabetes mellitus are more prone to the development of neuropathy, which affects the quality of life of up to 60% of patients [1]. STZ induced diabetes is a well-established animal model, which reports the involvement of oxidative stress in neuropathy.

Several studies have reported the preventive role of antioxidants in the progression of neuropathic pain [2]. α -lipoic acid (ALA), also known as thioctic acid, and its reduced dithiol form, i.e., dihydrolipoic acid (DHLA), are known for their antioxidant activities [3][4]. ALA is reported to possess various pharmacological activities in animals, such as participating in the regeneration of exogenous and endogenous antioxidants, reparation of oxidized proteins, chelation of metal ions, and quenching of reactive oxygen species [5-9]. Ferulic acid (FA) is a polyphenol compound with potent antioxidant and anti-diabetic activities [10]. On the other hand, pregabalin a well-known anti-diabetic drug and is reported as an effective agent for treating the pain associated with various conditions such as diabetic



neuropathy, post-herpetic neuralgia, and central neuropathic pain [11][12]. Combinations of antioxidants are reported to show synergistic activity [13-16]. Based on the available literature, this study was designed to investigate the combined effects of ALA and FA in streptozotocin-induced neuropathic pain in experimental animals by evaluating various behavioral and biochemical parameters.

2. Material and Methods

2.1. Animals

In the present study, albino rats of Wister strain were used. The rats were procured from (LACSMI Biofarm, Aundh, Pune, Maharashtra, India). All the rats were housed separately in polypropylene cages with paddy husk as bedding material. The rats were maintained at standard environment based on the guidelines provided by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. During the experiment, the rats had free access to water and standard laboratory food with proper nutrition value (Nutrivet Lab, Pune, India). The experimental protocols were reviewed and approved by the Institutional Animal Ethics Committee (IAEC) of the college with approval number SSDJ/ IAEC/2018-19/03.

2.2. Drugs and reagents

α -lipoic acid (ALA) and streptozotocin (STZ) were procured from Sigma Aldrich, USA. Ferulic acid (FA) was obtained from (Otto Kemmi PVT Ltd. Mumbai, India). Pregabalin (PG) was a gift sample from Sun Pharmaceuticals, Vadodara, Gujarat. Other chemicals used in the study were obtained from a standard supplier.

2.3. Preparation of drug solutions

All drug solutions were freshly prepared at the time of administration. STZ was dissolved in ice-chilled freshly prepared citrate buffer (0.1M, pH 5.5). Arachis oil was used as a vehicle for ALA, whereas FA and PG were dissolved in 0.9% normal saline.

2.4. Induction and assessment of Diabetes neuropathy

Diabetes neuropathy was induced in rats by administration of a single dose (60 mg/kg, i.p) of STZ. It was confirmed by estimating of 72 hours blood glucose level using Glucometer. Rats with blood glucose levels higher than 250mg/dl were considered as diabetic and used for the development of neuropathic pain[17].

2.5. Experimental Protocol

The day of which DN was developed is considered as basal readings (4 weeks). Since then, the treatment was carried out for two weeks, so the protocol was six weeks in total. The rats were divided into six groups

Rats in group-I were assigned as a control group (CN), while all the other groups were injected with STZ. Group II rats were injected with STZ and considered as Diabetic neuropathy (DN) group. Rats in group III received PG as a standard neuroprotective drug (30mg/kg i.p. once a day for two weeks). Group IV and V rats received ALA (25mg/kg, p.o. once a day for two weeks) or FA (10mg/kg, p.o. once a day for two weeks), respectively. Group VI received both FA(12mg/kg, p.o.) and ALA(05mg/kg) once a day for two weeks.

Blood glucose level was estimated using Glucometer (Nipro Diagnostics) by the end of the experiment. Food consumption was measured in terms of (g/day), water consumption was measured in terms of (ml/day), and body weight was measured in terms of (g) on the first week, basal week (4th week), and the final week (6th week).

