Biochemistry Letters, 20 (1) 2024, pages 76-90

Scientific Research & Studies Center-Faculty of Science- Zagazig University- Egypt

Biochemistry Letters

Journal home page:

Curcumin Nanoparticles effects in protection and reducing Alzheimer's Disease induced by Aluminum Chloride

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ARTICLE INFO

Received :2/7/2024
Accepted : 30/8/2024
Available online : 31/8/2024

Keywords:
Inflammation
Phosphorylated tau protein
oxidative stress
Cognitive impairment
Beta amyloid protein

ABSTRACT

Objective: The purpose of the current study was to use the curcumin nanoparticles in the protection and reduction of Alzheimer's disease (AD) induced by Aluminum chloride ($AlCl_3$) in male rats. *Materials and Methods:* 24 male albino rats were divided into four groups: group I (the negative control), group II (the diseased group), group III (the protective group), and group IV (the treated group). For each group, behavioral test was performed, biochemical evaluation of Malonaldehyde (MDA) levels and Catalase (CAT) activities, β-amyloid protein (APP) gene expression, immune-histochemical expression of p-Tau, and histological examination of the hippocampus were done. *Results:* Curcumin nanoparticles decreased the latency to escape box in the protective and therapeutic groups. Furthermore, there was reduction in the oxidative stress in MDA levels as well as downregulation of the relative expression of APP of brain tissue. It also ameliorated the histopathological alterations in the hippocampus with decreased immune expression of p-Tau protein. *Conclusion:* Nanocurcumin particles ameliorated the structural and functional alterations in the brain induced by $AlCl_3$ so, it could be considered one of the promising therapeutic compounds for treating AD.

Introduction:

Alzheimer's disease (AD) is an age-dependent disease that affects more than 10% of the elderly population 65 years of age and older. Around 50 million people worldwide suffer from AD. Cognitive impairment and dementia are hallmarks of AD. Ageing, head traumas, infections, mitochondrial dysfunction, oxidative stress, inflammation, and environmental factors like aluminum (Al) toxicity are some of the essential factors that contribute to the development of AD **[1]**. Symptoms of AD include swinging of mood, short-term memory loss,

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gradual decline in cognition, dementia, ending with losing body functions and death **[2]**. The etiology of AD is underpinned by two primary mechanisms; firstly, there is a build-up of tau protein inside brain neurons and beta-amyloid (Aβ) outside of them, which causes neuronal death. The second mechanism is believed to be calcium ion disruption-induced neuro-inflammation and cholinergic dysfunction. **[3]**. Aluminum (Al) inhibits long-term potentiation, induces inflammatory responses, affects the slow and fast axonal transports and causes synaptic structural abnormalities, resulting in profound memory loss **[4]**. Many researchers have made efforts in recent years to determine the effects of a whole plant extract on AD in addition to identifying the bioactive compounds that play a protective role. Natural compounds found in plants called phytochemicals offer a variety of biological and structural properties, making them a desirable starting point for the development of compounds that combat AD; one of these phytochemicals is curcumin **[5]**. Curcumin is characterized by unstable nature, quick metabolism, low water solubility, and low bioavailability in the central nervous system (CNS) **[6]**. Researchers developed novel methods for effectively administering drugs to the brain. Curcumin's water solubility can be increased by nanocarriers, which will increase the drug's bioavailability and tissue distribution, and ultimately its effectiveness in clinical applications **[7]**. The 100 nm size of nanocurcumin allows it to effectively break through the blood brain barrier (BBB) and release curcumin. Histopathological analysis revealed that the nanostructure protected against neuronal damage **[8]**. Experimental studies have shown that nanocurcumin has superior antioxidant, anti-proliferative, neuro-protective, and anti-inflammatory effects compared to curcumin **[9]**. Therefore, nanotechnology could provide new promises for the treatment of AD **[10].**

The aim of this study is to investigate the therapeutic and protective role of Curcumin nanoparticles on AD induced by $AlCl_3$ in male rats. Furthermore, to examine the histological and biochemical alterations that take place in the studied groups.

