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RESEARCH

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Alpha-lipoic acid-role in improving both reserpine toxicity and paroxetine treatment in the cerebral cortex of albino rats; histological, ultrastructural, immunohistohemical and biochemical studies

Samah M. Arisha*

Abstract

Background: Reserpine is a monoamine depletory drug cause oxidative damage and used to induce depression-like features in rodent model. Paroxetine is an antidepressant drug that exerts its effects by inhibiting dopaminergic neurons although it may exert much pathological damage. Alpha-lipoic acid (ALA) is an endogenous antioxidant co-factor of important enzymatic complexes. The present study was aimed to elucidate the possible protective effect of ALA in the improvement of the deleterious cerebral cortex injury after reserpine and paroxetine treatment. Forty adult male albino rats were equally divided into 5 groups. Group I served as control group orally treated with saline solution all the experiment period. Group II animals orally treated with ALA (200 mg/kg/day) for six weeks. The induction of depression-like features occurred when the rest of animals were intraperitoneally treated with 25 mg/kg of reserpine once daily for consecutive 14 day. Then these animals were divided into; Group III (reserpine group) animals in this group were sacrificed on 15th day. Group IV; reserpine-treated animals were treated with paroxetine (20 mg/kg) daily for 6 weeks. Group V, animals in this group were received paroxetine and ALA daily for 6 weeks.

Results: Reserpine-treated rats showed disorganized layers of cerebral cortex with degenerative, apoptotic and necrotic changes. Ultrastructure changes include both pyramidal and granule cells with severe degenerative, necrotic and apoptotic features. The nuclei appeared pyknotic; irregular with chromatin condensation as well as the cytoplasm of these cells contained many degenerated organelles. In addition, a significant increase in total oxidative stress and decrease in total antioxidant capacity, norepinephrine, dopamine and serotonin levels were recorded. The same treatment showed significant decrease in proliferating cell nuclear antigen (PCNA) expression and significant increase in caspase-3 expression in the granule and pyramidal cells. After paroxetine-treatment these parameters were more or less similar to those observed in reserpine-treated ones. While an obvious improvement was appeared when animal treated with both paroxetine and ALA and; all parameters restored its normal features.

Conclusions: This study concluded that; ALA treatment attenuated the cerebral injury induced by reserpine and improved the effects of paroxetine in rats due to its anti-inflammatory, anti-apoptotic and antioxidant activities.

*Correspondence: arishasamah@yahoo.com

Zoology Department, Faculty of Science, Menoufia University, Shibin El-Kom, Egypt

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Keywords: Alpha lipoic acid, Reserpine, Paroxetine, Cerebral cortex, Histological, Ultrastructure, Caspase-3, PCNA, Neurotransmitters

1 Background

Reserpine is a monoamine depletory drug that blocks vesicular monoamine transporters during neuronal transmission and storage, as well as promotes dopamine autoxidation and oxidative catabolism through monoamine oxidase, causing oxidative stress [1]. Based on the theory that depression decreased the levels of neurotransmitters; the drugs such as reserpine decrease the concentrations of monoamines in the central nervous system (CNS), and used as pharmacological model for depressive-like behavior induced in rodents [2, 3].

Depression is a common psychological disorder characterized by low mood, slow thinking activity; reduced language action and pathological changes mainly involve CNS, immune function and neuroendocrine function [4]. Chronic stress exposure is associated with neurons degeneration that leads to a marked change in antioxidant enzymes, generation of free radicals and reactive oxygen species (ROS) which produced in very high levels leading to toxic effects on intracellular components such as DNA, proteins and lipids resulting in an increase in the incidence of apoptosis of cells of vital organs causing great damaging impact on the limbic system and hence on mood status and consequently may contribute to the dysfunction of serotonergic and noradrenergic systems [5, 6].

Paroxetine is one of the selective serotonin reuptake inhibitors (SSRIs) antidepressants and widely used in the treatment of generalized anxiety and patients with depressive disorders [7, 8]. It is generally more preferred, for animal studies and human clinical trials, due to lesser adverse effects, good compliance, and comparable efficacy with these older drug groups [9]. Moreover, SSRIs increase the level of extracellular serotonin (5-hydroxytryptamine, 5-HT) by inhibition of 5-HT reuptake. This effect is thought to be the main mechanism of SSRIs drugs [10].

Neurotransmitters are include glutamate, aspartate, D-serine, γ-amino butyric acid, glycine; monoamines, dopamine (DA), norepinephrine (NE, noradrenaline), epinephrine (adrenaline), histamine, and 5-HT [11]. Small amount of serotonin of the human body's is, biochemically derived from tryptophan, synthesized in serotonergic neurons of the CNS, concentrated in the raphe nuclei of the brainstem, where it has various functions such as the regulation of mood, appetite, sleep, emotion, temperature, and the motor system [12]. Meanwhile, NE is synthesized from DA by dopamine β-hydroxylase [13]. The adrenal medulla released NE into the blood as a hormone, and as a neurotransmitter in the CNS and sympathetic nervous system where it is released from noradrenergic neurons. The noradrenergic neurons in the brain, when activated, exert effects on large areas of the brain, producing alertness and arousal [14].

Alpha-lipoic acid (ALA) is a disulfide compound naturally occurring in the human body and produced in the mitochondria from acetic acid, it contains 2 thiol groups which can be oxidized or reduced; therefore it is a redox couple so it is an ideal or universal antioxidant that protects the mitochondrial membranes against oxidative injury in various disorders such as atherosclerosis, diabetes mellitus, multiple sclerosis, and dementia, wherein oxidative stress plays a major role in pathogenesis [15–17]. In vivo, ALA is reduced to dihydro-LA (DHLA), which neutralizes ROS, chelates metal ions and promotes regeneration of endogenous antioxidants such as glutathione (GSH), vitamin E, and C [18]. Moreover**,** it is reported to inhibit the release of pro-inflammatory cytokines as well as improve cognitive function and reduce brain-oxidative stress and helped in the treatment of neurodegenerative diseases [19, 20]. Moreover, it has been reported that ALA affected the glucose and lipid metabolism [21], regulated the appetite [22], and combated complications of diabetic origin [23] and myocardial and cerebral reperfusion injuries [24].

The exact pathogenesis of depression is still unclear so this study aimed to investigate the effects of paroxetine and ALA on depressive pathological changes and the related biochemical markers in the cerebral cortex of rats treated with reserpine through histological, ultrastructural, immunohistochemical and biochemical investigations.

2 Methods

2.1 Drugs

2.1.1 Reserpine

Reserpine was obtained from Novartis Co. (Cairo, Egypt) it was provided as brinerdin tablets each contains 0.1 mg reserpine. These tablets were dissolved in saline solution and intraperitoneally injected to animals' (0.25 mg/kg/day, i.p.) for consecutive14 days [25].

2.1.2 Paroxetine

Paroxetine HCl, it manufactured by EVA Pharm for Pharmaceutical, Egypt, it found as film coated tablet contains 20 mg paroxetine. The drug was freshly dissolved in

saline solution and orally given at a dose level of 20 mg/kg/day for 6 weeks.

2.1.3 Alpha lipoic acid
Alpha lipoic acid (thioctic acid) is a disulfide compound, and it was purchased from local pharmacy as coated capsules each contains 300 mg of thioctic acid and it manufactured by EVA Pharm for Pharmaceutical, Egypt. These capsules were dissolved in saline solution and orally given to animals at a dose level of 200 mg/kg/day for six weeks.

Unless otherwise stated, all other chemicals were obtained from Sigma-Aldrich (Saint Louis, MO, USA). All doses correspond to the base of the drug.

2.2 Animals
Forty adult healthy male albino rats (*Rattus norvegicus*) 3 months old weighing 120–140 g were purchased from the animal house at the National Research Center, Giza, Egypt. Animals were left about ten days for acclaimed before experiments begin. The rats were housed 4 animals per cage and maintained on a 12 h light/12 h dark cycle and allowed free access of normal rat chow diet and water ad libitum. Principles of animal care and use were carefully followed during conducting the present study according to the guide for the care and use of laboratory animals approved by Faculty of Science, Menoufia University, Egypt (Approval No. MUFS/F/HI/5/21), and according to the National Institutes of Health guide for the care and use of laboratory animals (NIH publications No. 8023, received 1978).

2.3 Experimental design
After ten days of acclimatization the rats were randomly divided into 5 groups eight animal each. Group I rats in this group were served as control group orally given 0.5 ml of saline solution daily for six weeks. Group II animals were treated with ALA (200 mg/kg/day) for six week. The induction of cerebral injury, depression-like features, occurred when the rest of animals were intraperitoneally treated with 25 mg/kg of reserpine daily for 14 days. Then these animals were divided into the following three groups. Group III (reserpine group) animals in this group were sacrificed on 15th day. Group IV the reserpinized animals were treated with paroxetine (20 mg/kg) daily for 6 weeks. Group V, animals in this group were received paroxetine and ALA daily for 6 weeks. During the six weeks of the experiment, daily health checks of the rats of all groups were observed.