2.6. Assessment of Neuropathic Pain

Assessment of NP was carried out using various behavioural methods:

1. Mechanical allodynia using Von Frey Hair Test [18]
2. Mechanical hyperalgesia using Pinprick Test [19]

3. Randall-Selitto Analgesiometer [20]
4. Thermal allodynia and hyperalgesia using Eddy's Hot Plate method [21]
5. Cold allodynia using acetone [22]

2.7. Assessment of Biochemical Parameters

At the end of the treatment period, rats were anesthetized using a high dose of thiopental sodium. Blood glucose levels were determined. The sciatic nerve was isolated, and a homogenate was made using ice-cold Tris hydrochloric buffered saline (10mM, pH7.4). After centrifugation, the supernatant was used for the estimation of various biochemical parameters. The cleared supernatant was used for the determination of lipid peroxidation (LPO)[23], reduced glutathione(GSH) [23], and nitric oxide level(NO) [24], and the sediment was used for the determination of ATPases. Na⁺/K⁺-ATPase[25], Ca²⁺ATPase[26] and Mg²⁺-ATPase[27].

2.8. Statistical Analysis

The results were analyzed using Graph Pad Prism, version 0.5. One way ANOVA followed by Dunnett's *t* test was applied in order to compare test groups for significant differences. The control group was compared with diabetic neuropathy ^a*P*<0.05, Diabetic neuropathy group was compared with treatments ^b*P*<0.05, group treated with a combination of antioxidants were compared with ALA treated ^x*P*<0.05, and FA treated group ^y*P*<0.05.

3. Results

3.1. Blood glucose level, body weight, food, and water intake

By the end of the experiment, blood glucose levels in all STZ groups (Group II-VI) were significantly higher than that of CN group. However, treatment with ALA+FA for two weeks displayed a significant reduction in blood glucose level when compared with PG, DN, ALA, and FA treated group. Bodyweight was reduced in all diabetic groups, whereas food and water intake were increased compared to CN group. However, treatment PG, ALA, FA, or ALA+FA combination showed significant improvement in body weight, food intake as well as water intake as compared to DN (Table1).

3.2. Behavioral changes

Mechanical allodynia was performed using von Frey Hair Test. It was found that 50% gram threshold of the left hind paw in DN rats was significantly (*P*<0.05) less compared to CN rats on basal evaluation (4th week), 5th and 6th week. Treatment with ALA+FA group showed a significant increase in 50% gram threshold of the left paw compared to individual antioxidant as shown in (Fig. 1 A).

Mechanical hyperalgesia was performed using the Randall-Selitto test. It was observed that paw withdrawal latency of left hind paw in DN group was significantly (*P*<0.05) reduced as compared to CN group. ALA+FA treated rats displayed a significant rise in paw withdrawal latency of left paw compared to DN, ALA, FA, and PG, as shown in (Fig. 1 B).

Inpinprick test, the paw withdrawal latency of left paw in diseased rats was significantly (*P*<0.05) less compared to CN rats. Treatment with ALA+FA showed a significant rise in paw withdrawal latency as compared to DN, ALA, FA alone group (Fig. 1 C).

Cold allodynia was performed using acetone method. it was found that paw withdrawal latency of left hind paw in DN rats was significantly (*P*<0.05) less than that of CN rats. The ALA+FA rats showed significant elevation in paw withdrawal latency compared to DN, ALA, and FA, as shown in (Fig. 1 D).

Eddy's Hot Plate Test was performed to check the response to thermal hyperalgesia, and it was expressed in % Maximal possible effect (%MPE). %MPE in DN group showed significantly (*P*<0.05) reduced compared to CN animal. Rats treated with ALA and FA together or separately showed a significant rise in %MPE as compared to DN and ALA alone, as shown in (Fig. 2).

Table 1. Effect of ALA, FA and their combination on blood glucose level, body weight, food, and water intake in diabetic neuropathy induced rats