II. Materials and Methods

II.1. Material:

II.1.1. Chemicals:

- Loba-Chemie sells curcumin with a 95% curcuminoid content, which is what we bought. Chem-Lab supplied the acetic acid, while Millipore Merck supplied the TPP, 100% ethanol, and low molecular weight chitosan**.**
- Aluminum chloride ($AlCl_3$) was provided by Sigma-Aldrich (UK). CAS number: 7446-70-0, lot number: BCCB0771.
- ***Biochemical Kits:*** (MDA) and (CAT) kits were purchased from (Biodiagnostic, Dokki, Giza, Egypt).
- **Total RNA Purification Kit, RNA extraction kit** (Thermo Scientific, Fermentas, #K0731) and **Reverse transcription kits** (Thermo Scientific, Fermentas, #EP0451).
- **Primary antibodies for immuno-histochemical examination:** Rabbit anti-rat polyclonal phosphorylated Tau (P-Tau) antibody (Biosource International, Inc. USA)

II.1.2. Animals:

24 adult male Albino rats weighed (170 -190 g) were purchased from the animal house of Faculty of

Medicine, Zagazig University, Egypt. They were housed in the cages in the animal house ,They had unrestricted access to food and *ad libitum* water.

II.1.3. Approval of the Ethics Committee*:*

The experimental animals procedures approved by Zagazig University Institutional Animal Care and Use Committee, Zagazig University guidelines number (ZU-IACUC/1/F/105/2019).

II.1.4. Experimental Groups:

The animals were divided into four groups, ***Group I (negative control group)****:* were fed only on normal diet. ***Group II (diseased group):*** $AlCl_3$ was injected daily intraperitoneal (IP) at a dose of 40 mg/kg for 45 days **[11]**. ***Group III (protective group):*** nanocurcumin was administered orally dose of 150 mg/kg for four weeks **[12]**, after the first week of nanocurcumin, $AlCl_3$ was injected at a dose of 40 mg/kg/day for 45 days. ***Group IV (treated group)*****:** rats were first injected (IP) daily with $AlCl_3$ at a dose of 40 mg/kg/day for 45 days then, nanocurcumin was daily administered as in group III, for another four weeks.

II.1.4. Sampling:

At the end of the experiment, the behavioral test was performed then rats were sacrificed. After dissection, the whole brains from all groups were removed carefully, washed with saline, and divided into two halves. The first half was used for histological and immunohistochmical investigation and the second half was prepared for biochemical investigations.

II.2. Methods:

II. 2.1. Synthesis of nanocurcumin particles:

4 M NaOH was applied to reduce the pH to 5.0 after mixing 0.2% w/v chitosan solution with 2% v/v diluted acetic acid. A magnetic stirrer was used to agitate the liquid at 500–1000 rpm prior to the dropwise addition of an ethanolic curcumin solution (100 or 500 μg/mL). Then, dropwise additions of the cross-linker (TPP, 0.1% w/v) solution were made to this mixture until the mass ratios of TPP to chitosan reached 3:1. Before the nanoparticles were pelletized using a Hermle Z32 centrifuge (Germany) and centrifuged for 20 minutes at 10,000 rpm and 20°C, the mixture was stirred for an additional 10 to 45 minutes at ambient temperature. Then, the particle was again dispersed in distilled water **[13]**.

II.2. 2. ***Behavioral Test***

The modified Barnes maze (MBM) used to asses memory and learning in rats, based on a previously method **[14].** The maze consists of woody white circular platform elevated (108 cm off the floor) with122 cm in diameter , 1 cm thickness with 20 equally spaced holes (diameter = 10 cm). A black woody chamber was placed in the center of maze (an opaque, 20 cm× 30 cm long .A removable black escape box 38.7 cm long × 12.1 cm wide was placed under one hole of platform. 24 hours before the acquisition trial (AT) ,habituation was done for all rats. On days 1-3 , six acquisition trials were done in a form of 2 trials per day until the rat enter escape box. On days 4 and 9 two probe trials (PT) were done each one was 2 min . For each trial the latency to reach escape box were calculated .

