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Acetyl-L-carnitine and α-lipoic acid affect rotenone-induced damage in nigral dopaminergic neurons of rat brain, implication for Parkinson's disease therapy

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ABSTRACT

Although the mechanisms of neurodegeneration in Parkinson's disease are not fully understood, mitochondrial dysfunction, oxidative stress and environmental toxins may be involved. The current research was directed to investigate the protective role of two bioenergetic antioxidants, acetyl-L-carnitine and α-lipoic acid, in rotenone-parkinsonian rats. Ninety six male rats were divided into five groups. Group I is the vehicle-injected group, group II is the disease control group and was injected with six doses of rotenone (1.5 mg/kg/48 h, s.c.). Groups III, IV and V received rotenone in addition to acetyl-L-carnitine (100 mg/kg/day, p.o.), α-lipoic acid (50 mg/kg/day, p.o.) or their combination, respectively. Results showed that rotenone-treated rats exhibited bradykinesia and motor impairment in the open-field and square bridge tests. In addition, ATP level was decreased whereas lipid peroxides and protein carbonyls increased in the striata of rotenone-treated rats as compared to vehicle-treated rats. Treatment with acetyl-L-carnitine or α-lipoic acid improved the motor performance and reduced the level of lipid peroxides in rat brains as compared to rotenone group. Further, ATP production was enhanced along with acetyl-L-carnitine treatments ($p \leq 0.05$). Taken together, our study reinforces the view that acetyl-L-carnitine and α-lipoic acid are promising candidates for neuroprotection in Parkinson's disease.

1. Introduction

Neuropathology of Parkinson's disease (PD) is characterized by loss of dopaminergic (DAergic) neurons in the substantia nigra pars compacta (SNpc) with diminishing DAergic neurotransmission in the terminal region (striatum) (Dauer and Przedborski, 2003). Mitochondrial dysfunction has been implicated in neurodegeneration in PD (Haas et al., 1995; Schapira et al., 1989). Both experimental and epidemiological studies revealed a strong correlation between the disease onset and previous exposure to exogenous mitochondrial toxins (Fleming et al., 2004; Seidler et al., 1996). Where a loss of oxidative metabolism and production of ATP through the electron transport chain can bring about rapid neuronal depolarization and a calcium-mediated cascade of cell death (Sherer et al., 2001).

Rotenone is a naturally occurring insecticide and a specific inhibitor of mitochondrial complex I. Systemic administration of rotenone in low doses (1.5–2.5 mg/kg) has been used to create a chronic progressive animal model of PD (Betarbet et al., 2000). Neurodegeneration in rotenone-induced Parkinsonism is not purely a bio-energetic defect due to complex I inhibition (Sherer et al., 2003). Rotenone causes neurodegeneration also via multiple mechanisms that include moderate inhibition of complex I, oxidative damage (Tada-Oikawa et al., 2003), induction of apoptosis (Samantaray et al., 2007), activation of microglia (Gao et al., 2002), and acceleration of α-synuclein aggregation and fibrillation (Diaz-Corrales et al., 2005). Recently, it was reported that rotenone model is highly reproducible and may provide an excellent tool to test new neuroprotective strategies (Cannon et al., 2009).

Acetyl-L-carnitine is a member of the family of carnitines, a group of natural compounds. It is essential for β-oxidation of fatty acids in mitochondria to generate ATP (Bieber, 1988). It has been reported that acetyl-L-carnitine effectively prevents mitochondrial injury deriving from oxidative damage in vivo (Chang et al., 2002). Due to their intrinsic interaction with the bioenergetic processes, they play an important role in mitochondrial-related disorders. It was suggested that carnitines may have neuroprotective actions in conditions of mitochondrial dysfunction and oxidative stress and possibly in neurodegenerative disorders, such as PD (Beal, 2004). α-Lipoic acid is another antioxidant that is found naturally in mitochondria and involved in mitochondrial ATP production. Aside from their co-enzymatic roles, both α-lipoic acid and dihydrolipoic acid exhibit antioxidant activity and can readily cross the blood brain barrier (Smith et al., 2004). Its reduced form can recycle other antioxidants such as vitamin C, vitamin E and glutathione

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(Jones et al., 2002). All the above-mentioned properties makes acetyl-L-carnitine and α-lipoic acid very promising agents for treatment of neurological diseases whose etiology is related to mitochondrial dysfunction and oxidative stress (Farr et al., 2003).

Using the hypothesis that mitochondrial dysfunction and oxidative injury underlie neurodegeneration in PD; the inclusion of metabolic modifiers may provide an alternative and early intervention approach. The present study was designed to characterize the protective effect of the bioenergetic antioxidants, acetyl-L-carnitine and α-lipoic acid, in the rotenone model of PD. These agents may provide a potential neuroprotective therapy aimed for use as pharmacologic interventions to halt the progressive nature of PD.

2. Materials and methods

2.1. Chemicals and drugs

Rotenone (Sigma-Aldrich, MO, USA) was dissolved in 1:1 (v/v) dimethylsulfoxide (DMSO, Sigma-Aldrich, MO, USA) and polyethylene glycol (PEG-300; Sigma-Aldrich, MO, US). Acetyl-L-carnitine powder (Eva Pharmaceutical Co., Giza, Egypt) was dissolved in distilled water. α-Lipoic acid powder (Eva Pharmaceutical Co., Giza, Egypt) was dissolved in 5% Tween-80 solution. Monoclonal rabbit tyrosine hydroxylase antibodies were purchased from R&D systems® (Minneapolis, USA). Broad spectrum LAB-SA detection system from Invitrogen (Cat# 85-9043) was used to visualize any antigen-antibody reaction in the tissues. 3,3′-diaminobenzidine (DAB) was purchased from Sigma-Aldrich® (MO, USA).

2.2. Animals

Male albino rats were used in the present study. Their weight ranged between 200 and 250 g. Rats were housed in groups of four in stainless steel cages under hygienic controlled laboratory conditions (reversed light/dark cycle, temperature equals 25 °C and 55% relative humidity). Water and food pellets were given *ad libitum*. All the experimental protocols were approved by the Institutional Animal Care and Use Committee at the Faculty of Pharmacy, Suez Canal University.

2.3. Experimental design

Ninety six rats were randomly divided into five groups; sixteen rats each (except rotenone group, which started with thirty two rats). Rotenone (1.5 mg/kg/48 h/6 doses, s.c.) was employed to induce experimental Parkinsonism (Thiffault et al., 2000). Rotenone was prepared to be injected in a volume of 1 ml/kg body weight. However, vehicle-treated group received six subcutaneous injections of the vehicle (1:1, DMSO/PEG-300) on corresponding days in a volume of 1 ml/kg. The other three groups received rotenone in addition to daily protective doses of acetyl-L-carnitine (100 mg/kg/day/12 doses, p.o.) (Pisano et al., 2003), α-lipoic acid (50 mg/kg/day/12 doses, p.o.) (Maritim et al., 2003) or their combination in the same afore-mentioned doses. Control rats were treated daily with 1 ml/kg dose of distilled water. All the antioxidant therapies were administered using a feeding tube.

In another set of the experiment, twenty four rats were allocated into three groups. All of the groups were injected subcutaneously with the vehicle (1:1, DMSO/PEG-300). The first group received acetyl-L-carnitine, the second group received α-lipoic acid whereas, the third group received a combination of them in the same aforementioned doses. These three groups served as respective control groups; however, their results were not demonstrated in the illustrations to minimize overcrowding and to facilitate the interpretation of the results.

2.4. Functional assessment

Twenty four hours after the last injection of rotenone (day 12), rats were screened for motor impairments using the open-field test and square bridge test.

2.4.1. Open-field test

The open-field arena, with the measurements 113 × 113 × 44 cm, was made of dark glass. The floor was painted with white lines that formed a 5 × 5 cm pattern (Correa et al., 2004). Rats were introduced individually in the open-field arena and the locomotor behavior was video-recorded for 5 min. The film was later observed by a trained observer who was blinded to the treatment groups. The horizontal movement (ambulation frequency, number of stops and inactive sitting) and the vertical movement (rearing frequency) were registered. Furthermore, activity index (the number of squares passed in a locomoting interval = total number of squares divided by the total number of stops) was calculated for each animal (El-Awady et al., 2007).

2.4.2. Square bridge test

The square bridge assesses balance and motor coordination. A wooden bridge, 3 cm width and 60 cm long, between two wooden platforms was used. It was suspended 60 cm above a thick foam pad. A rat was placed in the middle of the bridge. The latency to fall was the number of seconds, to a maximum of 120 s, in which the rat remained on the bridge before falling. If the rat reached the platform before 120 s, the latency to fall recorded as 120 s (Markowska et al., 1990).

2.5. Processing of the brains

After assessment of the motor performance, rats were anesthetized by injection of ketamine (80 mg/kg, i.p.) and killed by decapitation. Brains were quickly dissected and washed with ice-cold saline. One hemisphere from each brain was weighed and rapidly frozen in liquid nitrogen. After that, the striata were isolated and weighed. One part from each striata were then homogenized in Tris buffer (10 mM Tris HCl, 1 mM EDTA, 0.32 M sucrose, pH = 7.8) as 10% (w/v) using a teflon homogenizer (Glas Col homogenizer system, Vernon hills, USA). The homogenate was sonicated and centrifuged at 20,000 × *g* for 10 min, then, supernatant (supernatant A) was kept at −80 °C until determination of the biochemical markers of oxidative stress. Other parts of the striata were used for determination of dopamine, extraction of ATP or extraction of mitochondrial DNA as described later.