One day after the last dose half of animals were anaesthetized and were perfused through the ascending aorta with 10% neutral formalin, for histological study. Then rest of animals in each experimental group were perfused with 2.5% gluteraldehyde, for ultrastructural study in 0.1 M sodium phosphate buffer at PH 7.4 The brain of each animal was removed and the cerebral cortex was dissected out and prepared for light, and electron microscopic examinations as well as immunohistochemical studies and biochemical analysis were performed.

2.4 Investigated parameters
2.4.1 Transmission electron microscopic study
For transmission electron microscopic study, very small pieces of cerebellar cortex about (1-2mm^3) immediately fixed in 2.5% gluteraldehyde for 48, then washed in phosphate buffer (PH 7.2) and post-fixed in a buffered solution of 1% osmium tetroxide for 2 h then washed in the same buffer 4 times for 20 min. the fixed tissues were dehydrated in ascending series of alcohol, cleared in two changed of propylene oxide and embedded in epoxy resin. The semi thin sections were prepared and stained with toluidine blue, then examined to select the areas that photographed with transmission electron microscope. The ultrathin sections (60–90 nm thick) were cut, mounted on grids and were stained with uranyl acetate and lead citrate. These grids were examined and photographed using 1400 plus-JSM transmission electron microscope (JEOL Ltd., Tokyo, Japan) in electron microscope unit, Faculty of Science, Alexandria University (Alexandria, Egypt).

2.4.2 Histological study
For light microscopic study, small pieces of the cerebral cortex were fixed in 10% neutral formalin and processed to prepare 5 micron sections stained with hematoxylin and counterstained with eosin (H&E).

2.4.3 Immunohistochemical study
The polyclonal primary antibody for the antibody for proliferating cell nuclear antigen (PCNA) and caspase-3 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The detection of PCNA and caspase-3 immune expressions was performed using a conventional avidin-biotin immune-peroxidase technique modified from published protocols [26].

2.4.4 Morphometric study
The morphometric measures were achieved by the touch account method using a computer assisted image analyzer (soft imaging system- An Olympus Company). The measurements were performed using an X400 objective lens in five non-overlapping fields in ten randomly chosen sections from eight different animals for each group. The areas percent for PCNA and caspase-3 immunoreactions in cerebral cortex were measured.

2.5 Biochemical analysis
After complete the perfusion process small pieces of each cerebral cortex were carefully excised, washed with ice-cold saline. Cerebral cortex specimens were homogenized in a cold 50 mM phosphate buffered saline (pH 7.4) by means of Ultra Turrax T25 homogenizator (Omni International, Kennesaw, GA, USA). Subsequently, the cerebral cortex homogenates were centrifuged at 20,000 rpm for 15 min (4 °C). The supernatants were collected and stored at −20 °C for further analysis. The tissue total antioxidant capacity (TAC) and total oxidative stress (TOS) were determined in the brain tissue using kits provided by Bio diagnostics, Cairo, Egypt. According to the method of Koracevic et al. [27] TAC was determined. The protein content of homogenate samples was measured using a commercially available kit. Total oxidative stress of brains samples were measured using a method developed by Erel [28].The results of TAC and TOS in brains were calculated according to the total tissue protein level and the absorbance of each sample were read spectrophotometrically at 560 nm as a sample blank. The measurement unit of TOS and TAC in tissue is μmol/g tissue protein.

2.6 Detection of monoamine neurotransmitters levels in the brains
For measuring the levels of 5-HT, NE, and DA the cerebral cortex homogenates were centrifuged at 12,000 rpm for 10 min at 4∘C. Supernatants were carefully transferred to 96-well plates and then injected onto the LC–MS/MS system by an auto sampler for subsequent analysis. A standard curve was generated via the preparation of 1 μg/mL stock solutions of standard 5-HT, NE, and DA, which were diluted to a concentration of 100 μg/mL of injection solution [29].

2.7 Statistical analysis
The data obtained were analysis with SPSS statistical software (SPSS for windows, revision 23.0) the data were expressed as the mean ± standard deviation (mean ± SD) and the compression between the experimental groups was done using ANOVA test. Significances of the obtained data were classified three categories $P < 0.0001$, $P < 0.001$, $P \leq 0.001$, $P \leq 0.0001$, and $P \leq 0.05$, according to the obtained P values.

3 Results
3.1 Clinical observations
Both the control and ALA-treated rats were appeared to have normal activity and normal adequate food and water intake. Reserpine-treated rats exhibited general weakness, decrease locomotors activities and loss of appetite. Some animals, especially after paroxetine treatment, showed twitching of body muscles. On the other hand, ALA and reserpine-treated rats didn't show any abnormality.

3.2 Light microscopic results
The light microscopic examination of the cerebral cortex obtained from both the control and ALA groups showed well known normal structure. The cerebral cortex covered externally with thin fibrous tissue that allows the blood vessels to pass through and nourish the brain, pia matter. The grey matter surrounding the cerebrum is known as the cortex of the brain where the actual processing is done. The neurons in the grey matter is consists of neuronal cell bodies, dendrites, and axons terminals where all synapses present. These neural cells were organized in six distinguished layers, with no sharp boundaries, in association small blood vessels in between. These layers are; an outer molecular layer, an external granular later, an external pyramidal layer, an inner granular layer, an inner pyramidal and polymorphic layers (Fig. 1a). The white matter is made up of axons which connect different parts of the grey matter to each other.

The outer molecular layer appeared thick and contained dense plexus of nerve fibers with few neuroglia cells. The external granular layer revealed granule cells characterized by rounded cell bodies and large prominent nuclei. Also, small sized pyramidal cells were present in this layer. The external pyramidal layer showed medium sized pyramidal cells with triangular shaped cell bodies, basophilic cytoplasm, rounded nuclei and apical dendrites. Also, the blood vessels were seen. The internal granular layer showed rounded granule cells with open face nuclei. The internal pyramidal layer contained large sized pyramidal cells with triangular shaped cell bodies, basophilic cytoplasm, rounded nuclei and long apical dendrites. The polymorphic layer was characterized by the presence of cells of various size and shapes. The ground substance between the nerve cells was normally occupied with homogenous eosinophilic neuropil with blood vessels and nuclei of glial cells in between (Figs. 1 and 2).

On the contrary, the cerebral cortex of reserpine-treated rats showed neuronal cells disorganization, in the different cerebral layers, degenerative vacuolization, and intercellular edema (Fig. 3a, b). Some areas of the cortex showed necrosis of some neurons and neuroglia contained pyknotic nuclei or karyrrhetic ones. Apoptotic pyramidal or granule cells characterized by neuronal shrinkage and chromatin condensation either marginated or clumped as well as diffuse chromatolysis of nuclear chromatin also observed. Moreover, absence of nucleoli was detected in the same cerebral cells. The pyramidal cells showed thick, tortuous cellular