Groups	Blood Glucose Level (mg/dl)	Change in bodyweight (g)			Change in food intake (g)			Change in water intake (ml)		
		1st week	Basal	6th week	1st week	Basal	6th week	1st week	Basal	6th week
CN	138.8 ±2.10	230.8±1.87	234.3±1.41	236.8±1.17	38.67±1.05	39.67±1.05	42.00±1.50	140.7±2.65	141.0±3.29	139.8±2.37
DN	379.0 ±8.66 ^a	232.0±1.51	173.2±1.01 ^a	162.2±2.21 ^a	37.67±1.14	70.00±3.93 ^a	89.17±4.53 ^a	140.3±2.91	236.0±1.38 ^a	324.3±5.22 ^a
PG	340.5 ±4.17 ^b	232.2±1.86	176.3±2.27 ^a	183.3±1.48 ^b	35.00±4.61	55.50±1.73 ^a	45.83±1.51 ^b	139.8±2.37	291.7±4.67 ^a	195.7±5.85 ^b
ALA	310.0 ±3.55 ^b	234.8±1.38	179.5±3.63 ^a	184.8±3.34 ^b	39.33±1.61	56.33±2.12 ^a	43.67±1.02 ^b	139.5±3.04	297.2±4.26 ^a	196.5±8.15 ^b
FA	327.3 ±20.98 ^b	235.5±1.45	180.5±1.45 ^a	185.5±1.25 ^b	39.17±1.47	59.33±1.23 ^a	47.13±1.33 ^b	141.2±2.71	290.2±4.36 ^a	213.7±5.32 ^b
ALA+FA	300.3 ±13.14 ^{bxy}	236.0±1.69	177.7±1.82 ^a	188.0±1.51 ^{bxy}	39.77±1.40	57.67±2.04 ^a	43.50±1.23 ^{bxy}	141.2±3.29	296.6±2.30 ^a	198.8±10.52 ^{bxy}

Values are expressed as mean ±SEM. Means were compared using Dunnett 't' test. Control compared with diabetic neuropathy ^a*P*<0.05, Diabetic neuropathy group compared with treatments (ALA/FA) ^b*P*<0.05, group treated with a combination of antioxidants were compared with ALA ^x*P*<0.05 and FA ^y*P*<0.05.