II.2.3. Biochemical analysis**:**

II.2.3.1. preparation of tissue homogenate :

Tissue homogenate was prepared according to method of **Keshta *et al.*, [15]**.

***II.2.3.2. Assessment of* MDA and CAT in the studied groups**: tissue levels of Malonaldhyde (MDA) were estimated according to method described by Ohkawa *et al.*,**[16]** and that of catalase (CAT) activity were measured according to the method of **Aebi;** respectively.**[17]**

II.2.3.3. Quantitative real-time PCR analysis of β-amyloid precursor protein (APP) gene:

Brain tissue were stored in liquid nitrogen for the PCR procedure **[18]**. The β-amyloid precursor protein (APP) genes' mRNA expression was determined using RNA Purification Kits. Following the extraction of total RNA from brain tissues using a kit (Thermo Scientific, Fermentas, #K0731), the RNA was genetically modified M-MuLV RT (Thermo Scientific, Fermentas, #EP0451) to convert it into complementary DNA (cDNA). Semi- quantitative real-time PCR was performed using PCR equipment (Stratagene MX3005P) and SYBR Green PCR Master Mix (Quanti Tect SYBR Green real time PCR Kit; Thermo Scientific, USA, # K0221). The relative mRNA expression of the target genes was evaluated using the 2Ct technique **[19]**. **Table (1)** contains a list of primer sequences that were employed.

II.2.3.4. Histological examination:

Sections of the brain tissue were fixed in Bouin's solution, dehydrated, and embedded in paraffin wax. 5 μm thick sections were stained with hematoxylin and eosin (H&E) for routine histological examination **[20]**.

***II.2.3.5.*Immunohistochmical examination:**

Immunohistochmical detection of anti P-Tau antibody was performed using the streptavidin–biotin complex immunoperoxidase technique. Sections of brain tissue were deparaffinized on positive charged slides then incubated in 0.1% hydrogen peroxide for 30 min to block the endogenous peroxidase activity then, they were incubated overnight with the corresponding primary antibody. After several washing with PBS, sections were incubated with secondary antibody (biotinylated goat anti-rabbit IgG, Zymed Laboratories, South San Francisco, CA, USA) for 30 min at room temperature then with streptavidin–peroxidase conjugate. After that, sections were washed with PBS and incubated with diaminobenzidine (DAB; Sigma–Aldrich, St. Louis, MO, USA) for 5 min and counterstained with Mayer's hematoxylin. PBS was utilized in place of the primary antibody for the negative controls **[21]**.

II.2.3.6. Morphometric study:

The Medical Histology and Cell Biology department at Zagazig University used the Leica Qwin 500 (Leica Ltd., Cambridge, UK) image analyzer computer equipment and the Fiji image J (1.51n, NIH, USA) program to assess the area percentage of immune-positive cells for P-TAU. Measurements were taken using the interactive measure menu. Each group's rat's ten measurements from five different locations were assessed.

***II.4.* Statistical Analysis**

Data were expressed using the means ±S.D. The statistical significance was evaluated using a one-way analysis of variance (ANOVA) and individual comparisons were produced using Duncan's multiple range test (DMRT) using SPSS, 18.0 software from 2011. Values were considered statistically significant when $p<0.05$.

The test for the least significant difference (LSD) was also used to assess significance across groups **[22]**.

III. Results

III.1. *Barnes maze test (Cognitive changes):*

The present study showed that the AD group (group II) took more time in finding the escape box during training and test days, compared to other groups, which indicates the protective role of nanocurcumin against AD, but protective and treated group spent less time (significant) on the test day compared to the control group. The protective group (group III) showed a significant improvement compared to the treatment group (IV). Groups during trials AT1 - AT6 showed a better performance in both protected and treated groups in **Figure (1A)**. Latency to escape box from AT1-AT6, increased in group II which ranged from (249.38±31.68-111.44±27.87), compared to control group (82.04±26.82- 10.82±2.38), but latency in the protected groups (91.44±10.55- 17.91±5.08) and treated group (154.09±30.43 - 40.99±19.31) both scored less time to reach escape box, All groups differed considerably from each other (P<0.001).