The second hemisphere of each brain was rinsed with phosphate-buffered saline (PBS), fixed using 4% paraformaldehyde in 0.1 M phosphate buffer, pH = 7.2 overnight and then embedded with paraffin. All paraffin-embedded tissues were sectioned at 4 μm thicknesses at the SN and left to dry overnight at 37 °C. Sections were then deparaffinized, rehydrated and prepared for histopathological staining with hematoxylin & eosin (H&E) or cresyl violet for Nissl-staining or for immunohistochemical staining for tyrosine hydroxylase (TH).

2.6. Biochemical analyses in the brain homogenate

2.6.1. Determination of striatal dopamine level

2.6.1.1. Sample preparation. Frozen tissue was homogenized for 20 s using a tapered motorized pestle in 500 ml of ice-cold mobile phase (50 mM sodium acetate buffer, pH 4.3, containing 35 mM citric acid, 0.13 mM disodium EDTA, 0.45 mM 1-octansulphonate and 10% methanol) containing known amount of 3,4-dihdroxybenzylamine (DHBA) as an internal standard. The homogenized tissue suspension was centrifuged at 10,000 × *g* for 1 min, and the supernatant

was removed and filtered through a 0.2 mm Teflon syringe filter for immediate HPLC analysis.

2.6.1.2. HPLC analysis. Levels of dopamine were measured in the reverse-phase HPLC coupled to ECD. First, the mobile phase was oxidized by a guard cell (V = +/450 mV). The guard cell serves to perform a partial clean up of the mobile phase prior to sample injection. The samples were introduced into the autosampler carrousel (CMA 200, CMA Microdialysis, Solna, Sweden) with the flow rate of mobile phase being 0.8 ml/min (ESA 2200 Analytic Pump, Bischoff, Leonberg, Germany). Separation of the neurotransmitters was carried out by means of reverse-phase column (Prontosil 33 ×/4.0 mm, pore diameter: 3.0 mm; Bischoff). The substrates were detected by an ESA detector (ESA Coulochem 5100A, Analytical cell ESA 5010). Typical values of applied potential used in the present experiment were +/20 mV at Electrode 1 and +/320 mV at Electrode 2.

2.6.2. Determination of ATP

ATP was extracted from the tissues with 10 ml of 0.6 M perchloric acid in the ice bath for 1 min by the method of Yang et al. (2002). The extraction mixture was centrifuged for 10 min at 6000×*g* and 4 °C, and 6 ml of the supernatant was taken and quickly neutralized to pH = 6.5 with 1 M KOH solution. The neutralized supernatant was then allowed to stand for 30 min in an ice bath to precipitate most of the potassium perchlorate, which was removed by paper filtration. The filtrate solution was filtered again through a 0.45 mm filter. The final filtrate (supernatant B) solution was made up to 8 ml and then stored at −30 °C prior to the analysis. The concentration of ATP was determined using the external standard method of Liu H et al. (2006). Data were expressed as means of six replicate determinations.

2.6.3. Determination of malondialdehyde and protein carbonyls

Tissue malondialdehyde (MDA) were estimated according to the spectrophotometric method of Ohkawa et al. (1979); using 1,1,3,3-tetramethoxypropane as a standard. The assay of protein carbonyls depends on the reaction of protein carbonyl groups with 2,4-dinitrophenylhydrazine to yield a colored product (Levine et al., 1990).

2.6.4. Determination of reduced glutathione (GSH)

Concentration of total glutathione (GSH and GSSG) and oxidized glutathione (GSSG) was measured spectrophotometrically using commercial kits according to the instructions of the manufacturer (Griffith, 1980; Tietze, 1969). Total GSH content was expressed in μM per g protein.

2.6.5. Determination of superoxide dismutase and catalase activity

Superoxide dismutase (SOD) activity was estimated as illustrated previously by Nishikimi et al. (1972). Catalase (CAT) activity was assessed as reported by Aebi (1984). Protein content was determined following the colorimetric method of Lowry et al. (1951). The absorbencies were measured using a UV–visible spectrophotometer (UV-1601PC, Shimadzu, Japan).

2.7. Assessment of mitochondrial DNA integrity

To investigate the possible involvement of mtDNA injury in the pathogenesis of rotenone-induced injury of the brain, mtDNA samples isolated from brain tissues were analyzed.

2.7.1. Isolation of mitochondria

Mitochondria were extracted by differential centrifugations following Chappel and Hansford (1969). Tissues were homogenized in 0.25 M sucrose in 0.7 M Tris–HCl buffer (pH = 7.4) at 1 g tissue 9 ml of Tris-sucrose. EDTA was added to aid disruption of cells. Tissue homogenate was spined at 2500×*g* for 10 min to remove nuclei and unbroken cells. Supernatant fluid was decanted into centrifuge tubes and spined at 10,000×*g* for 10 min to form primary mitochondrial pellet. Supernatant fluid was decanted and the pellet is gently resuspended in 10 ml Tris-sucrose for washing. Pellet was recentrifuged and supernatant fluid was decanted. This washing cycle was repeated several times to improve degree of mitochondrial purity. Final mitochondrial pellet is resuspended (1 ml Tris-sucrose/1 g of original sample).

2.7.2. Isolation of mitochondrial DNA (mtDNA)

Mitochondrial DNA (mtDNA) was isolated by using mitDNA isolation kit (Bio Vision, USA) (Chang et al., 2002). The mtDNA samples were subjected to 1% agarose gel electrophoresis at 4 V/cm using TAE solution (40 mM Tris-acetate, pH = 8.0, 1 mM EDTA) as a running buffer. The gel was stained with 0.5 μg/ml ethidium bromide and photographed by G:Box Gel Documentation system (Syngene, USA). Furthermore, mtDNA quantity and purity was determined using NanoDrop™ 1000 spectrophotometer V3.7 (Thermo Fisher Scientific Inc., Wilmington, DE, USA).

2.8. Histopathology and immunohistochemistry

Sections were fixed in a 65 °C oven for 1 h and then the slides were placed in a coplin jar filled with 60 ml of triology (Cell Marque®, CA-USA) working solution and the jar is securely positioned in an autoclave. The autoclave was adjusted so that temperature at 120 °C and maintained for 15 min after which pressure is released and the coplin jar is removed to allow slides to cool for 30 min. Sections were then washed and immersed in TBS to adjust the pH, this is repeated between each step of the immunohistochemical procedure. Quenching endogenous peroxidase activity was performed by immersing slides in 3% hydrogen peroxide for 10 min. Background staining was blocked by putting 2–3 drops of 10% goat non immune serum blocker on each slide and incubating them in a humidity chamber for 10 min. Without washing, excess serum was drained from each slide and 2–3 drops of the ready to use rabbit monoclonal tyrosine hydroxylase primary antibodies (R&D systems®). Then, slides were incubated in the humidity chamber for 1 h. Henceforward, biotinylated secondary antibody was applied on each slide for 20 min followed by another 20 min incubation with the enzyme conjugate. DAB chromogen was prepared and 2–3 drops were applied on each slide for 2 min. After that, DAB was rinsed and the slides were counterstaining with Mayer's hematoxylin and cover slipping was performed as the final step before slides were examined under a light microscope (Olympus CX21, Japan).

2.9. Neuronal cells quantification and image analysis

Neuronal cells stained with H&E or Nissl-stained neurons were quantified stereologically on three regularly spaced sections covering the entire surface of the SNpc as described (Hoglinger et al., 2004). Each midbrain section was viewed at low power (×10 magnification) and the SNpc was outlined. The cell numbers were counted at high power (×40 magnification). Neurons were counted only when their nuclei were clearly visualized within one focal plane. Nissl-stained neurons were differentiated from other non-neuronal cells by the clearly defined nucleus, cytoplasm and a prominent nucleolus (Vijitruth et al., 2006). After determination of the cell number in each slide, the percentage increase in the cell number relative to rotenone group was calculated and compared.

The SNpc slides were examined to measure the TH immunopositive cells using a computer assessed image analysis system "ImageJ 1.45F" (National Institute of Health, USA) as described previously (Bezard et al., 2001). The boundaries of the SNpc were chosen on three consecutive sections corresponding to a representative median plane of the SNpc by examining the size and shape of the different TH-immunoreactive neuronal groups. Cells that were clearly stained for TH with a visible nucleus were counted. After determination of

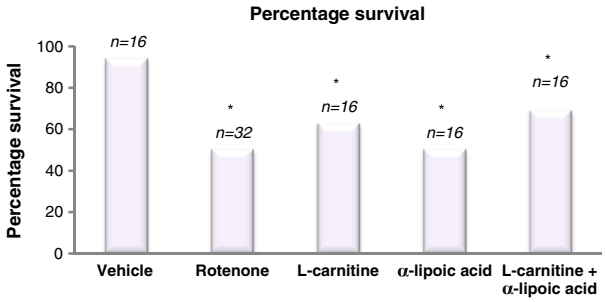

Fig. 1. Percentage survival in the experimental groups. Rotenone group (1.5 mg/kg/48 h/6 doses, s.c.) showed 50% survival which is significantly lower than the percentage survival observed in vehicle group. Treatment with acetyl-L-carnitine (100 mg/kg/day/12 doses, p.o.), α-lipoic acid (50 mg/kg/day/12 doses, p.o.) or their combination did not improve the percentage survival as compared to rotenone group. Results are expressed as means and analyzed using Chi square test. $^{*}p \leq 0.05$ compared to vehicle group.

the TH positive cells in all tissue sections, the percentage increase in TH positive cells relative to rotenone group was calculated as percentage. All histological examinations were performed by an experienced pathologist who was blinded to the experiment groups.