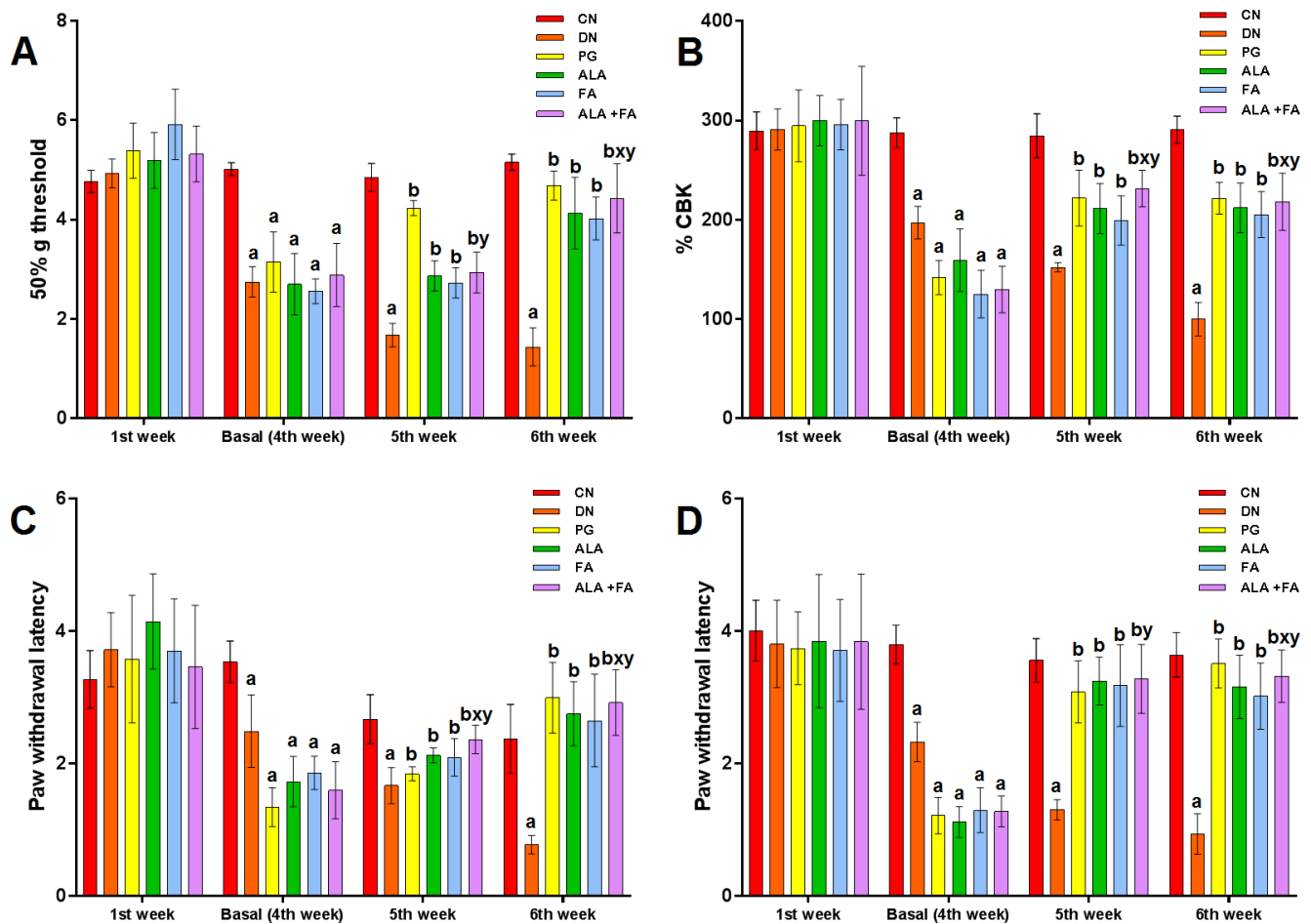


Figure 1. Effect of ALA, FA and their combination on 50% gram threshold of the left hind paw (A) and paw withdrawal latency using mechanical hyperalgesia with Randall-Selittoan algometer (B) and Pinprick Test (C), in addition to cold allodynia using acetone (D) in diabetic neuropathy induced rats. Values are expressed as mean± CI (%95). Means were compared using Dunnett ‘t’ test, Control compared with diabetic neuropathy ^a*P*<0.05, Diabetic neuropathy group compared with treatments (ALA/FA) ^b*P*<0.05, group treated with a combination of antioxidants were compared with ALA ^x*P*<0.05, and FA ^y*P*<0.05.

3.4. Endogenous antioxidants

The endogenous antioxidants markers such as LPO, GSH, and NO were measured in sciatic nerve homogenates. LPO level significantly increased (*P*<0.05), whereas the level of GSH was reduced considerably in DN rats compared to CN rats (table 2). Treatment with ALA, FA, PG, and ALA+FA for two weeks showed a significant (*P*<0.05) reduction in the level of LPO, and a significant (*P*<0.05) rise in GSH level as compared to DN (Table 2). NO level was significantly (*P*<0.05) higher in DN group as compared to CN. Combined treatment of ALA+FA showed a significant (*P*<0.05) reduction in NO level compared to DN rats (table 2).

Table 2. Effect of ALA, FA, and their combination on the level of Lipid peroxidation (LPO), Reduced glutathione (GSH), and Nitric oxide level (NO) (measured on the sixth week).

Groups	LPO (nM of MDA/mg)*	GSH (μ g of GSH/mg)*	NO (mg/mg)*
CN	260.3 \pm 15.620	0.950 \pm 0.088	07.69 \pm 0.363
DN	155.18 \pm 4.163a	3.767 \pm 0.417a	20.98 \pm 0.585a
PG	238.6 \pm 10.700b	1.067 0.3638b	09.05 \pm 0.361b
ALA	188.5 \pm 23.050b	1.836 0.244b	11.88 \pm 0.782b
FA	158.0 \pm 7.071b	1.720 \pm 0.169b	12.98 \pm 0.132b
ALA+FA	219.1 \pm 39.00bxy	1.204 \pm 0.128bxy	11.15 \pm 0.5452bxy

* All concentrations are represented in mg of tissue.

Values are expressed as mean \pm SEM. Means were compared using Dunnet 't' test. Control compared with diabetic neuropathy ^a $P < 0.05$, Diabetic neuropathy group compared with treatments (ALA/FA) ^b $P < 0.05$, group treated with a combination of antioxidants were compared with ALA ^x $P < 0.05$ and FA ^y $P < 0.05$.

3.5. Membrane-bound enzymes

The activity of membrane-bound phosphatases such as Na⁺/K⁺, Ca²⁺, and Mg²⁺ATPase was found to be significantly ($P < 0.05$) reduced in DN group compared to the control group. Treatment with ALA, FA, and PG for two weeks showed significant ($P < 0.05$) increased Na⁺/K⁺, Ca²⁺, and Mg²⁺ATPase levels compared to the non-treated DN group. Combined treatment with ALA and FA showed further improvement in the level of membrane-bound phosphatases compared to other treated groups (Table 3).

Table 3. Effect of ALA, FA, and their combination on membrane-bound phosphatases (Na⁺/K⁺, Ca²⁺ and Mg²⁺ ATPase) of DN induced rats (measured on the sixth week).