Escape hole latency in Probe Trial (PT1), was significantly increased in group II than all other groups, (P<0.001) ,latency to escape hole in control group took an average of (31.35±17) compared to group II which took longer time but Protected group (47.05±9.57) scored less time than Treated group (91.21±12.67), (P<0.001).

Control group in PT2 took average time (29.55±16) compared to rats in group II (104.19±27.42) whereas rats took less time in both protected **(**32.11±1.41) and treated groups **(**95.49±6.64) , all groups were significantly different from each other, (P<0.001) as seen in **Figure (1B)**.

III.2. Effect of nanocurcumin treatment on the levels of MDA and the activity of CAT in the brains of $AlCl_3$-treated rats.

MDA level was significantly higher in group II with mean value (10.55±0.44) compared to control group (4.05±0.20),while treatment with nanocurcumin in group III (6.49±0.26), and group IV (8.79±0.37) showed significantly decreased levels of MDA, ($p < 0.001$) compared to group II, as shown in **Figure (2A)**

The mean activity of CAT was found to be suppressed in AD group with mean value (85.1±4.59) compared to control group (445.35 ± 23.62) , but protective doses of nanocurcumin in group III (278.85± 13.62) elevated than treated group (157.69± 7.80) compared to AD group, ($p < 0.0001$),as seen in **Figure(2B)**

III.3. β-amyloid relative expression by Real time PCR:

Expression levels of beta amyloid protein in the brain tissue were significantly increased in group II (16.11± 0.86) compared to the control group (1.00±0.00). However, beta amyloid levels were significantly decreased in both protected and treated groups (3.66±0.16), and (7.11± 0.36); respectively, when compared to AD only group ($p < 0.05$) as shown in **table (2)**.

III.4. Histological rasults:

Sections stained with hematoxylin and eosin (H&E) from the control group demonstrated that the dentate

gyrus (DG) and the Cornu Ammonis (CA) areas comprised the hippocampus formation. Granular, polymorphic, and molecular layers came together to form the dentate gyrus. Closely spaced granule cells with vesicular nuclei made the granular layer. Normal pyramidal cells with vesicular nuclei were also visible in the other CA levels. Hippocampal areas in Group II displayed significant distortion along with a noticeable loss of cells, and the cells displayed black apoptotic nuclei. Areas of the hippocampal tissue showed some vacuolations. Group III had a small number of cells with dark nuclei and a majority of normal-looking cells. Group IV sections revealed that many cells were still shrunken and had dark apoptotic nuclei **(Fig. 3).**

III.5. Immunohistochmical results:

Sections stained with an anti-p-Tau antibody from group I showed a mild brown staining of the perikaryal cytoplasm of the neuronal cell bodies and their apical dendrites, which showed up as long, thin threads in the granule cells of DG and the pyramidal cells of the cerebral cortex. The cytoplasm and apical dendrites of group II's neural cells were stained a dark brown color, indicating a high expression of p-Tau in the cytoplasm and processes of neuronal cells, Group III showed a weak expression of p-Tau. The apical dendrites and cytoplasm of neurons in Group IV showed significant brown staining, indicating moderate expression of p-Tau **Fig(4).**

III.4.3. Morphometric results of histopathological examination:

In comparison to the control group, the mean area percentage of P-Tau immuno-expression in the hippocampal tissue was significantly higher in the AD group (Group II; $p < 0.0001$), but it was much lower in the treated and protected groups, with group III showing the greatest improvement. Group IV had a statistically significant difference from the other groups ($p < 0.0001$) (**Table 3).**

IV. Discussion

The pathology of AD is characterized by the presence of neurofibrillary tangles composed of hyperphosphorylated tau proteins and β-amyloid plaques in the brain. Widespread neuronal injury and atrophy have been linked to the buildup of these protein aggregates **[23]**.