2.10. Electron microscopy

For electron microscopic study, a piece of tissues was trimmed into few mm pieces in the presence of 2.5% of glutaraldehyde solution in which it was left for 24 h. Specimens were post-fixed in 1% osmium tetroxide, dehydrated in ascending grades of cold ethanol, cleared in propylene oxide, infiltrated and embedded in Spurr's resin at temperature of 60 °C for 48 h. Ultrathin sections (50 nm) were prepared on Leica Ultramicrotome (Ultra Cut) and subsequently stained for 10 min with aqueous uranyl acetate and for 10 min in lead citrate (Hayat, 1986). The substantia nigra sections were then examined and photographed using JEOL 1200EX II transmission electron microscope (Electron Microscope Unit, Faculty of Science, Ein Shams University) at 80 kV as described previously (Hoglinger et al., 2004).

2.11. Statistical analysis

Results were collected, tabulated and expressed as mean ± S.E.M. Quantitative measurements were analyzed using one-way analysis of variance, ANOVA, followed by Bonferroni's multiple comparisons test. However, qualitative measurements were analyzed using Chi square test. All statistical tests were done employing SPSS program version 17 (SPSS Software, SPSS Inc., Chicago, USA). Differences were considered significant at $p \leq 0.05$.

3. Results

In the present study, repeated administration of rotenone (1.5 mg/kg/48 h/6 doses, s.c.) in rats produced functional impairment in the form of bradykinesia in the open-field test and motor in-coordination in square bridge test. This was accompanied by biochemical deficits and histopathological changes in the SNpc dopaminergic neurons.

3.1. Percentage survival

In the current study, repeated treatment with rotenone resulted in death in rats. Death often occurred within 2 h after injection of rotenone. At the end of the experiment, percentage survival in the rotenone group was found to reach 50% (16 out of 32). This value was significantly lower than that observed in vehicle group, 93.75% (15 out of 16, Fig. 1). Treatment with the current antioxidants did not improve the survival of rats as compared to rotenone group ($p \leq 0.05$, Fig. 1). Rats dead during the course of the experiment were excluded from statistical analysis.

3.2. Assessment of the motor function

Treatment with acetyl-L-carnitine, α-lipoic acid or their combination significantly, to variable degrees, ameliorated the behavioral deficits due to administration of rotenone (Fig. 1). However, treatment with these agents in vehicle-treated rats did not induce a significant change in motor performance as compared to vehicle-treated controls (data not shown).

3.2.1. Open-field test

3.2.1.1. Ambulation frequency. The present results revealed a significant decrease in ambulation frequency in rotenone group as compared to vehicle group (Table 1). Acetyl-L-carnitine, α-lipoic acid and their combination significantly increased the ambulation frequency as compared to rotenone group. The combination group showed higher ambulation frequency in comparison to either acetyl-L-carnitine or α-lipoic acid groups ($p \leq 0.05$, Table 1).

3.2.1.2. No. of stops. Rotenone-treated rats showed lower no. of stops as compared to vehicle-treated rats. The implemented agents produced a significant increase in the number of stops in comparison to rotenone group ($p \leq 0.05$, Table 1).

3.2.1.3. Activity index. Activity index calculated in rotenone group was significantly lower than the value calculated in vehicle group (Table 1). Treatment with acetyl-L-carnitine, α-lipoic acid or their combination significantly elevated the activity index for rats in the

Table 1
The open-field test and square bridge test performed for the experimental groups.

Groups	Open-field test					Square bridge test
	Ambulation frequency	No. of stops	Activity index	Rearing frequency	Inactive sitting duration	Latency to fall
Vehicle (n = 15)	324 ± 29	20 ± 1	16 ± 1	18 ± 2	111 ± 10	118 ± 1.4
Rotenone (n = 16)	42 ± 7*	7 ± 1*	5.3 ± 1*	4 ± 1*	420 ± 32*	34 ± 6*
Acetyl-L-carnitine (n = 10)	217 ± 24*a	15 ± 1*a	15 ± 1.4a	15 ± 2a	184 ± 15*a	60 ± 5.8*a
α-Lipoic acid (n = 8)	144 ± 30*a	14 ± 3*a	10 ± 1*a,b	6 ± 1*	349 ± 29*a,b	45 ± 3.6*
Acetyl L-carnitine + α-Lipoic acid (n = 11)	298 ± 28a,b,c	19 ± 2a	15.6 ± 2a	16 ± 2ac	145 ± 15a,b,c	85 ± 6a,b,c

Rats were screened for motor impairment in the open-field test and square bridge test at day 12. Rotenone group (1.5 mg/kg/48 h/6 doses, s.c.) showed decreased ambulation frequency, No. of stops, activity index, rearing frequency and inactive sitting duration in the open-field test and decreased falling time in the square bridge test. Co-treatment with acetyl-L-carnitine (100 mg/kg/day/12 doses, p.o.), α-lipoic acid (50 mg/kg/day/12 doses, p.o.) or their combination improved the motor performance of the rats. Results are expressed as mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's multiple comparisons test.

* $p \leq 0.05$ compared to vehicle group.
[a] $p \leq 0.05$ compared to rotenone group.
[b] $p \leq 0.05$ compared to acetyl-L-carnitine group.
[c] $p \leq 0.05$ compared to α-lipoic acid group.

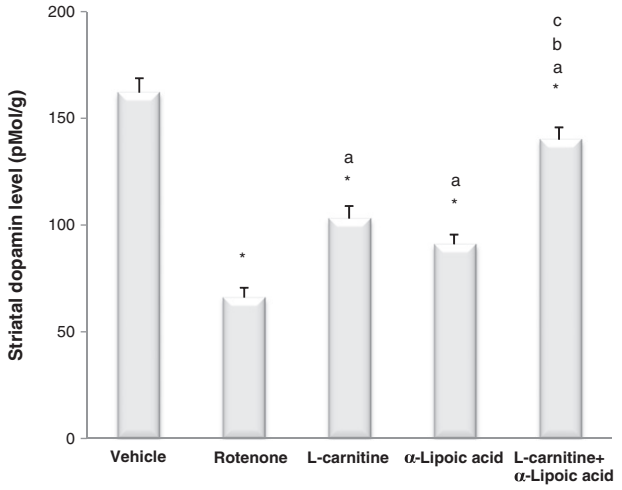

Fig. 2. Striatal dopamine level in the experimental groups. Rotenone group (1.5 mg/kg/48 h/6 doses, s.c.) showed a significant decreased in dopamine level compared to vehicle group. Treatment with acetyl-L-carnitine (100 mg/kg/day/12 doses, p.o.), α-lipoic acid (50 mg/kg/day/12 doses, p.o.) or their combination improved the striatal dopamine level as compared to rotenone group. Results are expressed as mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's post-hoc test. $^{*}p \leq 0.05$ compared to vehicle group. $^{a}p \leq 0.05$ compared to rotenone group. $^{b}p \leq 0.05$ compared to acetyl-L-carnitine group. $^{c}p \leq 0.05$ compared to α-lipoic acid group.

open-field test as compared to rotenone group. The activity index in groups treated with acetyl-L-carnitine alone or in combination with α-lipoic acid was not significantly different from the vehicle group ($p \leq 0.05$, Table 1).

3.2.1.4. Rearing frequency. The present results showed that the rearing frequency was significantly lower in rotenone group in comparison to the vehicle group (Table 1). In addition, the current results indicated that acetyl-L-carnitine alone or in combination with α-lipoic acid significantly increased the rearing frequency as compared to rotenone group ($p \leq 0.05$, Table 1). In contrast, treatment with α-lipoic acid failed to produce a similar effect.

3.2.1.5. Inactive sitting. The duration of inactive sitting was significantly high in rotenone-treated animals as compared to vehicle-treated control. Different treatment regimens significantly lessened the inactive sittings in comparison to rotenone group ($p \leq 0.05$, Table 1).

3.2.2. Square bridge test

In the present study, rotenone group showed rapid fall from the square bridge test as compared to the vehicle group which remained longer on the wooden bridge (up to 120 s, Table 1). Treatment with acetyl-L-carnitine alone or in combination with α-lipoic acid protected the rats from falling in the square bridge test as compared to rotenone group ($p \leq 0.05$, Table 1).

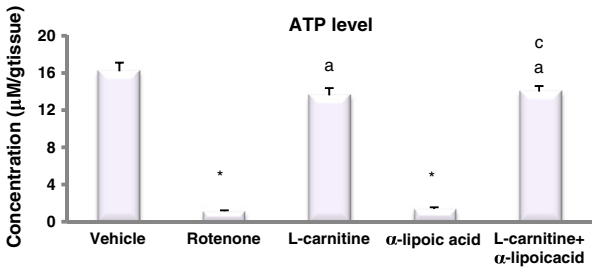

Fig. 3. ATP level in the striata of the experimental groups. Rotenone group (1.5 mg/kg/48 h/6 doses, s.c.) showed lower level of ATP as compared to vehicle group. Treatment with acetyl-L-carnitine (100 mg/kg/day/12 doses, p.o.) alone or in combination with α-lipoic acid restored ATP level. Results are expressed as mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's post-hoc test. $^{*}p \leq 0.05$ compared to vehicle group. $^{a}p \leq 0.05$ compared to rotenone group. $^{b}p \leq 0.05$ compared to acetyl-L-carnitine group. $^{c}p \leq 0.05$ compared to α-lipoic acid group.

3.3. Biochemical measurements

3.3.1. Dopamine level

Striatal dopamine level was significantly decreased in rotenone group as compared to vehicle-treated group. Treatment with acetyl-L-carnitine, α-lipoic acid, or their combination increased the striatal dopamine level in comparison to rotenone group (Fig. 2). However, treatment with these agents in vehicle treated rats did not increase striatal dopamine level (data not shown).