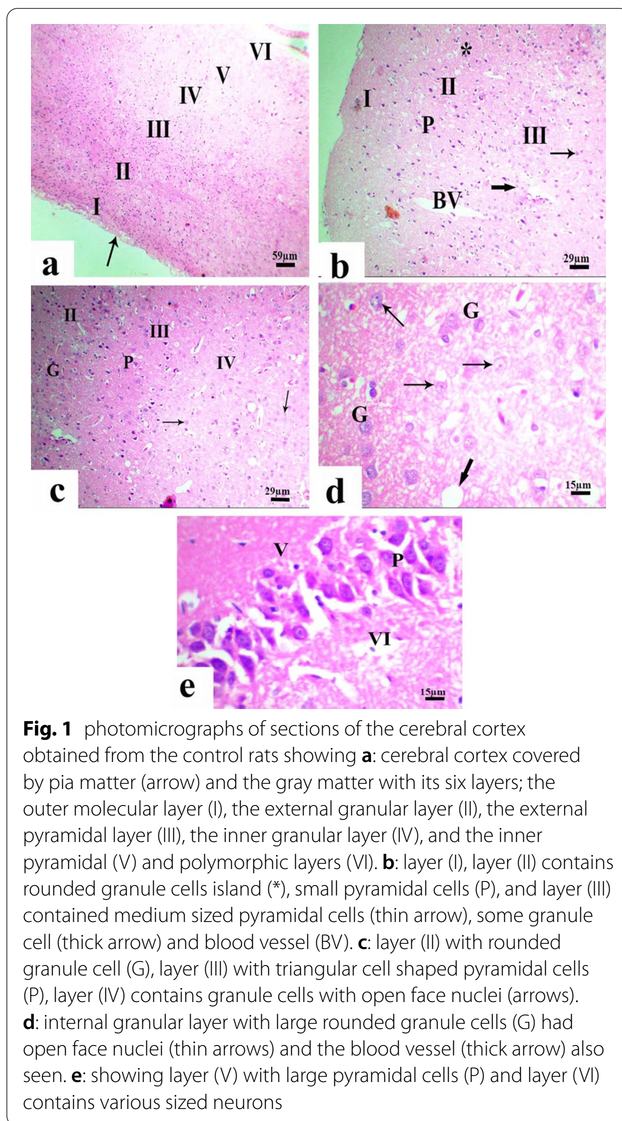

Fig. 1 photomicrographs of sections of the cerebral cortex obtained from the control rats showing **a**: cerebral cortex covered by pia matter (arrow) and the gray matter with its six layers; the outer molecular layer (I), the external granular layer (II), the external pyramidal layer (III), the inner granular layer (IV), and the inner pyramidal (V) and polymorphic layers (VI). **b**: layer (I), layer (II) contains rounded granule cells island (*), small pyramidal cells (P), and layer (III) contained medium sized pyramidal cells (thin arrow), some granule cell (thick arrow) and blood vessel (BV). **c**: layer (II) with rounded granule cell (G), layer (III) with triangular cell shaped pyramidal cells (P), layer (IV) contains granule cells with open face nuclei (arrows). **d**: internal granular layer with large rounded granule cells (G) had open face nuclei (thin arrows) and the blood vessel (thick arrow) also seen. **e**: showing layer (V) with large pyramidal cells (P) and layer (VI) contains various sized neurons

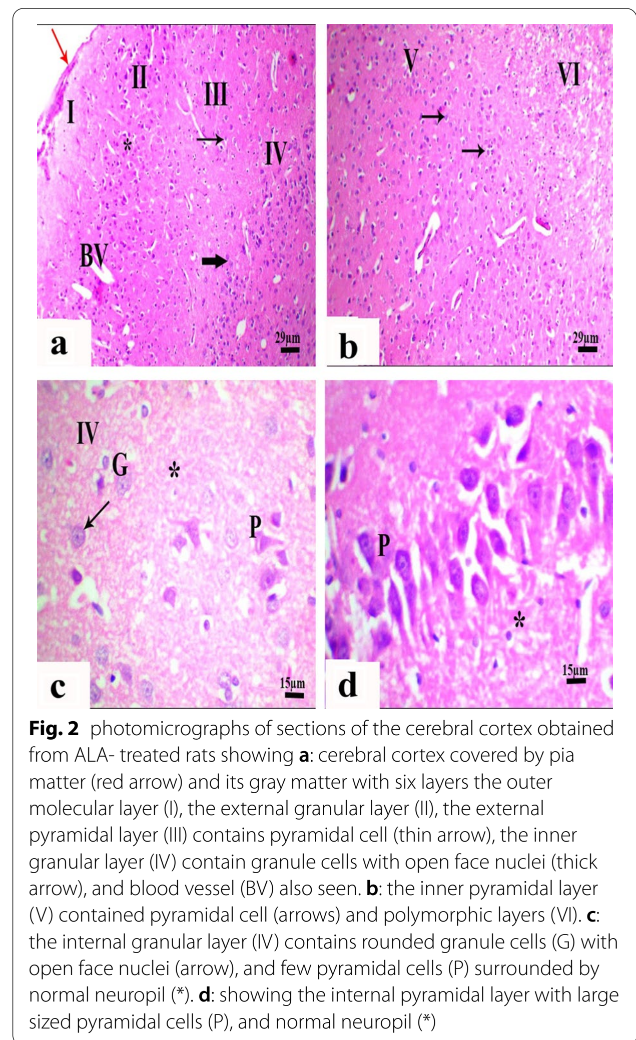

Fig. 2 photomicrographs of sections of the cerebral cortex obtained from ALA- treated rats showing **a**: cerebral cortex covered by pia matter (red arrow) and its gray matter with six layers the outer molecular layer (I), the external granular layer (II), the external pyramidal layer (III) contains pyramidal cell (thin arrow), the inner granular layer (IV) contain granule cells with open face nuclei (thick arrow), and blood vessel (BV) also seen. **b**: the inner pyramidal layer (V) contained pyramidal cell (arrows) and polymorphic layers (VI). **c**: the internal granular layer (IV) contains rounded granule cells (G) with open face nuclei (arrow), and few pyramidal cells (P) surrounded by normal neuropil (*). **d**: showing the internal pyramidal layer with large sized pyramidal cells (P), and normal neuropil (*)

processes with darkly stained, shrunken pyknotic nuclei surrounded by unstained areas. Moreover, the degenerative granule cells appeared surrounded with pericellular halos. There was vacuolization of the neuropile surrounding the affected nerve cells in addition to dilated congested blood capillary was noticed (Fig. 3c–e).

The degenerative changes observed in cerebral cortex of depressed animals treated with paroxetine were more or less similar to those observed in reserpine-treated ones. Figure 4a showed disorganized cerebral cortex layers contained degenerated neurons as well as some blood vessels appeared congested. In addition, the pyramidal cells showed eosinophilic degeneration appeared by contracted cells, lost their processes with eosinophilic cytoplasm and pyknotic nuclei (Fig. 4b). Some apoptotic neurons presented with diffused chromatin lysis of nuclei and complete absence of nucleoli. The internal granular layer appeared degenerated contained multicellular of granule cells with pyknotic nuclei. Dilated or congested blood vessels and excess vacuoles in the neuropil were noticed (Fig. 4c, d).

Examination of the specimens obtained from depressed animals treated with paroxetine concomitantly with ALA for six week showed an obvious improvement in the different types of neurons in many layers of the cerebral cortex. Most of the pyramidal and granule cells were more or less similar that of the control group except few cells showed picture of eosinophilic degeneration and others appeared as ghost; it appeared faint stained lost their nuclei. Moreover, the neuropil appeared normal homogenous contained few vacuoles (Fig. 5a–e).

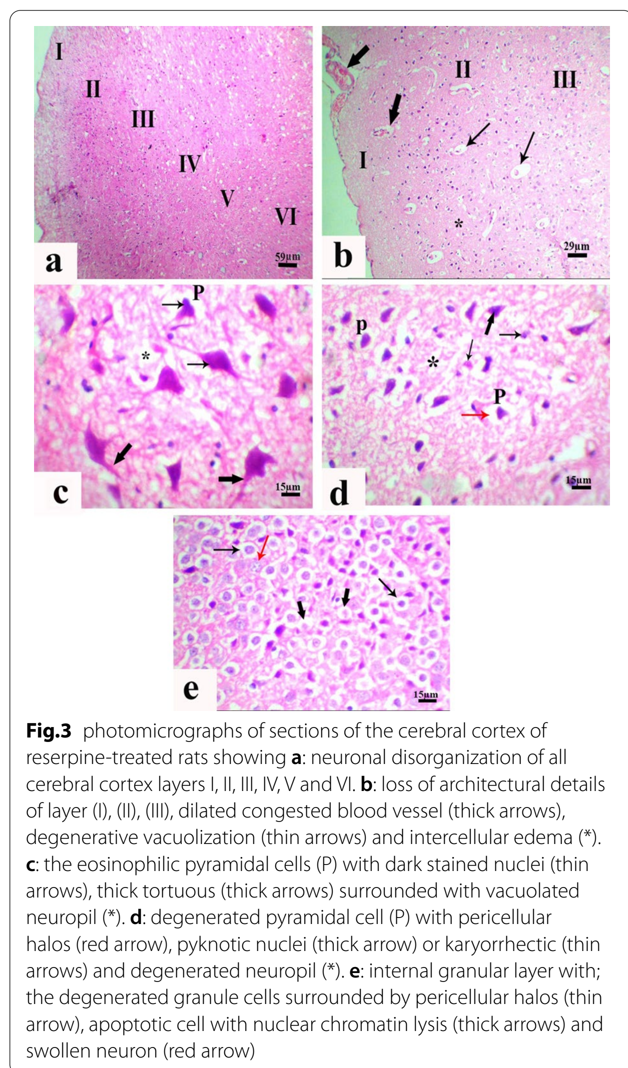

Fig.3 photomicrographs of sections of the cerebral cortex of reserpine-treated rats showing **a**: neuronal disorganization of all cerebral cortex layers I, II, III, IV, V and VI. **b**: loss of architectural details of layer (I), (II), (III), dilated congested blood vessel (thick arrows), degenerative vacuolization (thin arrows) and intercellular edema (*). **c**: the eosinophilic pyramidal cells (P) with dark stained nuclei (thin arrows), thick tortuous (thick arrows) surrounded with vacuolated neuropil (*). **d**: degenerated pyramidal cell (P) with pericellular halos (red arrow), pyknotic nuclei (thick arrow) or karyorrhectic (thin arrows) and degenerated neuropil (*). **e**: internal granular layer with; the degenerated granule cells surrounded by pericellular halos (thin arrow), apoptotic cell with nuclear chromatin lysis (thick arrows) and swollen neuron (red arrow)