Curcumin Nanoparticles with small size 100 nm have higher bioavailability and hence, greater efficacy in a possible clinical application **[24],** Thus, the purpose of this study is to evaluate the therapeutic and preventive effects of nanocurcumin in a rat model of AD caused by $AlCl_3$. Curcumin nanoparticles enhanced the recognition and learning ,This is due to nanocurcumin has antioxidant effect that increased the memory and learning **[25]**. The recognition memory of aged rats has been significantly enhanced by both short-term and long-term curcumin which support previous research assessed the effects of (6-week, 12-week) curcumin diet (480 mg/kg) on the proliferation of hippocampal cells showed a greater improvement in spatial memory and learning **[26]**. This study corroborated those of Helli *et al*., and Matias *et al*., showed the advantages of curcumin therapy for neuropsychiatric disorders and cognitive deficits **[27-28]**.

The study evaluated the role of oxidative stress in the pathophysiology of AD in the hippocamous which responsible for

recognition and spatial memory **[29]**. MDA recorded a significant decrease in nanocurcumin tested groups (protective &therapeutic) ,While CAT showed a significant increase in the same groups ,This is due to Al is well recognized to easily cross the BBB and accumulate in various regions of the brain and it could also trigger the free radical's production that could cause the brain injury particularly regions responsible for the memory and learning which is consistent with **Lakshmi *et al*.**, **[30]**. On another hand curcumin is a potent antioxidant that plays a vital role in defense versus reactive oxygen species (ROS) damage and regenerating other antioxidants, showing a neuroprotective effect against cognitive impairment and aluminum-induced oxidative stress **[31-32]**. It is able to thwart lipid peroxidation **[33]**. This was in agreement with **Ibrahim *et al*.**, **[34]** and **Kahya *et al*.**, **[35]** who showed that $AlCl_3$ injection decreased CAT activity and caused oxidative stress which can be inferred through the increase in lipid peroxidation, and also agreed with **Tee *et al*.,[36] ,**who proved a significant elevation in MDA caused by the $AlCl_3$ treatment due to Al-induced reduction in both synaptic vesicles resulting in the release of oxidative products in neurons. But in contrast, Rajeswari's study found that curcumin increased lipid peroxidation (MDA) in mice's midbrain **[37],** another study reported high activity of CAT enzyme, this may be due to tissue specificity, duration or disease severity **[38]**. APP gene expression levels were significantly down-regulated in nanocurcumin tested groups with greatest improvement, which may be due to Al is considered as an inducer of neurotoxicity via brain ROS induction. Al accumulation induced oxidative stress in CNS, stimulating amyloidogenic fragments **[39]**.The immature APP was significantly retained in the endoplasmic reticulum by curcumin **[40]**. Curcumin thus reduced the production of amyloid protein **[41],** these results are in line with our study .

Tau is a marker of neurodegeneration, elevated in many neurodegenerative diseases and brain conditions **[42-43]**. p-tau showed a significant decreased in neuronal cells after administration of nanocurcumin in both protective and treated groups because nanocurcumin was able to down-regulate the p-tau deposition and induced activation of microglia **[44-45]**. Tau-phosphorylation is closely associated with tau-related brain atrophy **[46]**. Tau oligomers have the potential to alter anatomical features and disrupt synaptic transmission.

V. Conclusion

Nanocurcumin particles reduced the reactive oxygen species resulted from $AlCl_3$ toxicity induced Alzheimer disease. Consequently, nanocurcumin helps in protecting our brain from neurodegenerative disorders.

VI. Acknowledgments

The authors express their gratitude to the National Institute of Laser Enhanced Sciences, Cairo University, Giza, Egypt, Animal House, the faculty of science, Zagazig University Researchers Supporting, and all of them together.