3.3.2. ATP level

In the present study, striatal ATP level was decreased in the brains of rotenone group as compared to vehicle group. Treatment with acetyl-L-carnitine significantly increased ATP level as compared to rotenone group. α-Lipoic acid had no significant effect on striatal ATP level (Fig. 3). Treatment with these agents in vehicle-treated rats did not change striatal ATP level as compared to vehicle-treated controls (data not shown).

3.3.3. Oxidative stress parameters

In the present study, monotherapy with acetyl-L-carnitine or α-lipoic acid or their combination significantly ameliorated some of the oxidative stress parameters as compared to rotenone treated rats. However, treatment with these agents in vehicle-treated rats did not induce a significant change in as compared to vehicle-treated controls (data not shown).

3.3.3.1. Lipid peroxides and protein carbonyls. Rotenone group showed approximately five-fold higher concentration of tissue MDA as compared to the vehicle group ($p \leq 0.05$). In comparison with rotenone group, both of the bioenergetic antioxidant treatments induced significant reductions in the elevated MDA level ($p \leq 0.05$, Fig. 4). Similarly, rotenone-treated rats exhibited a three-fold increase in the tissue protein carbonyls level as compared to the vehicle group. Protein carbonyls concentration was not significantly influenced by any of the single treatments, however, the combination of acetyl-L-carnitine and α-lipoic acid rendered the protein carbonyls level marginally lower than the rotenone group ($p \leq 0.05$, Fig. 4).

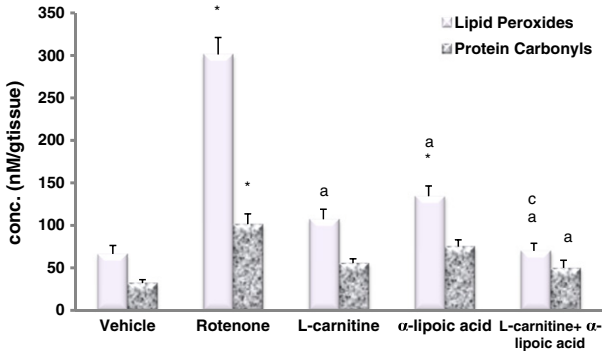

Fig. 4. Lipid peroxides and protein carbonyls level in the striata of the experimental groups. Rotenone group (1.5 mg/kg/48 h/6 doses, s.c.) showed higher levels of lipid peroxides and protein carbonyls as compared to vehicle group. Treatment with acetyl-L-carnitine (100 mg/kg/day/12 doses, p.o.) or α-lipoic acid (50 mg/kg/day/12 doses, p.o.) suppressed lipid peroxidation without influence on protein carbonyls production. The combination of them influenced lipid peroxides as well as protein carbonyls. Results are expressed as mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's post-hoc test. $^{*}p \leq 0.05$ compared to vehicle group. $^{a}p \leq 0.05$ compared to rotenone group. $^{b}p \leq 0.05$ compared to acetyl-L-carnitine group. $^{c}p \leq 0.05$ compared to α-lipoic acid group.

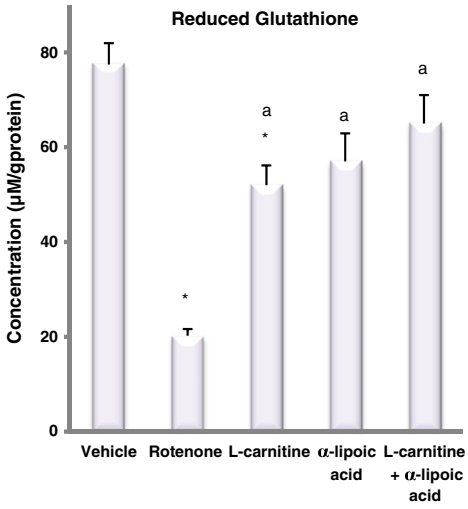

Fig. 5. Reduced glutathione (GSH) in the striata of the experimental groups. Rotenone group (1.5 mg/kg/48 h/6 doses, s.c.) showed low GSH level as compared to vehicle group. Co-treatment with acetyl-L-carnitine (100 mg/kg/day/12 doses, p.o.), α-lipoic acid (50 mg/kg/day/12 doses, p.o.) or their combination ameliorated GSH level. Results are expressed as mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's post-hoc test. $^{*}p \leq 0.05$ compared to vehicle group. $^{a}p \leq 0.05$ compared to rotenone group. $^{b}p \leq 0.05$ compared to acetyl-L-carnitine group. $^{c}p \leq 0.05$ compared to α-lipoic acid group.

3.3.3.2. Glutathione concentration. Rotenone administration induced a significant decrease in the tissue GSH content as compared to the vehicle group ($p \leq 0.05$, Fig. 5). Both acetyl-L-carnitine and α-lipoic acid, alone or in combination, induced significant increases in tissue GSH in comparison with rotenone group ($p \leq 0.05$, Fig. 5).

3.3.3.3. Superoxide dismutase and catalase activity. On the other hand, SOD and CAT activity was found to decrease in rotenone-treated rats as compared to vehicle-treated rats ($p \leq 0.05$, Fig. 6). Acetyl-L-carnitine group had a significant increase in SOD activity as compared to rotenone group; however, α-lipoic acid group did not show a similar effect. Both of the antioxidants failed to produce a significant increase in CAT activity. On the other hand, the combination of acetyl-L-carnitine and

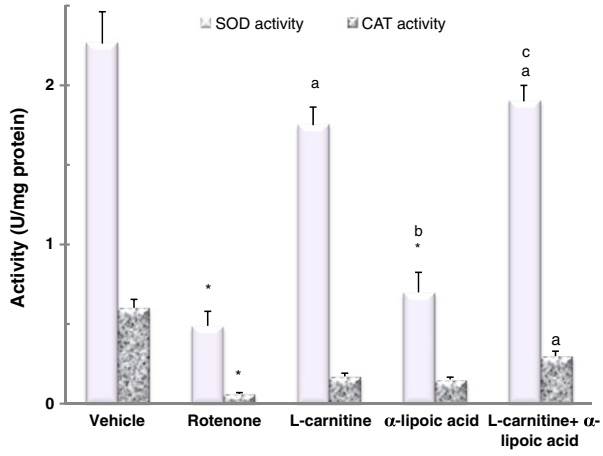

Fig. 6. Activity of SOD and CAT in the striata of the experimental groups. Rotenone group (1.5 mg/kg/48 h/6 doses, s.c.) showed decreased activities of SOD and CAT as compared to vehicle group. Treatment with acetyl-L-carnitine (100 mg/kg/day/12 doses, p.o.) alone or in combination with α-lipoic acid (50 mg/kg/day/12 doses, p.o.) improved SOD activity. The combination therapy enhanced CAT activity. SOD: superoxide dismutase, CAT: catalase. Results are expressed as mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's post-hoc test. $^{*}p \leq 0.05$ compared to vehicle group. $^{a}p \leq 0.05$ compared to rotenone group. $^{b}p \leq 0.05$ compared to acetyl L-carnitine group. $^{c}p \leq 0.05$ compared to α-lipoic acid group.

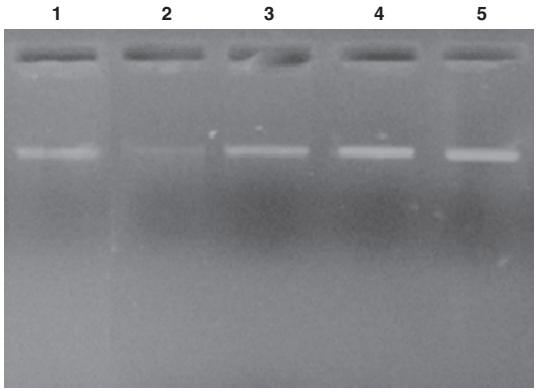

Fig. 7. Effect of acetyl-L-carnitine (100 mg/kg/day/12 doses, p.o.), α-lipoic acid (50 mg/kg/day/12 doses, p.o.) on rotenone-induced mtDNA damage in the striata of the experimental groups. Lane 1, shows mtDNA isolated from α-lipoic acid group, Lane 2, mtDNA sample from the rotenone group; lane 3, mtDNA sample isolated from acetyl-L-carnitine group; Lane 4, intact mtDNA isolated from vehicle group; Lane 5, shows mtDNA samples isolated from rats treated with a combination of acetyl-L-carnitine and α-lipoic acid.

α-lipoic acid enhanced SOD and CAT activities as compared to rotenone group ($p \leq 0.05$, Fig. 6).

3.4. Integrity of mitochondrial DNA

In vehicle-treated rats, intact form of mitochondrial DNA was electrophoresed as a major band of approximately 16.5 kb (lane 4, Fig. 7). On the other hand, administration of rotenone markedly decreased the amounts of intact mtDNA (lane 2, Fig. 7). However, mitochondrial DNA obtained from animals treated with acetyl-L-carnitine (lane 3, Fig. 7), α-lipoic acid (lane 1, Fig. 7) or their combination (lane 5, Fig. 7) was electrophoresed in its intact form (single band). This suggested that all the implemented agents markedly improved rotenone-induced injury to mtDNA.