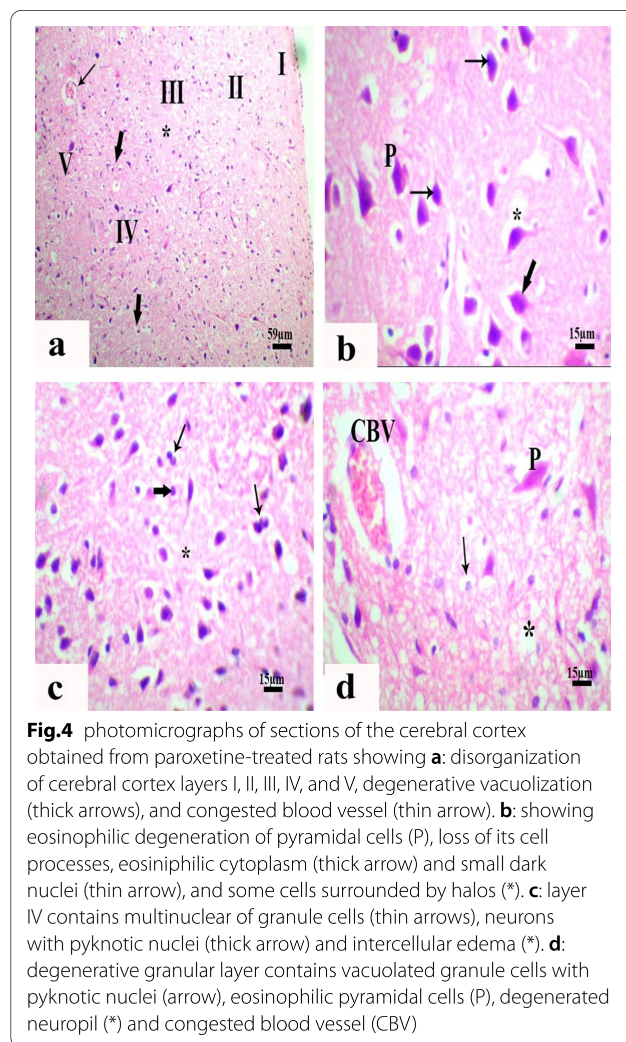

Fig.4 photomicrographs of sections of the cerebral cortex obtained from paroxetine-treated rats showing **a**: disorganization of cerebral cortex layers I, II, III, IV, and V, degenerative vacuolization (thick arrows), and congested blood vessel (thin arrow). **b**: showing eosinophilic degeneration of pyramidal cells (P), loss of its cell processes, eosiniphilic cytoplasm (thick arrow) and small dark nuclei (thin arrow), and some cells surrounded by halos (*). **c**: layer IV contains multinuclear of granule cells (thin arrows), neurons with pyknotic nuclei (thick arrow) and intercellular edema (*). **d**: degenerative granular layer contains vacuolated granule cells with pyknotic nuclei (arrow), eosinophilic pyramidal cells (P), degenerated neuropil (*) and congested blood vessel (CBV)

3.3 Electron microscopic results

Examination of the cerebral cortex ultrathin sections obtained from the control group showed well-known normal ultrastructural picture. The granular cells appeared with rounded nuclei with electron dense heterochromatin. The cytoplasm of these cells contained rough endoplasmic reticulum cisternae, numerous mitochondria and lysosomes. In addition, the myelinated nerve fibers were present (Fig. 6a). The pyramidal cells appeared with long apical dendrites and large oval euchromatic nuclei with fine granular chromatin and prominent nucleoli. The cytoplasm of these cells contains rough endoplasmic reticulum, multiple mitochondria, and excess Nissl's granules (Fig. 6b, c). The myelinated nerve axons showed regular smooth contour of its myelin sheath and contained normal-shaped mitochondria within hemogenous axoplasm (Fig. 6d).

Electron microscopic examination of the cerebral cortex of reserpine-treated rats showed obvious degenerative changes. The pyramidal cells with dark, shrunken irregular nuclei had a small amount of peripheral chromatin, and irregularities of nuclear envelopes were observed. The cytoplasm of these cells showed many degenerated features included electron-lucent vacuoles, scanty free ribosomes, lysosomes, disrupted mitochondria, and dilated rough endoplasmic reticulum (Fig. 7a–c). The apoptotic pyramidal cells had cytoplasmic blebs, shrinkage or intended oval nuclei with chromatin condensation or margination, and clumping, of electron dense heterochromatin and irregularity of nuclear envelope (Fig. 7d, e). The granule cells in the same group appeared either degenerated or apoptotic showing severe ultrastructure changes include, shrinkage of the rounded nuclei with chromatin condensation; margination and clumping of its electron dense heterochromatin. Their cytoplasm contained

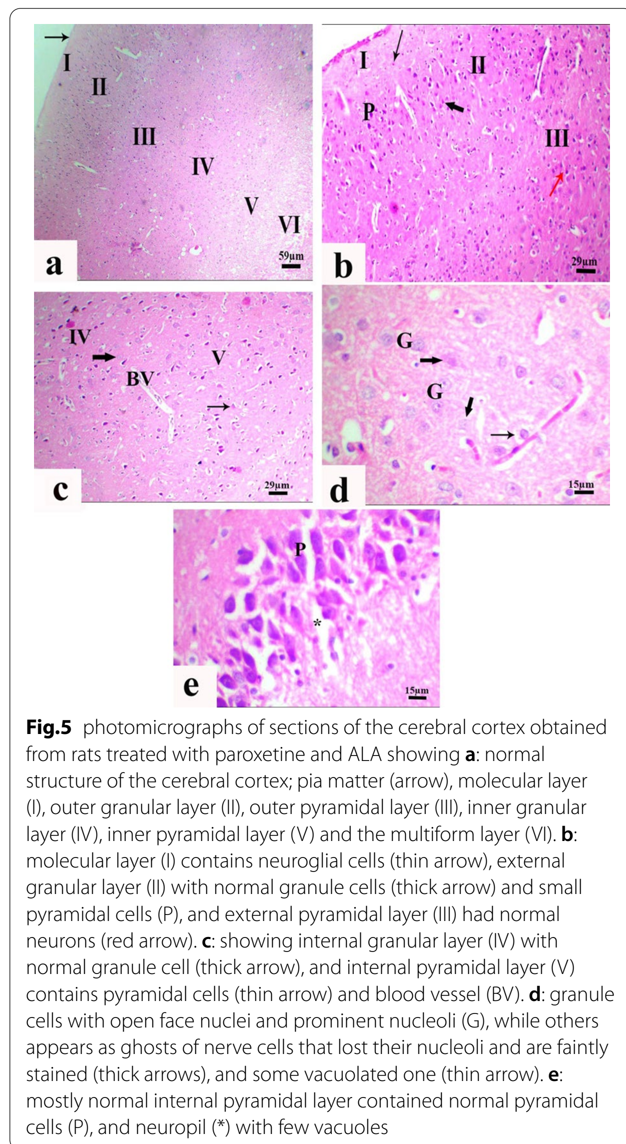

Fig.5 photomicrographs of sections of the cerebral cortex obtained from rats treated with paroxetine and ALA showing **a**: normal structure of the cerebral cortex; pia matter (arrow), molecular layer (I), outer granular layer (II), outer pyramidal layer (III), inner granular layer (IV), inner pyramidal layer (V) and the multiform layer (VI). **b**: molecular layer (I) contains neuroglial cells (thin arrow), external granular layer (II) with normal granule cells (thick arrow) and small pyramidal cells (P), and external pyramidal layer (III) had normal neurons (red arrow). **c**: showing internal granular layer (IV) with normal granule cell (thick arrow), and internal pyramidal layer (V) contains pyramidal cells (thin arrow) and blood vessel (BV). **d**: granule cells with open face nuclei and prominent nucleoli (G), while others appears as ghosts of nerve cells that lost their nucleoli and are faintly stained (thick arrows), and some vacuolated one (thin arrow). **e**: mostly normal internal pyramidal layer contained normal pyramidal cells (P), and neuropil (*) with few vacuoles