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Table (1): Forward and reverse primers sequence for primers used in qPCR

Gene	**Forward primer (′5 ------ ′3)**	**Reverse primer (′5 ------ ′3)**
APP	CAACCGTGGCATCCTTTTGG	CGTCGACAGGCTCAACTTCA **[47]**
(β-Actin)	AAGTCCCTCACCCTCCCAAAAG	AAGCAATGCTGTCACCTTCCC **[48]**

Table (2): Relative expression of *β-amyloid* precursor protein (APP) gene

Group	**Fold change mean± SEM**		**%change**
Group I	**1.00 ±0.00**		**_**
Group II	**16.11±0.86**	***p* < 0.0001*** **	**1.51**
Group III	**3.66±0.16**		**-77.28**
Group IV	**7.11±0.36**		**-55.86**

SEM: Standard error mean, * ** : highly significant (P<0.0001), Post hoc: Tukey test

Table (3): Statitical results of the mean area % of P-TAU immunoexpression in the different groups

	Group I	**Group II**	**Group III**	**Group IV**	***P***
Area % P-TAU	8.4±2.37	35.2±3.61[a]	17.5±1.09[a,b]	25.2±2.7[a,b,c]	< 0.0001

Data are presented as mean ± SD.
a: significant with group I b: significant with group II c: significant with group III

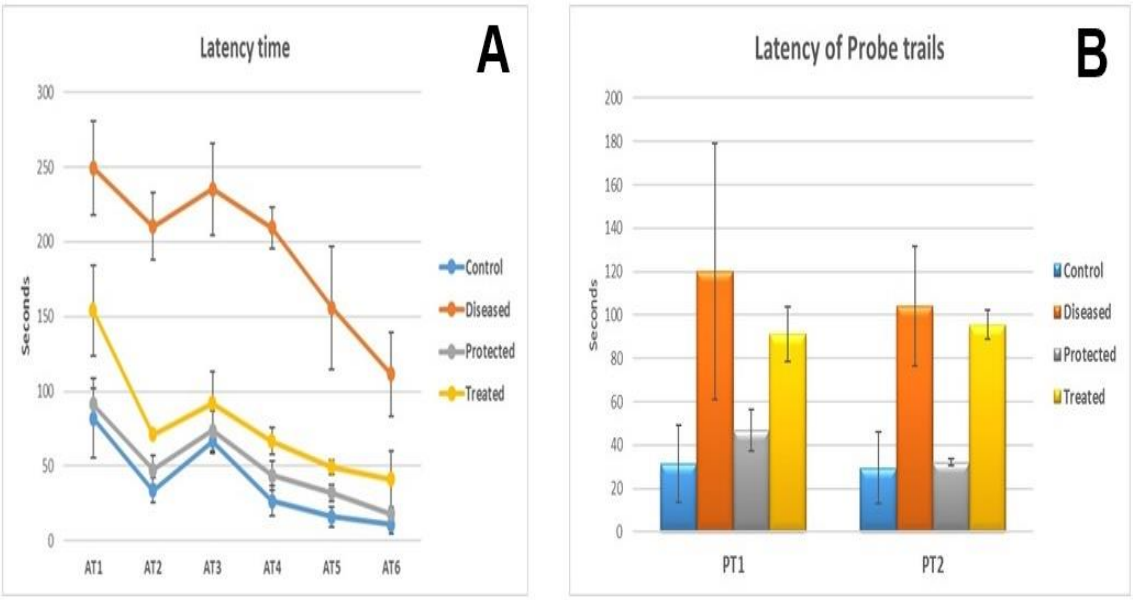

Figure (1): Latency to escape in all the studied groups **A:** at different trails, **B:** at Probe trails.