3.5. Mitochondrial DNA (mtDNA) quantity

In rotenone-treated group, a significant decline was observed in the content of mitochondrial DNA, as compared to that of control group ($p \leq 0.05$, Table 2). However, normalization of the mtDNA content was observed upon supplementation of acetyl-L-carnitine alone or in combination with α-lipoic acid. Furthermore, administration of α-lipoic acid partially reversed the rotenone-induced decline in mtDNA content in the tissue homogenates (Fig. 7). Hence, the current data emphasized that rotenone-induced mtDNA damage was significantly decreased upon administration of the current treatments, suggesting a possible protective potential. All the implemented agents did not induce a significant change in mtDNA content as compared to vehicle-treated controls (data not shown).

Table 2
The concentration of mitochondrial DNA (mtDNA) extracted from brain tissues for the experimental groups.

Groups	mtDNA concentration (ng/µl)
Vehicle *(n = 15)*	112.5 ± 2.75
Rotenone *(n = 16)*	11.9 ± 0.81*
Acetyl-L-carnitine *(n = 10)*	105.8 ± 4.34
α-lipoic acid *(n = 8)*	68.8 ± 2.35*a,b
Acetyl-L-carnitine + α-lipoic acid *(n = 11)*	110.5 ± 3.42a,c

Results are expressed as mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's multiple comparisons test.
* $p \leq 0.05$ compared to vehicle group.
[a] $p \leq 0.05$ compared to rotenone group.
[b] $p \leq 0.05$ compared to acetyl-L-carnitine group.
[c] $p \leq 0.05$ compared to α-lipoic acid group.

3.6. Histopathological evaluation and immunostaining

Histological assessment demonstrated that vehicle-treated rats showed normal SNpc neurons. On the other hand, the SNpc of rotenone-treated rats showed marked neuronal degeneration (Fig. 8-A). The mean number of SNpc neurons in was higher in vehicle group in comparison to rotenone group. Single or combined therapy with acetyl-L-carnitine and α-lipoic acid showed higher percentage increases in number of SNpc dopaminergic neurons as compared to rotenone group (Fig. 8-B). Nissl staining showed a similar trend and revealed that vehicle treated rats showed a high number of Nissl stained neurons in the SNpc, whereas, rotenone treated rats showed lower number of Nissl stained neurons (Fig. 9-A). Monotherapy with acetyl-L-carnitine or α-lipoic acid as well as their combined

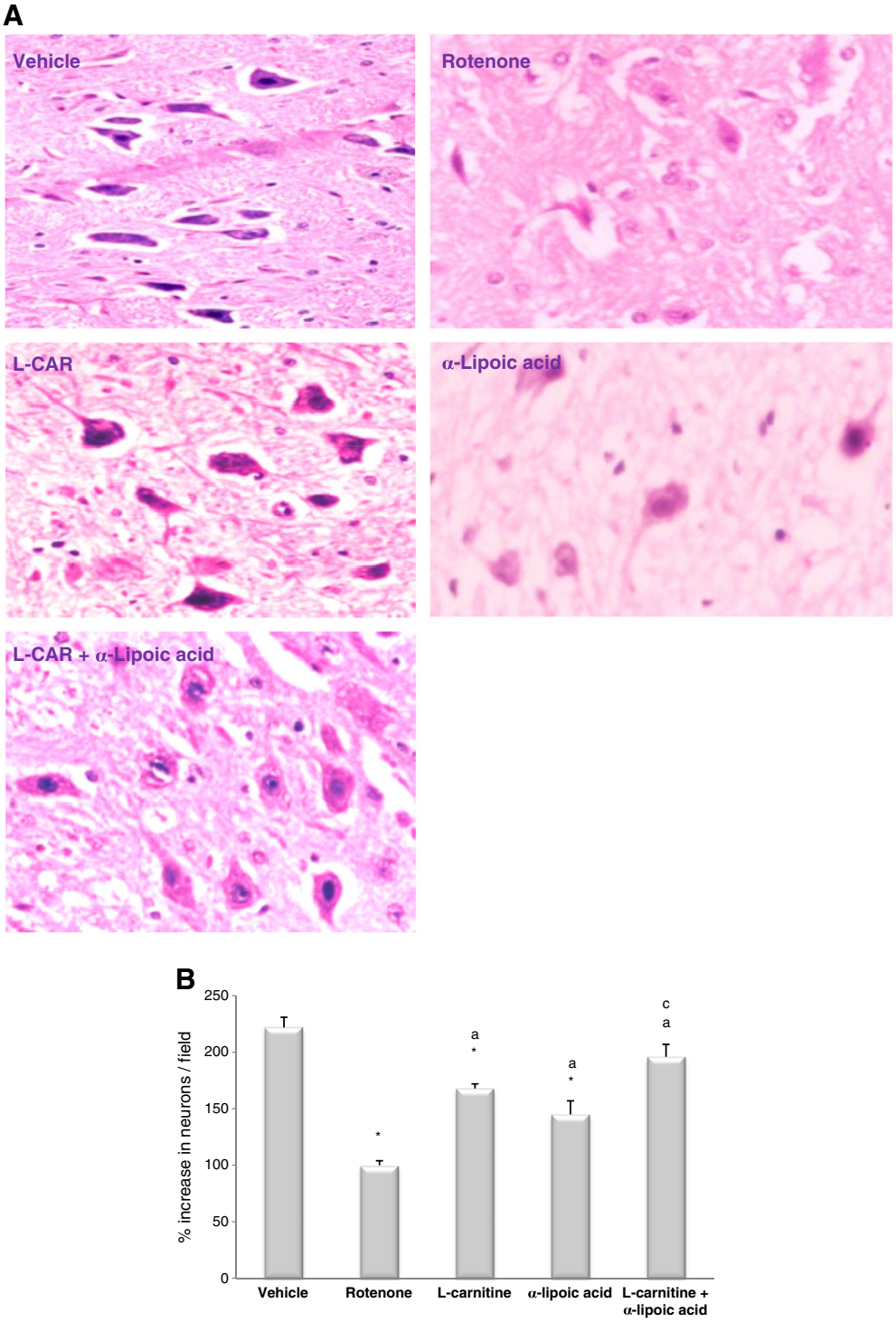

Fig. 8. A — The histological changes in the brain of the experimental groups. A — Histological sections in the substantia nigra of a vehicle-treated rat showing a number of intact neurons with visible nuclei. Nigral neurons in rotenone-treated rats (1.5 mg/kg/48 h/6 doses, s.c.) shows indistinct neuronal boundaries and invisible nuclei. Treatment with acetyl-L-carnitine, α-lipoic acid or their combination significantly increased the number of SNpc neurons (H&E×240). B — Percentage increase in the number of dopaminergic neurons in the SNpc in the experimental groups. Results are expressed as mean ± SEM. $^{*}p \leq 0.05$ compared to vehicle group. $^{a}p \leq 0.05$ compared to rotenone group. $^{b}p \leq 0.05$ compared to acetyl-L-carnitine group. $^{c}p \leq 0.05$ compared to α-lipoic acid group.