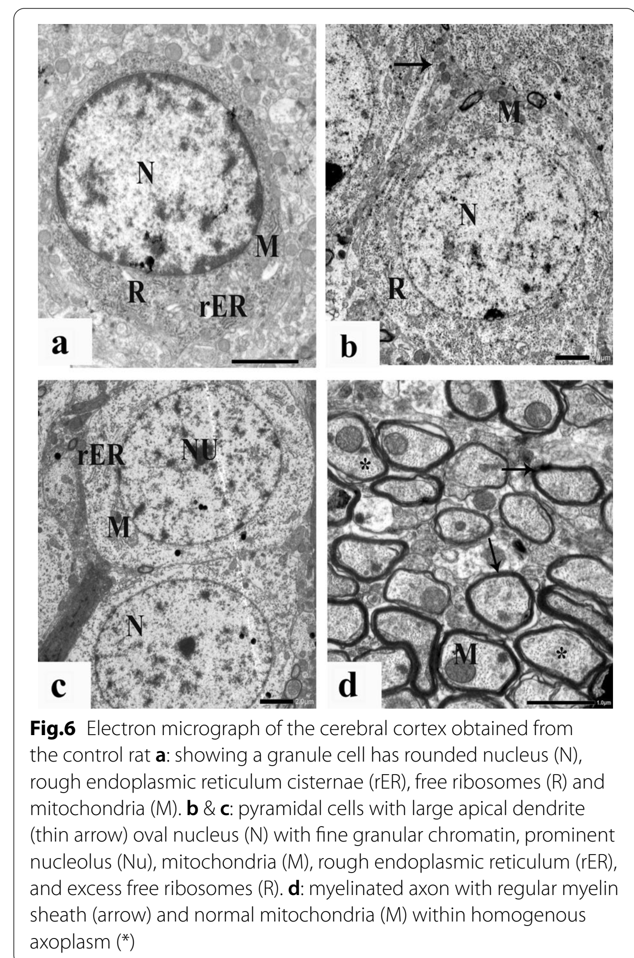

Fig.6 Electron micrograph of the cerebral cortex obtained from the control rat **a**: showing a granule cell has rounded nucleus (N), rough endoplasmic reticulum cisternae (rER), free ribosomes (R) and mitochondria (M). **b** & **c**: pyramidal cells with large apical dendrite (thin arrow) oval nucleus (N) with fine granular chromatin, prominent nucleolus (Nu), mitochondria (M), rough endoplasmic reticulum (rER), and excess free ribosomes (R). **d**: myelinated axon with regular myelin sheath (arrow) and normal mitochondria (M) within homogenous axoplasm (*)

free ribosomes, distorted or dilated rough endoplasmic reticulum, vacuoles, lysosomes and disorganized mitochondria (Fig. 8a, b). The myelinted fibers appeared degenerated and swollen with splitting or discontinuity of their myelin lamellae and the axoplasm appeared rarified contained dense mitochondria (Fig. 8c).

Electron microscopic examination of the cerebral cortex of paroxetine-treated animals showed the granule cells had shrinkage nuclei with chromatin condensation and irregular nuclear envelope. The cytoplasm of these cells contained numerous vacuoles, lysosomes, mitochondria and swollen or distorted rough endoplasmic reticulum cisternae (Fig. 9a). The pyramidal cells of the same group showed degenerated, necrotic and apoptotic features. The nuclei of these cells appeared either with irregular nuclear envelope and abnormal chromatin distribution or shrunk with marked indentation, partial chromatin condensation and the apoptotic blebs were seen. The cytoplasm of these pyramidal cells contained dilated rough endoplasmic reticulum and swollen mitochondria with destructed cristae (Fig. 9b, c). Some axon of myelinated fibers exhibited irregular contour and multiple splitting of their myelin lamellae with wide spaces in between showed splitting in their walls in addition to axoplasmic vacuoles others contained degenerated mitochondria within axoplasm (Fig. 9d).

Examination of ultrathin sections obtained from animals treated with paroxetine and ALA at the same time revealed that most of pyramidal and granule cells had nearly normal nuclei, many lysosomes, mitochondria, few dilated rough endoplasmic reticulum in between normal ones (Fig. 10a–c). The myelinted fibers showed regular arrangement of their myelin sheath and the homogenous axoplasm contained normal mitochondria; few nerves still had splitting in their myelin sheath (Fig. 10d).

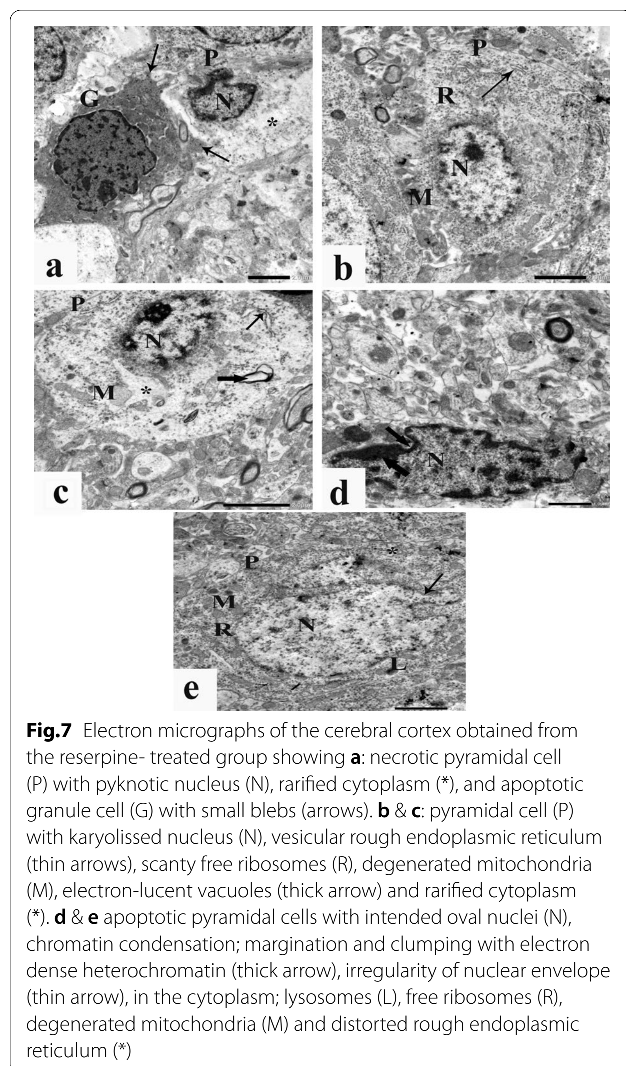

Fig.7 Electron micrographs of the cerebral cortex obtained from the reserpine- treated group showing **a**: necrotic pyramidal cell (P) with pyknotic nucleus (N), rarified cytoplasm (*), and apoptotic granule cell (G) with small blebs (arrows). **b** & **c**: pyramidal cell (P) with karyolissed nucleus (N), vesicular rough endoplasmic reticulum (thin arrows), scanty free ribosomes (R), degenerated mitochondria (M), electron-lucent vacuoles (thick arrow) and rarified cytoplasm (*). **d** & **e** apoptotic pyramidal cells with intended oval nuclei (N), chromatin condensation; margination and clumping with electron dense heterochromatin (thick arrow), irregularity of nuclear envelope (thin arrow), in the cytoplasm; lysosomes (L), free ribosomes (R), degenerated mitochondria (M) and distorted rough endoplasmic reticulum (*)

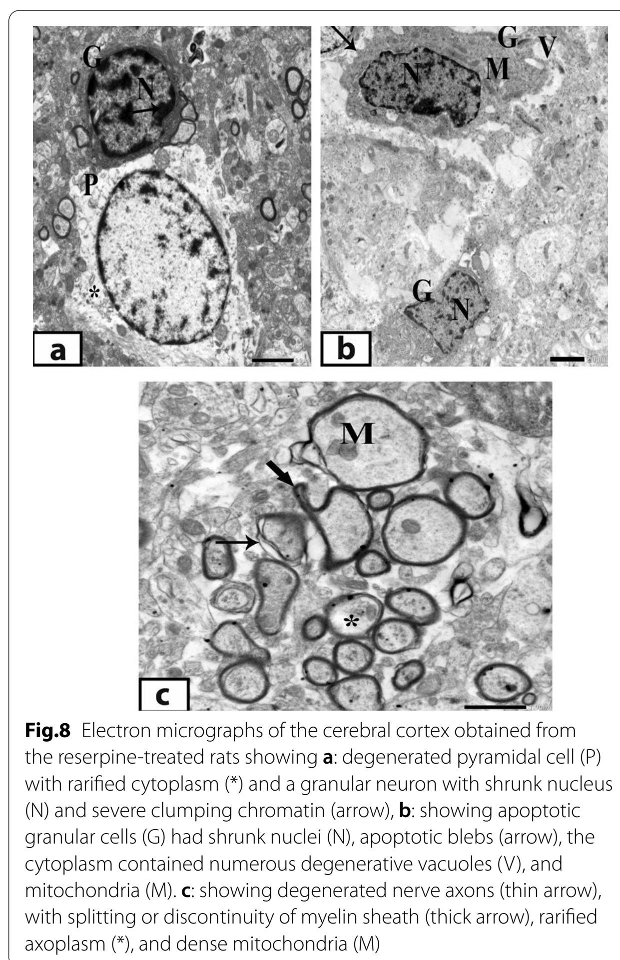

Fig.8 Electron micrographs of the cerebral cortex obtained from the reserpine-treated rats showing **a**: degenerated pyramidal cell (P) with rarified cytoplasm (*) and a granular neuron with shrunk nucleus (N) and severe clumping chromatin (arrow), **b**: showing apoptotic granular cells (G) had shrunk nuclei (N), apoptotic blebs (arrow), the cytoplasm contained numerous degenerative vacuoles (V), and mitochondria (M). **c**: showing degenerated nerve axons (thin arrow), with splitting or discontinuity of myelin sheath (thick arrow), rarified axoplasm (*), and dense mitochondria (M)

