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Attenuation of gibberellic acid-induced cerebellar toxicity by N-acetylcysteine in adult male rats; histological, ultrastructural, biochemical and immunohistochemical studies

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Key words: Gibberellic acid, N-acetyl cysteine, Cerebellar toxicity, Ultrastructure, Oxidative stress and antioxidants, Bax, Bcl-2

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Abstract

Gibberellic acid (GA3) is one of the plant growth regulators that are not nutrients, but chemicals used to promote, influence, growth development and plant availability. N-acetyl cysteine (NAC) exerts protective effects in the brain via its antioxidant activity that gives it the neuroprotective role. The present study aimed to evaluate the protective effects of NAC against GA3 induced cerebellar toxicity in adult male albino rats. Forty adult male albino rats were divided equally to four groups. Group I, is the control group. Group II, where rats received an oral dose of NAC (300mg/kg) three times per week. Group III, where rats received an oral dose of 200 ppm of GA3 (equivalent to 55mg/kg, three times per week). Group IV, where rats received GA3 simultaneously with NAC. Light and ultrastructural microscopic examinations of the cerebellar cortex of GA3-treated animals revealed prominent neurotoxic effects on its three layers. The brain content of antioxidant enzymes was significantly decreased while the malondialdehyde level was significantly increased. Moreover, the immunohistochemical results showed elevation in the expression of Bcl2 and reduction in the expression of Bax. While, GA3 and NAC- treatment retrained all the explained toxic changes and almost restored the normal structure.

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Introduction

Plant growth regulators (PGRs) are known as plant hormones or phytohormones that used as agricultural chemicals to regulate plant growth and increase seed production (Çelik *et al.*, 2002). Gibberellic acid (GA3) is one of PGRs produced by a naturally occurring fungus and sustained in the bioactive form in the soil for months. People can expose to residues of GA3 through drinking water or within their diets derived from the consumption of different types of fruits and vegetables treated with GA3 (Schwechheimer and Willige, 2009; Hussein *et al.*, 2011). Moreover, PGRs may induce oxidative stress, leading to the generation of free radicals, causing hazardous effects and cells damage in many organs including the heart, kidney, stomach, liver and spleen (Celik and Tuluce, 2006; Troudi *et al.*, 2009). Maternal exposure to GA3, during pregnancy and lactation, caused delayed development of the cerebellar cortex of offspring (Ali *et al.*, 2018). Many studies have reported that trans-placental passage of GA3 effects on fetal tissue as well as the central nervous system which consider its main target organ (Alsemeh *et al.*, 2019). Also, the oral use of GA3 could reduce fertility by influencing the sperm number and the quality of sperm's chromatins (Hosseinchi *et al.,* 2013). Additionally, Abu Amra *et al.* (2020) concluded that the GA3-induced histopathological changes in the adult tissues (uterus, ovary) and developing tissues (liver, kidney, skin) of the treated mice.

N-acetyl cysteine (NAC) is a small molecule containing a thiol antioxidant group, glutathione precursor, and can pass the blood brain-barrier. Additionally, NAC promotes cell membrane and blood brain-barrier integrity and can prevent the induced oxidative damage through scavenging free radicals and directly reacts with hydroxyl radicals (Arakawa 2007; Zhou *et al.* 2013). Many studies confirmed that NAC has neuroprotective effects during the ischemic conditions (Pawlas *et al.*, 2009), improved memory retention and attenuated oxidative damage (Prakash and Kumar 2009). Also, it attenuates neuroinflammation in various disease models as ischemiare-perfusion injury (Sekhon *et al.*, 2003) and multiple sclerosis (Stanislaus *et al.*, 2005). In addition, high concentrations of NAC protect DNA from induced oxidative damage and can prevent neuron apoptosis (Olivieri *et al.*, 2001).

The supplement of NAC exerts antioxidant and anti-inflammatory actions by reduction of inflammatory cytokines and oxidative stress. It also has a neuroprotective role against excitotoxicity due to glutamate alterations that associated with the contribution of inflammatory cytokines (di Michele *et al.*, 2018). Moreover, NAC may decrease the inflammation associated with chronic obstructive pulmonary disease, influenza, and idiopathic pulmonary fibrosis (Millea 2009). In addition, the NAC-mechanism of vasodilation action is associated with the facilitation of the production and action of nitric oxide. So, it used in the treatment of neurological disorders via its ability to down-regulate inflammation, oxidative stress which supports the survival of existing neurons and can stimulate neurogenesis (Shahripou *et al.*, 2014; Bhatti *et al.*, 2018).