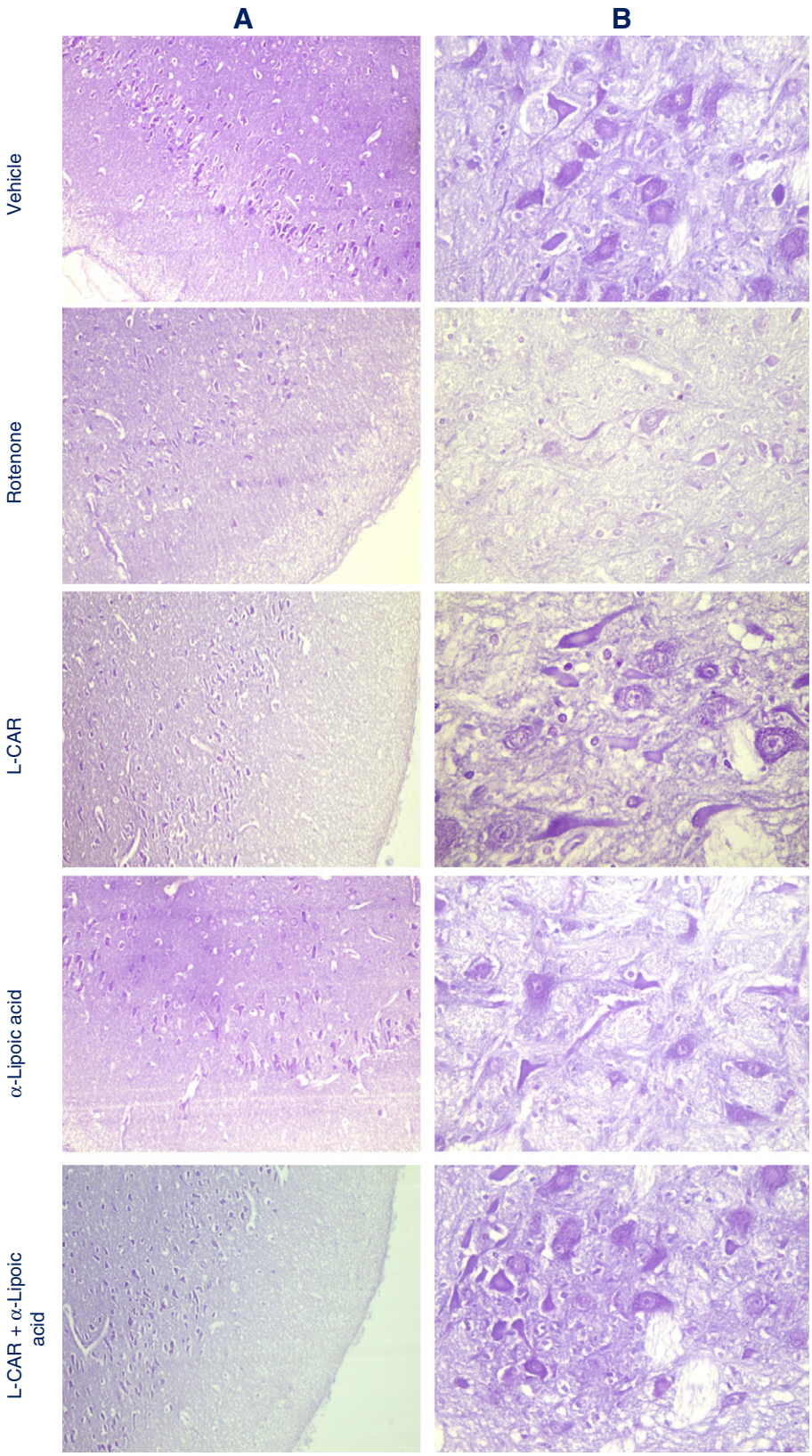

Fig. 9. Nissl staining for in the experimental groups. A and B — Vehicle treated group showed higher number of Nissl stained neurons whereas, rotenone treated rats showed lower number of Nissl stained neurons. Monotherapy with acetyl-L-carnitine or α–lipoic acid and the combination therapy increased the number of Nissl positive neurons in the SNpc (Nissl stain (A) ×20 and (B) ×630 magnification). C — Percentage increase in the number of Nissl stained dopaminergic neurons in the SNpc in the experimental groups. Results are expressed as mean ± S.E.M. $^{*}p \leq 0.05$ compared to vehicle group. $^{a}p \leq 0.05$ compared to rotenone group. $^{b}p \leq 0.05$ compared to acetyl-L-carnitine group. $^{c}p \leq 0.05$ compared to α-lipoic acid group.

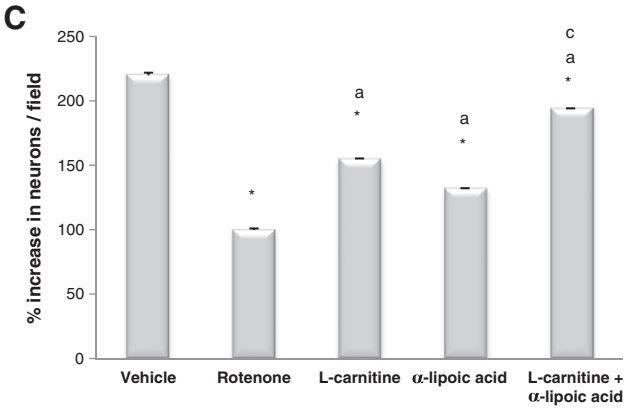

Fig. 9 (*continued*).

therapy significantly increased the number of Nissl stained neurons (Fig. 9-B).

Immunostaining for TH indicated that the administration of rotenone significantly decreased the number of TH immunostained dopaminergic neurons in the SNpc as compared to vehicle control (Fig. 10-A). Treatment with acetyl-L-carnitine, α-lipoic acid or their combination increased TH immunostaining in SNpc dopaminergic neurons as compared to rotenone group (Fig. 10-B). Regarding all the histopathological assessments, monotherapy with acetyl-L-carnitine or α-lipoic acid as well as their combination in the vehicle treated rats did not show differences from vehicle treated controls (data not shown).

Electron microscopic examination of the control ultrathin sections revealed that the neurons in vehicle group showing elongated mitochondria (Fig. 11-A) and on higher magnification the mitochondria appeared to have cristae (Fig. 11-A). The neurons in rotenone group have mitochondria that appeared elongated and cloudy (Fig. 11-B) while the cristae were destructed and disappeared (Fig. 11-B). On the other hand the neurons of in acetyl-L-carnitine group appeared to have spherical mitochondria (Fig. 11-C) with longer cristae (Fig. 11-C). However, in α-lipoic acid group, the neurons have mitochondria which appeared spherical and elongated (Fig. 11-D) and cristae were preserved but short (11-D). Finally, the neurons in combination group appeared to have elongated mitochondria (Fig. 11-E) with apparent cristae which appeared nearly like the vehicle control (Fig. 11-E).

4. Discussion

In the current study, repeated administration of rotenone resulted in a low survival rate (50%) and the surviving rats showed motor deficits in addition to histopathological changes in the SNpc neurons. The lethality due to rotenone occurred directly after injection of rotenone in more than 90% of the cases. This suggests that the mortality was mostly attributed to the acute toxicity of rotenone rather than the cumulative neurotoxic effect. It has been reported that rotenone may exert significant peripheral organ toxicity to the heart (Akpinar et al., 2005) as well as the liver and stomach (Ravenstijn et al., 2008). In agreement, peripheral mitochondrial inhibition was considered to play a critical role in the poor survival rate observed in rotenone treated rats (Fleming et al., 2004).

Consistently, it has been reported that a dose-dependent decrease in survival rate was observed in rats infused subcutaneously with rotenone (Fleming et al., 2004). In the current study, the lethality of rotenone was not rescued by treatment with the implemented agents, suggesting that also in surviving rats a high impact of non-DAergic mitophagy is contributing to the observed symptoms. In addition, Hoglinger et al. (2005) demonstrated that both nigral and striatal neurons degenerate in rotenone-treated rats, as observed in atypical Parkinson syndromes, but not in PD (Braak and Braak, 2000; Poewe and Wenning, 2002); this came in line with our findings.

Rotenone-treated rats exhibited impaired motor activity in the open-field test and square bridge test along with a marked decrease in striatal DA level. In agreement, rotenone-treated rats exhibit reduced mobility (Sindhu et al., 2005), progressive degeneration of the nigrostriatal pathway (Betarbet et al., 2002) and show depletion in striatal dopamine level (Alam and Scmidt, 2002). The profound striatal dopamine depletion observed in rotenone-treated rats might be due to an energy-related impairment in the presynaptic end terminal of DAergic neurons and their dendrites (Alam and Scmidt, 2002).

Tyrosine hydroxylase immunoreactivity in the SNpc dopaminergic neurons was affected by repeated administration of the current dose of rotenone. Consistently, in rotenone-treated rats, the number of TH-positive DAergic neurons in the SNpc was reduced by 23% and the density of TH-positive fibers was reduced in the striatum by 53% (Hoglinger et al., 2005). There has been controversy whether neuronal loss in rotenone-treated rats is restricted to the SNpc (Betarbet et al., 2000; Sherer et al., 2003) or also affects the striatum (Hoglinger et al., 2003; Lapointe et al., 2004; Zhu et al., 2004). Fleming et al. (2004) reported that there was no correlation between striatal TH immunostaining and behavioral impairment. These findings may indicate that when rotenone does not markedly alter TH immunoreactivity in the striatum, low doses of rotenone have a significant effect on SNpc neurons and some aspects of motor behavior; this seems to be compatible with the present results.

In the present study, ATP level was decreased in the brains of rotenone group as compared to vehicle group. Consistently, rats treated with rotenone showed a decrease in striatal complex-I activity and ATP (Abdin and Hamouda, 2008). In addition, rotenone caused a markedly decreased amount of mtDNA. DAergic neurons have a greater energy demand in order to sustain their metabolic activities that contribute to their greater sensitivity toward rotenone-induced ATP depletion (Kweon et al., 2004).

The current results demonstrated that striatal MDA and protein carbonyls levels were significantly higher in rotenone-treated rats as compared to vehicle-treated rats. It was reported that chronic administration of rotenone was capable of increasing MDA in the cortex and the striatum of rats (Bashkatova et al., 2004) and increased oxidative damage to lipids, DNA, and protein (Jia et al., 2010; Pearce et al., 1997). In accordance, rotenone was suggested to induce cell death in primary DAergic culture by increasing ROS production and inhibiting mitochondrial respiration (Radad et al., 2006).

In agreement, some investigators found that the greatly enhanced neurotoxicity of rotenone was attributed to the release of NADPH oxidase-derived superoxide from activated microglia (Gao et al., 2003; Radad et al., 2006). Superoxide and its downstream products (hydroxyl radicals, singlet oxygen species, and hydrogen peroxide) are quite reactive and can interact with proteins, DNA, or RNA to alter their functions or induce lipid peroxidation, leading to eventual cell death (Facchinetti et al., 1998). DAergic neurons exert greater oxidative stress than other neurons owing to the production of hydrogen peroxide (H_2O_2) during monoamine oxidase-driven dopamine metabolism (Kweon et al., 2004). Additional oxidative stress from rotenone-induced ROS could easily overload the intrinsic anti-oxidative system resulting in cell death (Leung et al., 2007). These findings are compatible with those reported that oxidative stress could be involved in the pathogenic mechanisms of PD (Jin-Peng et al., 2009).