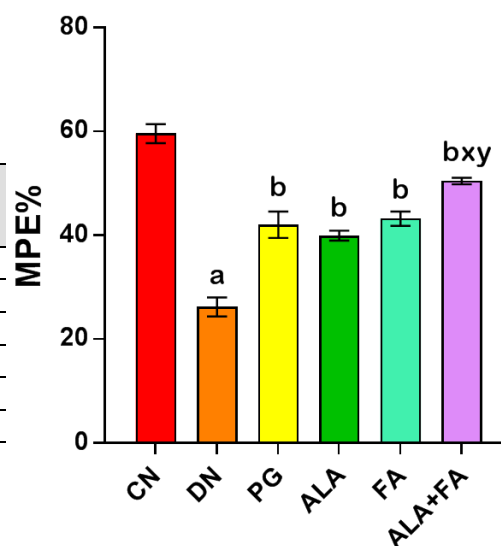
Groups	Na ⁺ /K ⁺ ATPase (mM of Pi liberated/mg)*	Ca ²⁺ ATPase (mM of Pi liberated/mg)*	Mg ²⁺ ATPase (mM of Pi liberated/mg)*
CN	698.1 \pm 11.29	409.0 \pm 7.591	568.1 \pm 11.29
DN	498.7 \pm 32.10a	245.1 \pm 22.87a	398.7 \pm 32.10a
PG	659.3 \pm 26.28b	345.8 \pm 22.69b	566.3 \pm 26.28b
ALA	598.8 \pm 36.30b	323.4 \pm 4.77b	523.8 \pm 36.30b
FA	560.6 \pm 32.04b	313.4 \pm 5.84b	521.6 \pm 36.04b
ALA+FA	611.7 \pm 31.77bxy	339.3 \pm 19.54bxy	548.7 \pm 31.77bxy

* All concentrations are represented in mg of tissue.

Values are expressed as mean \pm SEM. Means were compared using Dunnet 't' test. Control compared with diabetic neuropathy ^a $P < 0.05$, Diabetic neuropathy group compared with treatments (ALA/FA) ^b $P < 0.05$, group treated with a combination of antioxidants were compared with ALA ^x $P < 0.05$ and FA ^y $P < 0.05$.

5. Discussion

The present study was conducted to investigate the potential beneficial effects of two well-known antioxidants ALA and FA, in streptozotocin-induced diabetic neuropathy. ALA therapeutic effectiveness was previously reported in a broad spectrum of diseases such as heavy metal poisoning, ischemia-reperfusion injury, cardiovascular disorders, and neurodegenerative disease, which is probably due to its substantial antioxidant properties[4][28][29]. Additionally, FA is used as an antioxidant in the management of diabetes, aging, cancer, and is also used as a pulmonary protecting agent [30]. PG is a novel antiepileptic agent that primarily binds to the alpha-2-delta subunit of voltage-gated Ca²⁺ channels and exerts analgesic action.[31].

**Figure 2. Effect of ALA, FA, and their combination of heat (thermal) hyperalgesia using Eddy's hot plate in diabetic neuropathy induced rats (measured on the sixth week).**

Values are expressed as mean \pm SEM. Means were compared using Dunnet 't' test. Control compared with diabetic neuropathy ^a $P < 0.05$, Diabetic neuropathy group compared with treatments (ALA/FA) ^b $P < 0.05$, group treated with a combination of antioxidants were compared with ALA ^x $P < 0.05$ and FA ^y $P < 0.05$.

The current study showed that the administration of ALA and FA reduced the elevated level of glucose. This observation was in line with previous reports [28][32]. FA was reported to stimulate insulin secretion and to increase the utilization of glucose by extrahepatic tissues [27][33]. Rats with DN showed increased food and water consumption, which corresponds to the previously reported study [34]. The treatment with ALA and FA alone and their combination significantly reduced the food and water intake. Rats with DN showed decreased body weight compared to the control group, similar to the results reported by [35]. A decrease in body weight after STZ administration might be due to the degradation of structural proteins, which increases muscle wasting [31][38]. However, the combined ALA + FA treatment enhanced bodyweight significantly.

DN is associated with a decrease in paw withdrawal latency, which can be assessed by various behavioral nociceptive tests. Using Von Frey hair test, it was found that STZ induced a reduction in the 50 % gram paw withdrawal threshold, and the treatment with ALA and FA with their combination significantly elevated the 50% gram paw withdrawal threshold on the left hind paw.