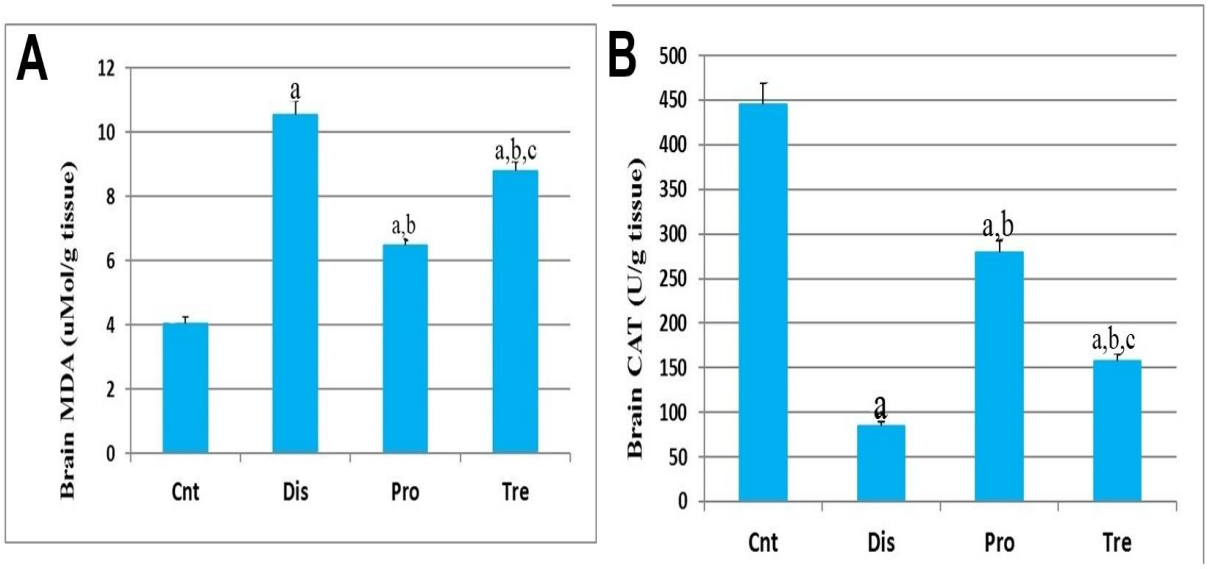

Fig (2): Graphical presentation of the statistical results showing:
A: The MDA level in the studied groups.
B: The CAT activity in the studied groups.