Rotenone group demonstrated depletion in striatal GSH content. Consistently, low density of GSH positive cells was found surrounding the parkinsonian DAergic neurons (Ebadi et al., 1996), which along with glutathione peroxidase (G-Px) form the primary defense system of DAergic neurons against ROS (Abd-El Gawad et al., 2004). The current study demonstrated diminished SOD and CAT activity in rotenone-treated rats as compared to vehicle-treated rats. Similarly, the

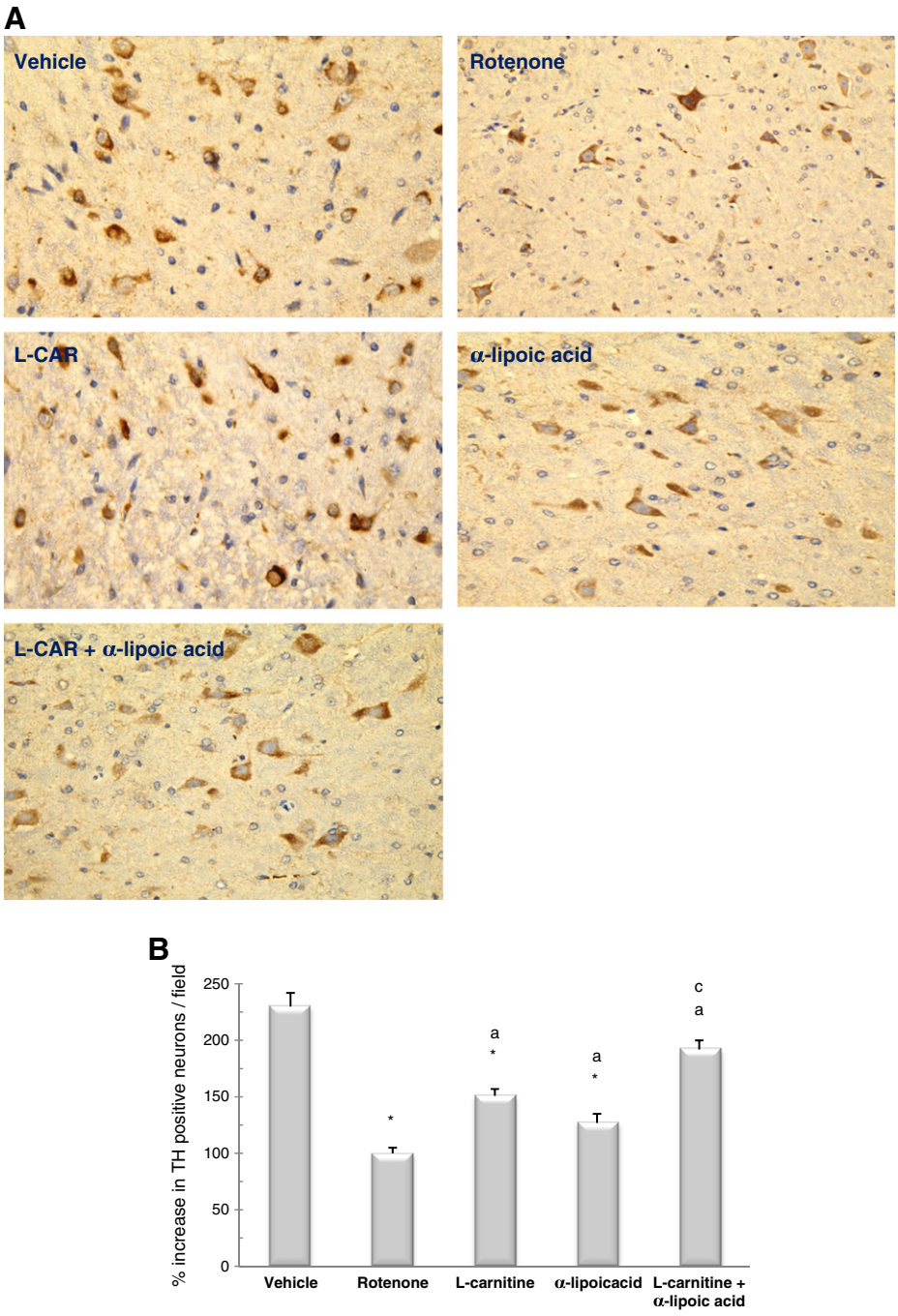

Fig. 10. A — TH immunostaining in the experimental groups. Vehicle-treated group showing high number of TH immunostained neurons. Rotenone group showed less immunostaining for TH (photomicrographs captured at ×20 magnification). B — Percentage increase in immunostaining for TH in the substantia nigra of the experimental groups. Results are expressed as mean ± S.E.M. $^{*}p \leq 0.05$ compared to vehicle group. $^{a}p \leq 0.05$ compared to rotenone group. $^{b}p \leq 0.05$ compared to acetyl-L-carnitine group. $^{c}p \leq 0.05$ compared to α-lipoic acid group.

SN of PD brains has a reduced level of the antioxidant enzymes such as CAT, SOD and G-Px (Sian et al., 1994a) and antioxidant molecules such as GSH (Sian et al., 1994b), suggesting the presence of a sustained burden of oxidative stress that overwhelmed the antioxidant capacity (Liu, 2006). Collectively, rotenone model recapitulates most of the mechanisms thought to be important in PD pathogenesis (Betarbet et al., 2002).

4.1. Acetyl-L-carnitine and α-lipoic acid

In the present study, single treatment with acetyl-L-carnitine or α-lipoic acid or their combination parallel to rotenone protected significantly against some of the neurotoxic effects of rotenone. Treatment with these agents improved the motor function of rats and increased ATP production as well as the amount of intact mtDNA. In addition, the implemented agents protected against rotenone-induced oxidative stress by suppressing the production of LPs and increasing GSH level. Additionally, these agents ameliorated the histopathological picture of the SNpc neurons as compared with rotenone group. Therefore, functional assessment came on line with biochemical and histopathologic assessment.

In the open field test, all the horizontal movement parameters were improved by the implemented agents; however, vertical movement (rearing) was not rescued by single treatment with α-lipoic

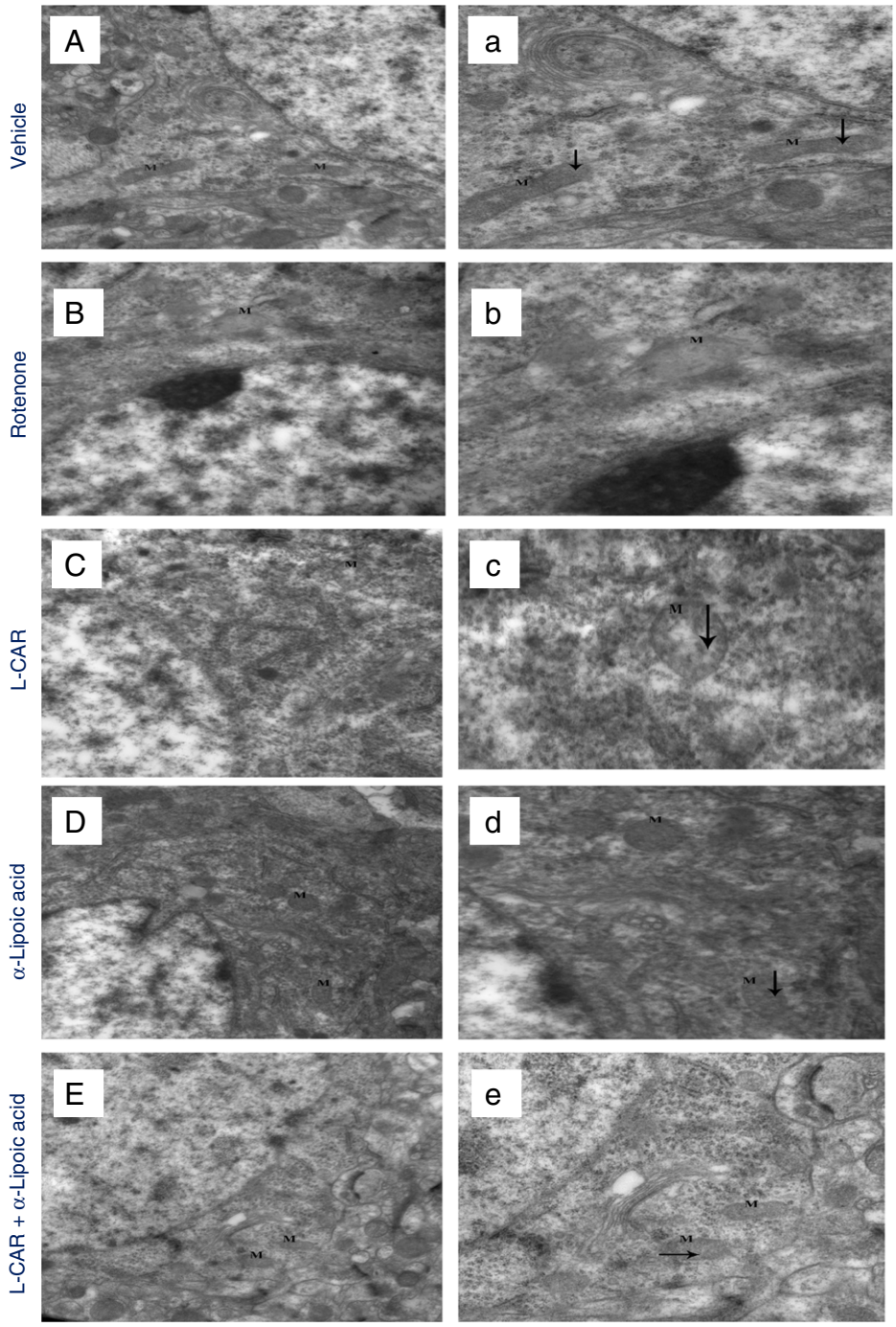

Fig. 11. Ultra structural image of the mitochondria in the experimental groups. An electron micrograph of rat SNpc neurons in vehicle treated group showing elongated mitochondria (M) with apparent cristae (arrows) (A&A). A neuron in SNpc of rotenone group showing an elongated cloudy mitochondrion without apparent cristae (B&B). An electron micrograph of one neuron in acetyl-L-carnitine group showing a spherical mitochondrion with cristae (arrows) (C&C). An electron micrograph for a neuron in lipoic acid group showing spherical and elongated mitochondria with less apparent cristae (arrow) as compared to acetyl-L-carnitine group (D&D). An electron micrograph of a neuron in rats treated with a combination of acetyl-L-carnitine and α-lipoic acid showing elongated mitochondria with apparent cristae (arrow) (E&E). Electron micrographs captured at ×4000 (A–E) or ×7500 (a–e).

acid. Rearing behavior is used to assess the innate drive to explore a novel environment by rearing and leaning their forepaws against the wall of the open-field. Indeed, acetyl-L-carnitine showed much more protection in comparison to α-lipoic acid in the majority of the measured parameters; e.g. DA level, ATP level, mtDNA integrity and histology. Therefore, these protective effects were reflected by the locomotor activity of the animals. This suggests that rearing behavior (vertical movement) is the most sensitive parameter to nigral DAergic degeneration in comparison to other measured parameters (horizontal movement) in the open-field test. In agreement, Fleming et al. (2004) reported that rearing was the only test consistently affected by rotenone. On the other hand, square bridge test assesses balance and motor coordination, the lack of effectiveness α-lipoic acid in the square bridge test further suggest that other brain systems may be affected by rotenone, which were not influenced by this agent. It has been shown that non-DAergic striatal neurons and the globus pallidus can be damaged by rotenone administration (Hoglinger et al., 2003; Fleming et al., 2004). Alterations to other

neurotransmitter systems and damage to basal ganglia structures may contribute to the observed motor impairment.