The B cell lymphoma protein family members are considered to be primary regulators of apoptosis. Among these, the best characterized are B-cell lymphoma 2 (Bcl-2) and associated X protein (Bax). Overexpression of Bcl-2 inhibits cell apoptosis and Bax has been implicated in the process of apoptosis in neurons (Abdullah *et al.*, 2007). The Bcl-2 family of proteins is comprised of inhibitors (Bcl- 2, Bl-xL, Bcl-w and Mcl-1) and promoters (Bax, Bik, Bok, Bak, Blk, Bid, Bad, Bim and Hrk) of apoptosis (Palowski and Kraft, 2000), which share structural domains, including BH1, BH2 and BH3. The C termini of the Bcl-2 and Bax proteins contain a domain that is necessary for mitochondrial insertion; its deletion blocks Bax-induced apoptosis (Nechushtan *et al.,* 1999). Bcl-2 modulates its anti-apoptotic property by forming heterodimers with the pro-apoptotic members of its family, predominantly Bax, blocking their key functions and exerts its influence by blocking programmed cell death and thereby enhancing cell survival, rather than by stimulating cell division. Through this mechanism, Bcl-2 protein is suspected to impart resistance to the induced apoptosis (Srinivas *et al.,* 2000).

According to the reports, amphetamine derivatives cause increased numbers of pro-death Bax proteins in addition to decreased numbers of anti-death Bcl-2 proteins (Spagnuolo *et al.*, 2006).

Therefore, the aim of this study was to evaluate the prophylactic role of NAC against neurotoxicity induced by GA3 through integrative parameters include histological, ultrastructural, immunohistochemical and biochemical studies.

Materials and methods

Chemicals

Gibberellic acid (GA3), (2, 4 a, 7-Trihydroxy-1-methyl-8-methylenegib-3-ene-1, 10- dicarboxylic acid 1, 4 a-lactone), in the form of white crystalline powder, was bought from Sigma -Aldrich chemical Company (St Louis, MO, USA). 4ml of 5% GA3 (equivalent to 200mg of GA3) was diluted with tap water to reach 1000 ml to obtain 200 ppm of GA3, equivalents to 55mg/kg. The rats were given the dose 3 times a week for 6 weeks. The chosen dose was used to provoke oxidative stress without the lethal effects (Troudi *et al.,* 2012).

N-Acetylcysteine (NAC) was purchased from the pharmacy of National Center of Clinical and Environmental Toxicology, Faculty of Medicine, Cairo University the drug was produced by the South Egypt Drug Industries Company, Egypt. NAC was dissolved in distilled water and administered to the rats in the following protocol: 300mg/kg, by gavage, 3 times per week for 6 weeks.

Experimental animals

Forty adult male albino rats (*Rattus norvegicus*) weight average from 130 to 140 g were used. These animals were obtained from the Animal House in Zagazig University, Faculty of Veterinary Medicine. In order to adapt the animals to a new environment and to ascertain from their physical wellbeing; all animals were subjected to 14 days of passive preliminaries. Animals were housed in separate well-ventilated plastic cages, under standard conditions, with free access to the standard rodents' pellet diet and water *ad libitum*. All the experiments were done in compliance with the guide of the care and use of laboratory animals, faculty of Science, Menoufia University, Egypt (Approval No. MUFS/F/HI/2/21) that according to the National Institutes of Health guide for the care and use of laboratory animals (NIH publications No. 8023, received 1978).

Animal grouping and experimental design

Forty adult male albino rats were divided randomly into four equal groups. Group I, animals of this group had been kept as normal without any treatment and considered as controls. Group II, (NAC-treated group), where rats received an oral dose of 300mg/kg of NAC, three times per week, via intra-gastric intubation. Group III, (GA3-treated group), where rats received an oral dose 200 ppm of GA3 (equivalent to 55mg/kg) three times per week. Group IV, (GA3 & NAC group), where rats received GA3 (55mg/kg) simultaneously with 300mg/kg of NAC.

At the end of the experimental period, after six weeks overnight fasted rats were anesthetized for profusion then rats were sacrificed and the cerebellum of each animal was quickly taken and prepared for histopathological and ultrastructural studies.

Histology

Small species of the cerebellum were fixed in neutral 10% formalin for 48 h. Later, they were dehydrated in graded concentrations of ethanol, cleared in two changes of xylene and embedded in paraffin wax for sectioning. Then 5-µm thick sections were cut, mounted on glass slides, and stained with hematoxylin and eosin stain. Chemicals used in the histological assay were purchased from Sigma-Aldrich Corp (St. Louis, MO USA).