3.4 Immunohistochemical results

Examination of sections of the cerebral cortex of the control as well as ALA-treated rats showed PCNA expression in few numbers of nuclei of granule and pyramidal cells and few caspase-3 expressions in the cytoplasm of these cells, both PCNA and caspase-3 expressions appeared as brown color, in the nucleus and the cytoplasm respectively. There was non-significant difference in the percentage areas of PCNA and caspase-3 expressions was recorded between control and ALA-treated animals. The treatment with reserpine showed few numbers of cells had positive PCNA expression and large number of cell appeared with positive caspase-3 expression. A significant decrease ($P \leq 0.0001$) in the percentage areas of PCNA expression and significant increase ($P \leq 0.00$) in caspase-3 expression were recorded in reserpine-treated rats when compared with the control group. While animals treated with reserpine and paroxetine showed considerable number of cells with PCNA expression and large numbers of cells with caspase-3 expression. A significant increase ($P \leq 0.05$ and $P \leq 0.0001$, respectively) was recorded in both PCNA and caspase-3 expressions comparing with reserpine group. On the other hand, nearly normal PCNA and caspase-3 expressions appeared after the treatment with paroxetine and ALA. A significant increase ($P \leq 0.0001$) in the percentage areas of PCNA and significant decrease ($P \leq 0.0001$) caspase-3 were recorded comparing with reserpine-treated rats (Figs. 11 and 12 and Table 1).

3.5 Biochemical results

Data in Table 2 showed the changes in the levels of TAC, TOS, NE, DA and 5-HT in the brains in the different experimental groups. Concerning the levels of TAC and TOS there was non-significant difference was appeared in ALA-treated animals comparing with the control ones. When animals treated with either reserpine or reserpine and paroxetine, significant increase ($P < 0.0001$) in TOS and significant decrease

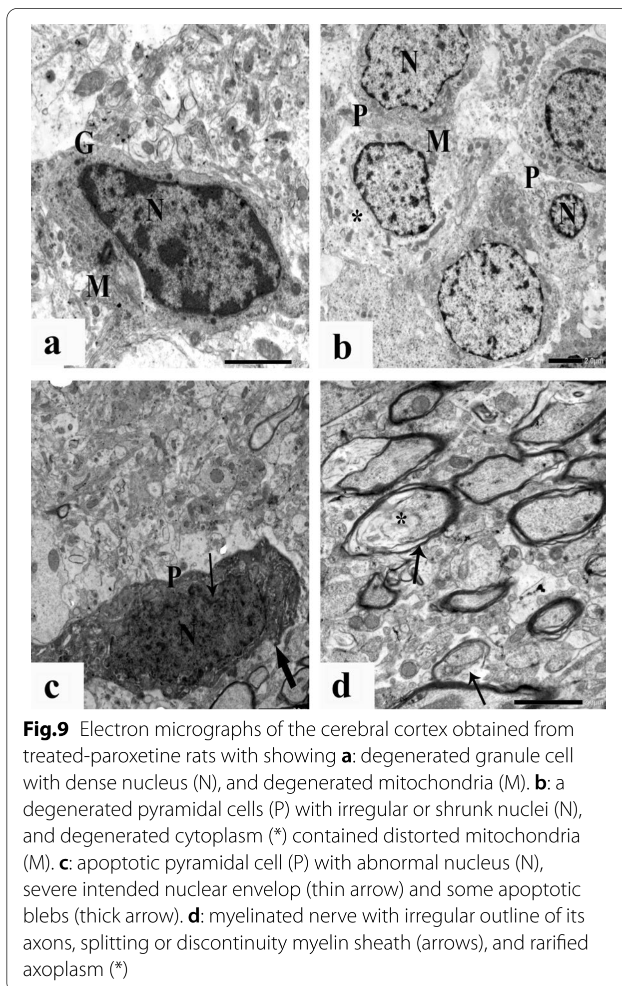

Fig. 9 Electron micrographs of the cerebral cortex obtained from treated-paroxetine rats with showing **a**: degenerated granule cell with dense nucleus (N), and degenerated mitochondria (M). **b**: a degenerated pyramidal cells (P) with irregular or shrunk nuclei (N), and degenerated cytoplasm (*) contained distorted mitochondria (M). **c**: apoptotic pyramidal cell (P) with abnormal nucleus (N), severe intended nuclear envelop (thin arrow) and some apoptotic blebs (thick arrow). **d**: myelinated nerve with irregular outline of its axons, splitting or discontinuity myelin sheath (arrows), and rarified axoplasm (*)

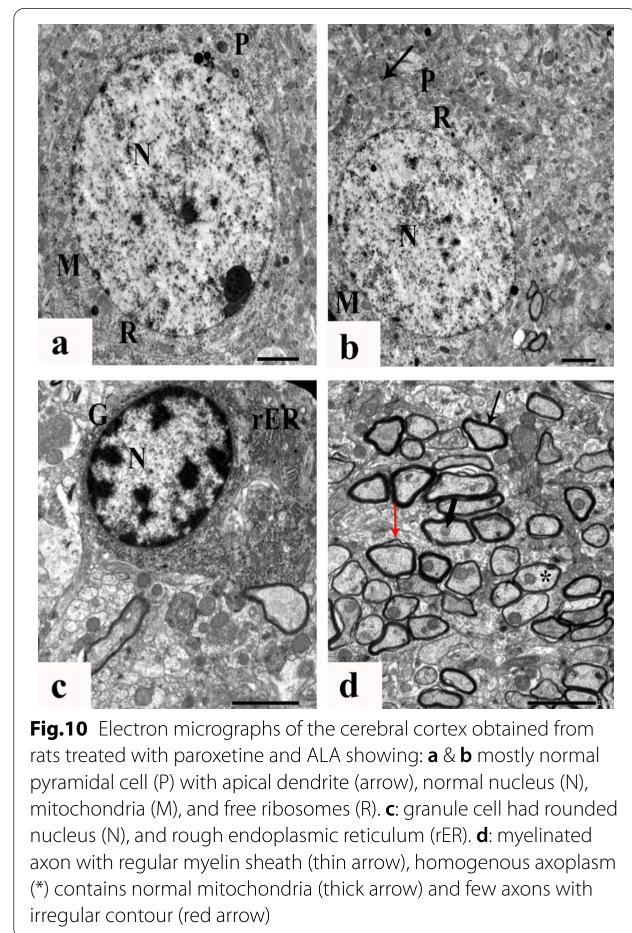

Fig. 10 Electron micrographs of the cerebral cortex obtained from rats treated with paroxetine and ALA showing: **a** & **b** mostly normal pyramidal cell (P) with apical dendrite (arrow), normal nucleus (N), mitochondria (M), and free ribosomes (R). **c**: granule cell had rounded nucleus (N), and rough endoplasmic reticulum (rER). **d**: myelinated axon with regular myelin sheath (thin arrow), homogenous axoplasm (*) contains normal mitochondria (thick arrow) and few axons with irregular contour (red arrow)

($P < 0.0001$) in TAC were recorded when compared with control group. On the other hand a significant increase ($P < 0.0001$) in TAC and significant decrease in TOS levels were recorded when depressed animals treated with both paroxetine and ALA when compared with reserpine group.

There was non-significant difference in brain levels of 5-HT, DA and NE between the control and ALA-treated animals. There was a significant decrease ($P < 0.0001$) was recorded in the brain NE, DA and 5-HT levels in reserpine-treated group when compared with the control group. The treatment with paroxetine after reserpine recorded a significant increase ($P < 0.0001$) in these neurotransmitters when compared with reserpine group. When depressed rats treated with ALA and paroxetine, significant increase ($P < 0.0001$) was recorded in 5-HT, DA and NE levels when comparing with reserpine group.

4 Discussion

Examination of the cerebral cortex of rats treated with ALA only showed no pathological changes; the six layers of the cerebral cortex appeared nearly normal without any inflammatory changes. Similarly, Perera et al. [30] and Abd-Ella et al. [31] confirmed these results and reported that ALA-treated animals showed histological findings were similar to those appeared in the control ones.