In addition, the restorative effect of the combination therapy on striatal dopamine was higher than single treatments. This suggests a complementary neuroprotective effect of both of the antioxidants that was reflected by the motor performance of the rats.

In the present study, acetyl-L-carnitine alone or in combination with α-lipoic acid increased the mitochondrial ATP level. In addition, all of the current treatments ameliorated the mtDNA integrity. Consistently, previous results demonstrated that rotenone inhibited mitochondrial function and caused cell death in vitro cultured rat cortical neurons that were, in part, protected by the co-incubation of the cells with 1 mM acetyl-L-carnitine (Virmani et al., 1995). Carnitine effectively inhibited mitochondrial injury induced by oxidative stress and mitochondria dependent apoptosis in various types of cells (Furuno et al., 2001; Hagen et al., 2002; Therrien et al., 1997).

Acetyl-L-carnitine has also been shown to improve lipid, oxygen and glucose delivery for enhanced energy metabolism and generation of ATP (Gabryel et al., 2002). In addition, acetyl-L-carnitine could involve a restoration of mitochondrial function and/or improved use of energy from glycolysis in cultured neuroblastoma cells treated with the neurotoxin, 1-methyl-4-phenyl tetrahydropyridine (Mazzio et al., 2003). In agreement with our results, Chang et al. (2002) reported that cisplatin-induced mtDNA injury in the kidney and small intestine was strongly inhibited by L-carnitine. The free radical scavenger activity of acetyl-L-carnitine might justify its protective effect. Moreover, L-carnitine improves the transcription of mtDNA probably by increasing oxygen consumption and therefore ATP synthesis; this energy is required for DNA repair. Furthermore, acetyl-L-carnitine enhances the activity of DNA repairing enzyme, poly (ADP-ribosyl) polymerase (Savitha and Panneerselvam, 2006).

In the study of Sharman and Bondy (2001), cerebral mitochondrial electron transport system enzyme activities were elevated, in aged animals supplemented with α-lipoic acid, above corresponding values obtained with basal diet. The current study revealed that α-lipoic acid administration significantly attenuated rotenone-induced mtDNA decay. Being a powerful antioxidant, dihydrolipoic acid reduced the formation of mitochondrial superoxide radicals. Dihydrolipoic acid controls mitochondrial superoxide formation indirectly by regulating redox-cycling ubiquinone. Suppressing this superoxide generator mitigates oxidative stress, and in turn reduces damage to mtDNA (Ramadan, 2008) and improves mitochondrial function (Hagen et al., 1999). Thus, the overall enhancement in the striatal ATP level in the combination therapy group in present study, was attributed to acetyl-L-carnitine rather than α-lipoic acid.

Remarkably, acetyl-L-carnitine and α-lipoic acid rescued mitophagy in DAergic neurons of brain but not in peripheral tissues, as indicated by low survival rates. Because of its particular biochemical characteristics, the CNS is considered to be a potential place where oxidative reactions catalyzed by ROS can occur (Bongarzone et al., 1995). In accordance, high amount of iron may predispose to generation of lipid peroxides in brains submitted to brain ischemia (Arabi et al., 1987). Additionally, high content of free fatty acids was reported to stimulate ROS production. It has been suggested that polyunsaturated fatty acids play a key role in membrane damage following ischemia (Chan et al., 1985). Overall, all the previous observations may explain the high susceptibility of the CNS to mitopathy and oxidative damage. The current antioxidants lessened this high level of oxidative damage in the rotenone parkinsonian rats but did not modify the locomotor function in vehicle treated controls.

Single or combined treatment with acetyl-L-carnitine and α-lipoic acid suppressed MDA production and increased GSH concentration in the striata of rotenone-treated rats. However, the protein carbonyls and CAT activity were influenced only by the combination treatment. The protective effects of acetyl-L-carnitine might reflect its activity to improve energy metabolism and repair oxidized membrane/lipid bilayers, thereby suppressing the release of free electrons from mitochondrial electron transport system, a prerequisite reaction to generate free radicals (Liu et al., 2002). Furthermore, acetyl-L-carnitine may have a direct effect on the membrane, and may prevent cell damage by stabilizing the membrane against free radical damage.

It was suggested that α-lipoic acid suppressed 6-hydroxydopamine-induced ROS generation and apoptosis through the stimulation of the intracellular levels of GSH and the expression of GSH synthesis-related genes (Fujitaa et al., 2008). Additionally, (R)-α-lipoic acid reverses the age-related loss in GSH redox status in post-mitotic tissues (Suh et al., 2004a). This dithiol compound effectively improved cellular redox balance (as indicated by a higher GSH/GSSG ratio) (Hagen, 2004) and protects biomembranes from the damage induced by oxidative stresses (Persson et al., 2001).

In vivo, α-lipoic acid markedly increased the concentration of GSH and decreased GSSG level in the striatum of parkinsonian rats (Bilska et al., 2007). Moreover, α-lipoic acid protects against peroxynitrite-induced tyrosine nitration and α1-antiproteinase inactivation (Whiteman et al., 1996). It also prevents age-related declines in long-term potentiation as well as decreases in antioxidants (Hagen et al., 2002). Further, α-lipoic acid acts as a potent inducer of phase II detoxication proteins in astroglia cells, a principal cell type responsible for GSH synthesis in the brain (Flier et al., 2002).

The present results highlighted that the protective effect of the combination therapy involves enhancing striatal GSH level; this effect seems to be attributed to both of the antioxidants. However, a greater contribution was due to α-lipoic acid. Because α-lipoic acid is readily taken up into neuronal tissues (McGahon et al., 1999), the results from our study reinforce the view that α-lipoic acid represents an effective, means to beneficially improve the toxin-related loss of cerebral GSH, which could be important to mitigate oxidative stress-induced pathophysiologies, such as PD. In addition, many reports have shown that α-lipoic acid promotes induction of antioxidant enzymes (Suh et al., 2004a, 2004b; Volobueva et al., 2005). The present results indicated that the effect on SOD activity was more related to acetyl-L-carnitine, however, the effect on CAT activity seems to be related to the combined effect of both of the antioxidants.

Collectively, mitochondrial injury may be prevented by acetyl-L-carnitine also by increasing energy production and decreasing the leakage of free radicals (Virmani et al., 2006). However, the molecular mechanism by which α-lipoic acid suppressed nigral injury and eventual neurodegeneration involves mainly antioxidant effect. The combination of acetyl-L-carnitine and α-lipoic acid induced a significant improvement in the locomotor behavior of the animals accompanied by improvement in the biochemical and histologic parameters as compared to either of the agents alone. In agreement, combined (R)-α-lipoic acid and acetyl-L-carnitine was found to exert efficient preventative effects in a cellular model of PD (Zhang et al., 2010).The complementary effect of α-lipoic acid and acetyl-L-carnitine on cognitive and mitochondrial dysfunction has been shown in aging rats (Hagen et al., 2002; Liu et al., 2002). One reason is that α-lipoic acid plus acetyl-L-carnitine act on different pathways necessary for mitochondria: acetyl-L-carnitine, although stimulating mitochondrial function, while α-lipoic acid, an effective mitochondrial antioxidant. The complementary effect may also come from the different functions of α-lipoic acid and acetyl-L-carnitine in the mitochondria (Shen et al., 2008). Further, the neuroprotective effect of the current antioxidant combination was significantly higher than that produced by α-lipoic acid alone but was not different from the effect exerted by acetyl-L-carnitine. Therefore, the neuroprotective effect of this combination seems to be more dependent on the action of the later antioxidant.

5. Conclusion

Based on the previous findings, agents that specifically modulate the mitochondrial dysfunction and oxidative stress would be promising

candidates for neuroprotection in early PD and they can slow down the progression of the disease. In addition, further studies are needed to explore other possible mechanisms involved in the neuroprotective effect of acetyl-L-carnitine and α-lipoic acid in the experimental models of PD.

Abbreviations

ALC	acetyl-L-carnitine
LA	α-lipoic acid
CAT	catalase
DMSO	dimethylsulfoxide
DAergic	dopaminergic
ETS	electron transport system
G-Px	glutathione peroxidase
LPs	lipid peroxides
mtDNA	mitochondrial DNA
MDA	malondialdehyde
GSSG	oxidized glutathione
PD	Parkinson's disease
PEG	polyethylene glycol
ROS	reactive oxygen species
PBS	phosphate-buffered saline
GSH	reduced glutathione
SNpc	substantia nigra pars compacta
SOD	superoxide dismutase
TH	tyrosine hydroxylase

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