Transmission electron microscope essay

Each cerebellum was cut into small pieces (1 mm^3) and immediately fixed in 2.5% glutaraldehyde for 48 hr. Then, specimens were washed in phosphate buffer (pH 7.2-7.4) 3-4 times for 20 min. and post-fixed in a buffered solution of 1% osmium tetraoxide for 2 hr, then washed in the same buffer 4 times for 20 min. each. Fixed specimens were dehydrated in ascending grades of ethyl alcohol (30% to 100%), cleared in two

changes of propylene oxide and embedded in Epoxy resin. Semithin sections (1µm thick) were prepared and stained with toluidine blue and examined under the light microscope to select the areas that photographed with electron microscope. Ultrathin sections were cut (60-90 nm thick), mounted on copper grids and double-stained with uranyl acetate and lead citrate. The grids were examined and photographed using a transmission electron microscope 1400 plus-JSM transmission electron microscope (JEOL Ltd., Tokyo, Japan) in the electron microscope unit, Faculty of Science, Alexandria University (Alexandria, Egypt). Chemicals used in the ultrastructure assay were purchased from Sigma-Aldrich Corp (St. Louis, MO USA).

Oxidative stress markers

Bio-diagnostic kits for estimation the cerebellar content of superoxide dismutase (SOD), glutathione (GSH) and malondialdehyde (MDA) were estimated using the commercial kits (Bio Diagnostic Co., Egypt) according to the manufacturer's recommendation.

Immunohistochemical assay

For immunohistochemistry techniques, 1-2 µm thickness sections of the cerebellar tissue were prepared and stained to determine the expression of Bcl-2-associated X protein (Bax) and B-cell lymphoma 2 (Bcl-2). The suitable antibody in each staining time, anti-Bax anti-Bcl-2 was used according to Hsu *et al.* (1981). To evaluate Bcl-2 and Bax labeling index, the percentage of stained cells was counted in 10 cerebellar sections from each animal group.

Statistical analysis

All data were analyzed using the SPSS for windows version 20 (SPSS Inc., Chicago, USA). Analysis of variance (one-way ANOVA) was performed to test for any significant differences among the control and the treated groups. The level of significance was set as P≤ 0.01 and at P≤0.001 for all statistical tests.

Results

Effect of the different treatments on the body weights

Data in fig. 1 showed that there was a non-significant change in the body weight of the control (135.5±4.6) and NAC-treated animals'(135.3±5.6). While the treatment with GA3 a significant reduction (111.3±12.3) was recorded in the body weight of rats when compared with the control group. When animals treated with GA3 and NAC, a significant increase (126.0±17.3) was recorded in the body weights of experimental animals compared with GA3 only treated group.

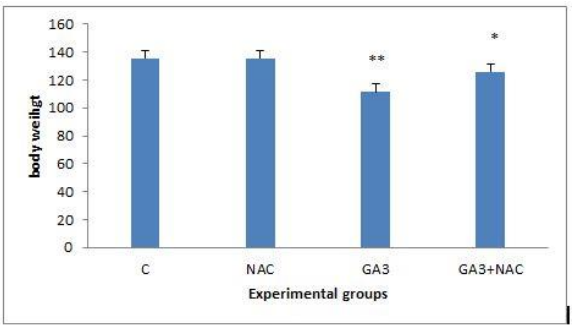

Fig. 1. Effects of GA3 and NAC and their combination on the body weight of different experimental groups.

The values are expressed as mean ± SD, n=8

(**) Significant comparing with control (P≤ 0.0001).

(*) Significant comparing with GA3 (P≤ 0.01).

Histological observations

Light microscopic examination of sections of the brain of control as well as the NAC group revealed the normal architecture of the cerebellar cortex. The cerebellar folium contains a central core of white matter covered by an outer cortical gray matter. The latter layer of the cerebellum was formed of three layers that arranged from the inward outside; the granular layer, Purkinje cell layer and molecular layer (Fig. 2a). The inner granular layer is consists of numerous small granular cell that appeared closely packed with darkly stained nuclei surrounded by very little cytoplasm and these cells separated by lightly stained areas called neuropil. The middle layer called the Purkinje cell layer that arranged in one row between granular and molecular layers. The Purkinje cell appeared with flask-shaped contains centrally located rounded open-face nucleus with prominent nucleolus and surrounded by cytoplasm contains basophilic Nissel's granules. The outer molecular layer formed of nerve fibers with few scattered stellate and basket cells (or formed of the sparse population of neurons).

It also contains the dendritic arbors of Purkinje neurons (Fig. 2b &c).