In the present work histological and ultrastructural observations of the reserpine-treated rats showed severe damage of the cerebral cortex layers that may be due to the ability of reserpine to initiate the oxidative stress in the brain via increasing the production of ROS that resulted in mitochondrial toxicity and consequently damage the cytoplasmic organelles. Similarly, the results of the previous studies confirmed that the depressed mood is often associated with an activation of immune system functions with a marked secretion of inflammatory cytokines that associated with damaging actions of exposure to oxidative stress, stimulated the synthesis of ROS, instability of antioxidant enzymes and down-regulation

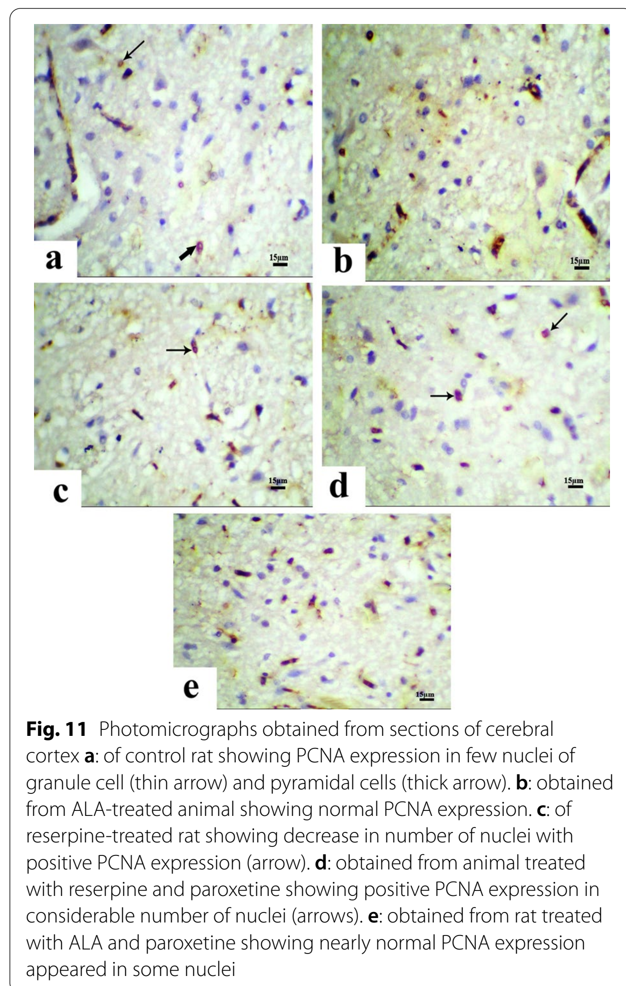

Fig. 11 Photomicrographs obtained from sections of cerebral cortex **a**: of control rat showing PCNA expression in few nuclei of granule cell (thin arrow) and pyramidal cells (thick arrow). **b**: obtained from ALA-treated animal showing normal PCNA expression. **c**: of reserpine-treated rat showing decrease in number of nuclei with positive PCNA expression (arrow). **d**: obtained from animal treated with reserpine and paroxetine showing positive PCNA expression in considerable number of nuclei (arrows). **e**: obtained from rat treated with ALA and paroxetine showing nearly normal PCNA expression appeared in some nuclei

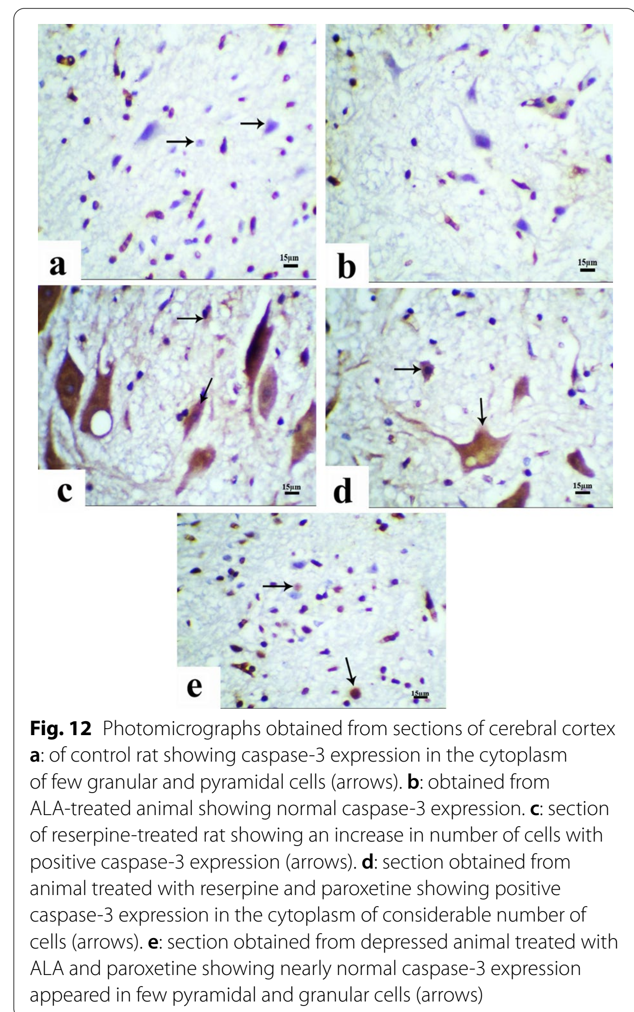

Fig. 12 Photomicrographs obtained from sections of cerebral cortex **a**: of control rat showing caspase-3 expression in the cytoplasm of few granular and pyramidal cells (arrows). **b**: obtained from ALA-treated animal showing normal caspase-3 expression. **c**: section of reserpine-treated rat showing an increase in number of cells with positive caspase-3 expression (arrows). **d**: section obtained from animal treated with reserpine and paroxetine showing positive caspase-3 expression in the cytoplasm of considerable number of cells (arrows). **e**: section obtained from depressed animal treated with ALA and paroxetine showing nearly normal caspase-3 expression appeared in few pyramidal and granular cells (arrows)

of their synthesis [32, 33]. The mechanism of cytokine involved in the depression may associated with the activation of the necrosis factor kappa B (NF-κβ) pathway peripherally and centrally resulted in reduced monoamine production, reduced neurotrophic factors, and increased excitotoxicity [34]. Tanga et al. [35] reported that reserpine is a vesicular monoamine transporter (VMAT) inhibitor that produced pathological effect by blocking and decreasing VMAT results in the accumulation of neurotoxic dopamine oxidation byproducts. Similarly, Liu et al. [36] found that reserpine caused extensive destruction of the cerebral tissue in treated mice which included severe degeneration, edema, and necrosis.

In the present study, the levels of 5-HT, DA, and NE in the cerebral cortex showed significant decrease in animals treated with reserpine which may be attributed to the cytokines activation induced by reserpine resulted in reduced the neurotransmitters store. Similarly Tsao et al. [37] reported that several cytokines are able to activate hypothalamus–pituitary–adrenal axis and able to influence the serotonergic and norepinephrine systems, which have been observed in depressed patients. Ribas et al. [38] attributed similar results to the capability of reserpine to pass the blood brain barrier, depleting the peripheral monoamine stores, and cause depletion of central stores of neurotransmitter amines which responsible for the antipsychotic effects and consequently its adverse side effects such as depression. The chronic reserpine treatment induced significant decreased in DA, NE, and 5-HT levels in the brain associated with an increase in lipid peroxide level that confirm the inflammatory potential effect of reserpine [39, 40].

A significant increase in TOS and significant decrease in TAC levels in the brains tissue were recorded in animals treated with reserpine that confirmed the ability of reserpine to induce oxidative stress status. Similarly, Fernandesa et al. [41] found that reserpine induced motor abnormalities and raised TOS levels, reduced TAC that indicated the oxidative stress-induced neuronal damage.

Table 1 Percentage area of PCNA and caspase-3 expressions in the cerebral cortex of rats in the different experimental groups

Groups	Parameter	
	The percentage areas of PCNA expression (Mean ± SD)	**The percentage areas of caspase-3 expression (Mean ± SD)**
Control	2.15 ± 0.46	1.63 ± 0.03
ALA	2.02 ± 0.09	1.71 ± 0.26
Reserpine	1.08 ± 0.91**	13.50 ± 1.78**
Reserpine + paroxetine	2.99 ± 0.94*[a]	14.26 ± 2.43**
Reserpine + paroxetine + ALA	2.10 ± 0.77[a]	4.24 ± 0.62**[a]

$n = 8$ animals for each group

(*) $P \leq 0.05$ and (**) $P \leq 0.0001$ significant comparing with control group

[a] $P \leq 0.0001$ significant comparing with reserpine group

Table 2 Effect of different treatments on the levels of brains TOS, TAC, serotonin, dopamine and norepinephrine in the different experimental groups