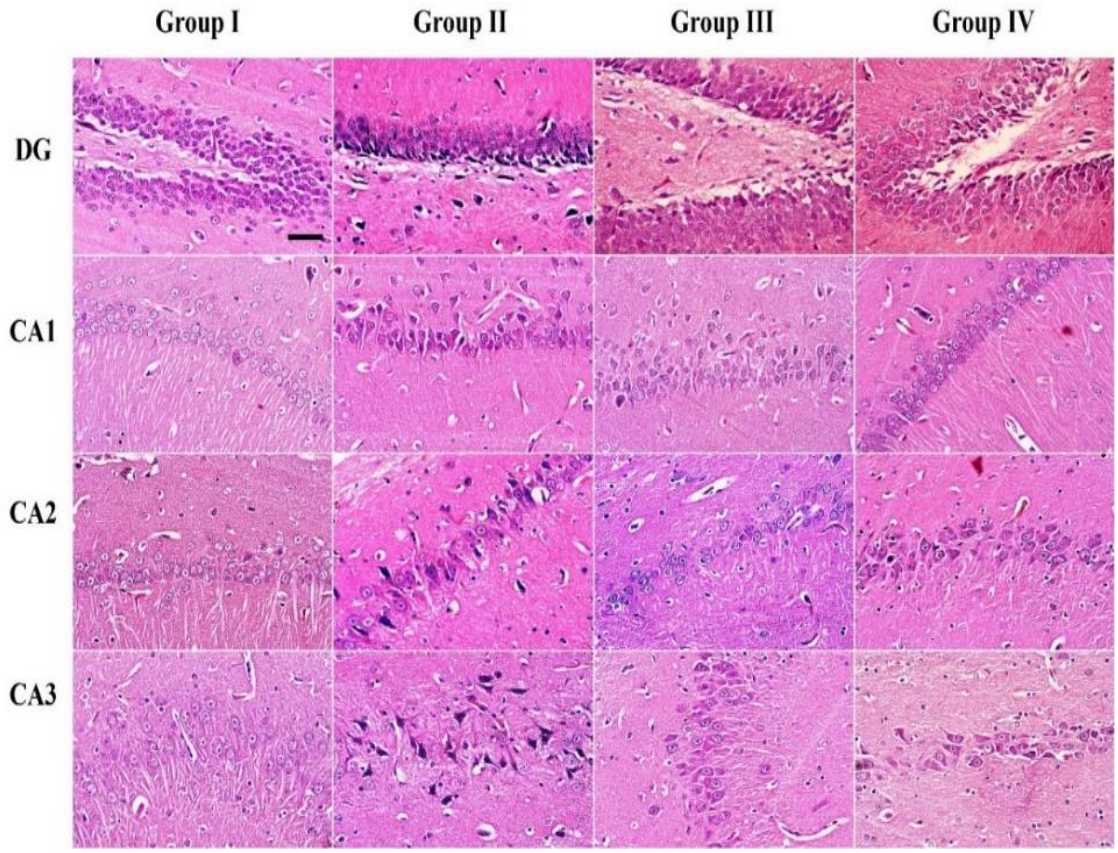

Fig (3): H&E-stained section of the control hippocampus (Group I) shows normal appearance of DG, CA1, CA2 &CA3 regions. Group II shows marked cell loss and most cells are shrunken with apoptotic nuclei. Group III shows normal appearance of many granule cells of DG and pyramidal cells of CA regions that have vesicular nuclei. Group IV shows many cells are shrunken with dark apoptotic nuclei **(H&E stain x400; Scale bar30 µm).**

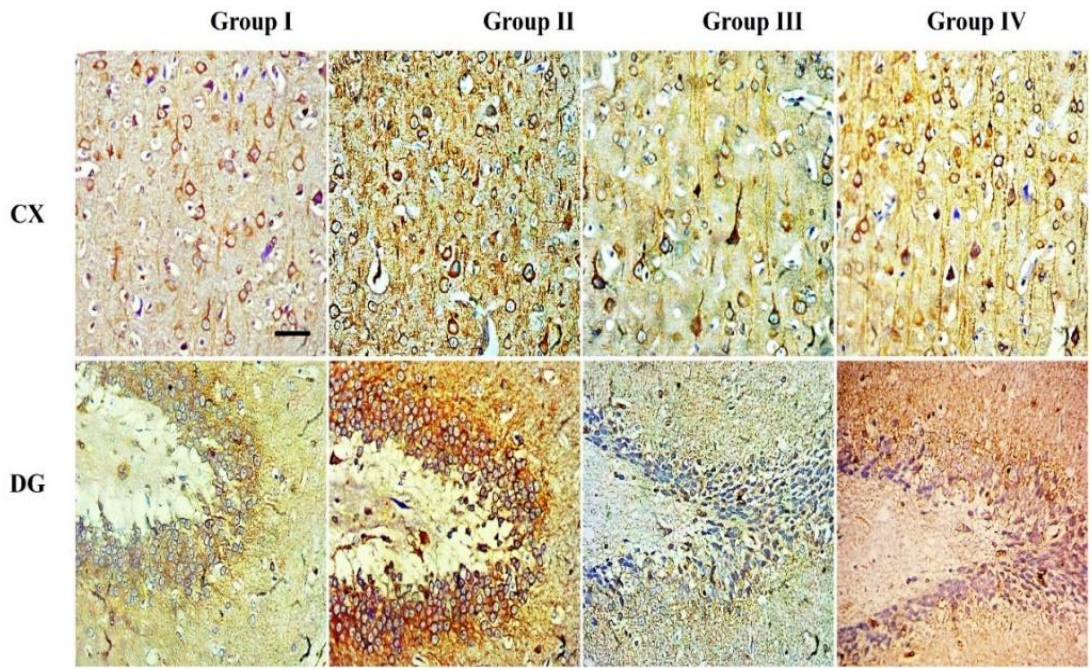

Fig (4): Immunohistochemical staining of p-Tau in the hippocampus shows faint brown staining of the perikaryon cytoplasm of the neuronal cell bodies and their processes in groups I and III in cerebral cortex and DG. Group II exhibits an intense brown staining of the perikaryon cytoplasm of the neuronal cell bodies and their processes. Group IV reveals a moderate brown staining of the perikaryal cytoplasm of the neuronal cell bodies and their processes. **(Avidin biotin Peroxidase system X400, Scale bar30 µm).**