The paw withdrawal threshold in terms of %CBK was measured using Randall-Selitto analgesiometer. The treatment with ALA, FA, and their combination significantly elevated the paw withdrawal threshold on the left hind paw compared to non treated diabetic rats [37]. In the present study, the mechanical hyperalgesia was evaluated using Pinprick test, which showed a significant increase in paw withdrawal threshold in the left hind paw of the combination group compared to individual drugs in both treatment weeks. As for acetone induced cold allodynia, the ALA and FA combination significantly elevated the paw withdrawal latency compared to the non-treated diabetic group. Additionally, the treatment with antioxidants combination normalizes the threshold in a better way compared to single antioxidant treatments. Change in nociception was well reported in earlier studies of diabetic neuropathic pain. The changes in the nervous system associated with neuropathic pain include a critical up-regulation of the calcium channel subunit $CaV\alpha2\delta-1$ [38]. PG effect mechanism includes the binding with $\alpha2\delta$ subunit of voltage-gated calcium channel and leads to reduced release of neurotransmitters. These effects are linked in the attenuation of neuronal hyperexcitability and the development of analgesic effect [39].

STZ produces free radicals species, thereby disturbing the endogenous balance between oxidants and antioxidants. In the present study, oxidative stress markers (LPO, GSH, and NO) were monitored to obtain a better idea of oxidative status. An increase in free radicals may react with a polyunsaturated fatty acid in the cell membrane leading to LPO formation; thus, the uncontrolled lipid peroxidation leads to damage of islet cell, which might be responsible for hyperglycemia [40]. Other studies showed that there was a high level of LPO in DN conditions [41]. GSH is a critical intracellular antioxidant, and its function is to suppress lipid peroxidation [44]. The decrease in GSH level and its metabolism impairments in the diabetic condition is due to competition between aldose reductase and glutathione reductase for NADPH and the increased oxidative stress [43][44]. The current study showed that ALA might have increased intracellular GSH levels. On the other hand, FA is a hydroxyl radical scavenger [45], which might have assisted under oxidative conditions. Increased levels of NO might be due to the stimulation of iNOS. Interaction of O_2^- with NO is rapid and leads to the formation of potent oxidant radical, i.e., peroxynitrite ($ONOO^-$), which stimulates arachidonic acid metabolism, lipid peroxidation, and prostanoid production [47][48]. The NO level in the combination group was significantly decreased as compared to DN. This observation might be the result of FA neutralizing NO radical and the reversing effect of ALA on NO- cGMP system as noted in previous studies; hence the decrease in the NO levels [46][49][50].

Membrane-bound enzymes (ATPase) are essential players in maintaining membrane integrity and thereby ionic balance. Na^+/K^+ -ATPase is critical for the membrane potential and many types of transport. In the present study, membrane-bound ATPases (Na^+/K^+ -ATPase, Ca^{2+} -ATPase, and Mg^{2+} -ATPase) were estimated in rat's sciatic nerve homogenate and was found to be significantly altered in STZ treated rats. The alteration in membrane-bound ATPases activities in the study might be due to depletion of the intracellular pool of *myo*-inositol, increased flux through the aldose reductase pathway, and alteration in PKC [51]. Treatment with ALA or FA alone and in combination increased the level of ATPases compared to non-treated DN rats. The results were in line with previous reports [52]. ALA effect on Na^+/K^+ -ATPase may be due to amelioration of nerve *myo*-inositol and taurine depletion, which could reflect on the restoration of Na^+/K^+ -ATPase by Na^+ - dependent uptake [53][54][55].

It was found that ALA increased GLUT4 translocation to the cell membrane and increase glucose uptake in cultural adipose and muscle cells. It also has the capacity to scavenge ROS directly and regenerate endogenous antioxidants [19]. The mechanism of FA action as an antioxidant was due to its resonance stabilization. It neutralizes the free radicals produced by STZ in the pancreases, and it also increases insulin secretion, which helps lowering blood glucose levels. Therefore, due to its antioxidant property, FA has a neuroprotective effect. Therefore, scavenging free radicals reduced the above effects in sciatic pain models.

6. Conclusion

In the present study, the concomitant administration of ALA and FA in diabetic induced neuropathic rats showed better effects compared to single ALA or FA treatments. These protective effects might be due to the potent antioxidant activities of the combined antioxidants, with each one of them affecting an independent pathway.

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