Groups	Parameters				
	TOS µmol/g tissue	**TAC µmol/g tissue**	**Serotonin µg/g**	**Dopamine µg/g**	**Norepinephrine µg/g**
Control	5.51 ± 0.68	64.83 ± 10.06	94.17 ± 12.31	210.50 ± 33.17	287.00 ± 25.06
ALA	5.31 ± 1.03	65.33 ± 11.20	93.33 ± 12.94	207.33 ± 27.63	298.00 ± 22.38
Reserpine	27.68 ± 4.20**	34.17 ± 5.49**[b]	45.17 ± 9.17**	108.17 ± 15.58**	110.00 ± 16.21**
Reserpine + Paroxetine	21.83 ± 4.62**[a]	33.50 ± 5.28**[b]	84.67 ± 13.50**a	156.67 ± 39.02** [a]	232.17 ± 43.37*[a]
Reserpine + Paroxetine + ALA	6.25 ± 0.59[a]	54.67 ± 5.08*[a]	104.17 ± 27.65**[a]	195.50 ± 23.88[a]	258.83 ± 47.91**[a]

$n = 8$ animals for each group

(*) $P < 0.01$ and (**) $P < 0.0001$ significant comparing with control group

[a] $P < 0.0001$ and [b] $P < 0.001$ significant comparing with reserpine group

In the present study, treatment with reserpine revealed significant decrease in PCNA expression and significant increase in caspase-3 expression. These results may be attributed to the ability of reserpine to inhibit DNA repair and cell proliferation and activate the caspase path way. Ramu et al. [42] found that reserpine oral administration markedly reduced the expression of PCNA and inhibited DNA repair factor and cell proliferation via inhibits transforming growth factor-β (TGF-β) mediated phosphorylation and nuclear translocation. The same authors added that reserpine induced apoptosis through blockade of the Ku70 function, is DNA repair subunit protein, and up regulated the apoptotic protein thereby activate Bax, mediated programmed cell death and activated cytochrome C release and caspase-3. The activation of caspases destroys important cellular machinery, preventing the synthesis of new proteins, which ultimately leads to irreversible cell injury [43].

In the present study, treatment with paroxetine showed no improvement in the histological and ultrastructural changes and the tissues still unhealthy and faraway from control picture. Moreover, there was significant decrease in TAC and significant increase TOS in levels comparing with the control animals, but recorded significant decrease in TOS comparing with reserpine group. These results mirrored the ability of paroxetine to mild improve, minute effect, against the induced pathological damage.

In other studies, similar pathological effects were confirmed our findings. Testicular damage appeared in the paroxetine-treated animals that may be attributed to the ability of paroxetine to alter hormonal homeostasis that disrupt spermatogenesis and damage cell membrane [44, 45]. Clear tissue degeneration and necrosis were observed in the brain tissue of paroxetine-treated mothers and their embryo which may be resulted from the ability of antidepressant drug to decrease antioxidant levels and increase oxidant levels by elevating superoxide anion, lipid peroxidation and induced oxidative stress and these consequences may cause neuronal damage of the brain [46]. Moreover, Kumar et al. [47] reported

that SSRIs might affect ATP synthesis inhibitory oxidative phosphorylation in mitochondria. Hamad et al. [48] found that SSRIs had minute effect on histological structure of cerebral cortex include dead neurons in the second and third layers of the grey matter. Paroxetine is often associated with nonspecific toxicity (necrosis) due to alteration processes mainly of cell membrane [49].

In the present study the treatment with paroxetine showed significant increase in the percentage area of both PCNA and recorded caspase-3 expressions which may be due to the ability of paroxetine to increase both caspase-3 activity and DNA fragmentation. Similarly, Kamarudin and Parhar [50] reported that treatment with paroxetine led to a concentration-dependent reduction in cell viability that was followed by a concentration-dependent increase in DNA fragmentation and apoptotic by increased caspase-3 activity that was preceded by the rapid and transient activation of phospho-c-Jun levels and subsequent mitochondrial release of cytochrome C. Additionally, paroxetine was shown to induce an increase in cytosolic free $Ca2+$concentrations [51]. Paroxetine induces apoptosis of rats' astrocytes through increase $Ca2+$influx, decrease mitochondrial membrane permeability and production of ROS [52]. The excessive generation of ROS caused oxidative stress which contributes to adverse event inducing neuronal cell death and characterized the cleavage of caspas-8 and 9 and mitochondrial permeability and increase Bax/Bcl-2 ratio [52, 53].

A significant increase in NE, 5-HT, and dopamine levels was recorded after paroxetine treatment comparing with reserpine-group which still recorded significant decrease comparing with control group. Paroxetine enhanced serotonergic activity via the inhibition presynaptic reuptake of serotonin by the serotonin receptor. This inhibition raises the level of serotonin in the synaptic cleft, relieving various symptoms [54]. Kamal [55] and Zhong et al. [56] demonstrated that paroxetine treatment may increase 5-HTT and NE levels which may disrupt oligodendrocytes maturation.

In the present study, ALA and paroxetine treatment showed normal architecture of cerebral cortex that confirmed by histological, ultrastructural results and restoration the normal ranges of TAC, TOS, NE, 5-HT, and DA. The significant increase in antioxidant activity coupled with the histological evidence may be attributed to the ability of ALA to reduce the induced oxidative damage in rat's brains via its scavenging ability to intracellular superoxide improving mitochondrial function and DNA damage which supported the ability of ALA to decrease apoptosis as observed in this study. The mechanism by which ALA improved cerebral necrosis and decrease the intensity of damage include angiogenesis with sprouting of numerous capillaries and arterioles within areas of necrosis which resulted in the atrophic neurons were infrequent and the majority of neurons became healthy with visible axons and dendrites [30]. Treatment with ALA significantly improved the oxidative stress condition, histological and morphometric picture of the pancreas, and restored normal expression of related genes, including Nrf2, caspase-3, and Bcl-2 [57]. The efficacy of ALA against different insults induced toxicity could be attributed to its antioxidant, anti-apoptotic, and anti-inflammatory activities, regulated inflammatory cell infiltration into the central nervous system, that prevent the oxidative damage through direct scavenging of ROS, regeneration of other antioxidants such as vitamin C and E and by increasing intracellular GSH level as well as it down-regulate inflammatory processes by reducing pro-inflammatory redox-sensitive signal transduction processes including NF-κβ translocation, leading to lower release of other free radicals, cytotoxic cytokines and improves neuron degeneration [58–61].

The treatment with ALA in neurodegenerative disease models, can improve the function of DA, 5-HT and NE [62]. Moosazadeh et al. [63], shown that ALA has a positive intervention in the neurodegenerative processes, by reducing neuronal apoptosis and supporting a neuron protective role mediated by the mitochondrial cell death process and it able to inhibit the formation of amyloid β fibrils, thereby improving the consequent neurological damage, and it significantly restores acetyl choline esterase activity which support the hypothesis that it could be used as a supplementary treatment in neurodegenerative diseases. Sun et al. [58] demonstrated that ALA treatment significantly reduced caspase-3 activity. Also, it was reported that the nuclear translocation of apoptosis-inducing factor is involved in protective effect of lipoic acid [64]. Recently, Bahaa El-Dein et al. [65] found that Alpha-lipoic acid treatment showed evident improvement of the histological picture of the islets of Langerhans, revealed little caspase-3 immunoreactivity and significant elevation in TAC compared to the stressed rats. In addition, diabetic rats treated with ALA showed markedly reduced plasma levels of inflammatory markers, plasma and cardiac tissue malondialdehyde, and significantly increasing cardiac glutathione level and superoxide dismutase activity [66]. As well as Tanbek et al. [67] recently concluded that ALA improved the histological and ultrastructural damage include the degeneration findings observed in the diabetic animals and attributed that to the effective role of ALA in restoring cell damage and cognitive functions in brain tissue via its antioxidant and neuroprotective properties.

5 Conclusions

The increased vulnerability of rat's brains to the induced oxidative stress and the improvements in the histopathological, biochemical, and immunohistochemical analyses as well as ALA could improve the effect of paroxetine in the present study. These findings confirmed the free radical scavenger, anti-apoptotic and anti-inflammatory hypothesis of ALA against reserpine that induced cerebral injury.

Abbreviations

5-HT: Serotonin; ALA: Alpha-lipoic acid; Aß: Amyloid β; CNS: Central nervous system; DHLA: Dihydro-lipoic acid; GSH: Glutathione; NE: Norepinephrine; NF-κβ: Necrosis factor kappa B; PCNA: Proliferating cell nuclear antigen; ROS: Reactive oxygen species; SSRIs: Selective serotonin reuptake inhibitors; TAC : Total antioxidant capacity; TGF-β: Transforming growth factor-β; TOS: Total oxidative stress; VMAT: Vesicular monoamine transporter.

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Author contributions

SMA performed the experimental design,performed all experiment practically, the biochemical, histological, ultrastructural, and immunohistochemical assessments. Then collect data, analyzed and interpreted the results, finally write, review and approved the final manuscript.

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Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

Principles of animal care and use were carefully followed during conducting the present study according to the guide for the care and use of laboratory animals approved by Faculty of Science, Menoufia University, Egypt (Approval No. MUFS/F/HI/5/21), and according to the National Institutes of Health guide for the care and use of laboratory animals (NIH publications No. 8023, received 1978).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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