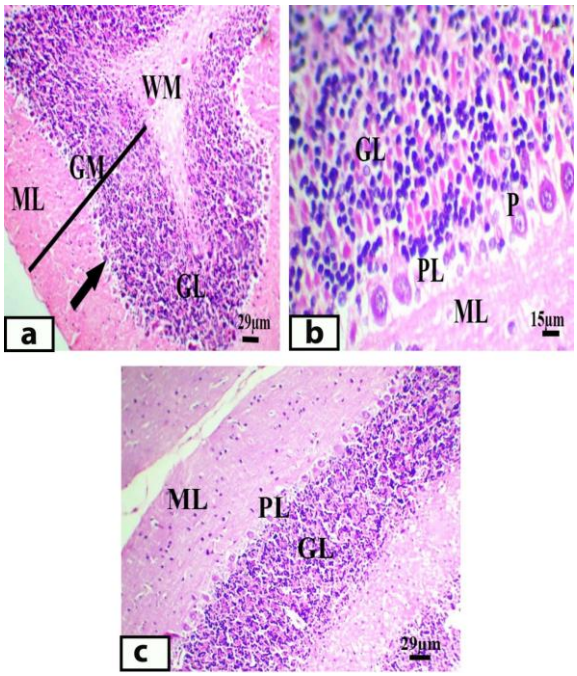

Fig. 2. photomicrographs of sections of cerebellar cortex of the control rats (a) showing cerebellar folium with inner white matter (WM) and outer gray matter (GM) consisting of granular layer (GL), Purkinje layer (arrow) and molecular layer (ML), (X 200), (b) showing outer molecular layer (ML), the middle Purkinje cell layer (PL) contains flask shape Purkinje cell (P) and inner granular layer (GL), (X 400). 4c: A photomicrograph of section of cerebellum of rat treated with NAC showing normal molecular layer (ML), Purkinje cell layer (PL) and granular layer (GL), (X 200).

Examination of the cerebellum of GA3 treated rats revealed marked pathological features that appeared more obvious in the Purkinje cell layer and the granular layer while the molecular layers were less affected. The monolayer arrangement of these cells was disrupted in many areas; most of the Purkinje cells appeared scattered between molecular layer and granular one revealed multilayer deposition. Some of these cells were absent leaving empty spaces while others lose their normal pyriform shape, appeared shrunk with irregular size and shape contains deep homogenous cytoplasm and surrounded with vacuolated neuropil.

Concerning their nuclei, few cells appeared with vesicular nuclei and others showed pyknotic or completely karyolitic ones. The granular layer appeared degenerated; these cells contain dark cytoplasm and surrounded by halos of empty space. In addition, areas of degenerations surrounded by inflammatory cells were detected. The molecular layer appeared degenerated with multiple vacuolated perineural spaces associated with increased microglial nuclei were seen (Fig. 3a-d).

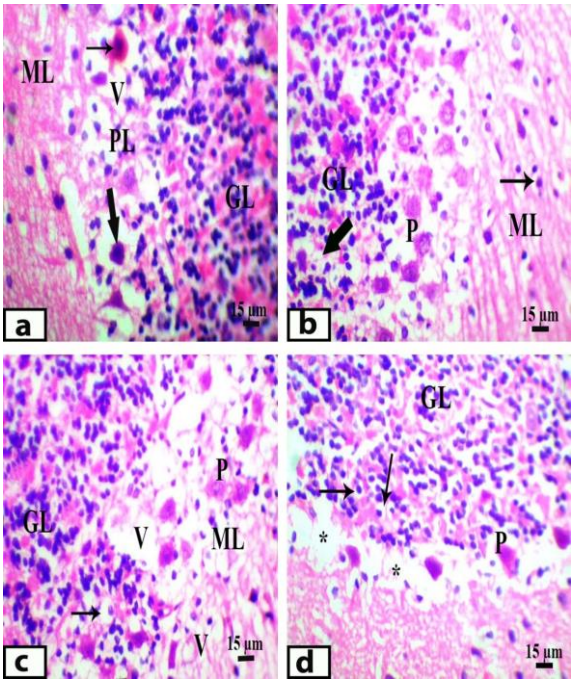

Fig. 3. photomicrographs of sections of cerebellar cortex of the rats treated with GA3 (a) showing Purkinje layer (PL) with shrunk irregular Purkinje cells with either pyknotic nucleus (thick arrow) or eosinophilic cytoplasm (thin arrow) surrounded by vacuolated neuropil (V) and both the granular layer (GL) and molecular layer (ML) appeared degenerated. (b) Showing scattered Purkinje cells (P) forming many layer of distorted one between degenerated inner granular layer (GL) with vacuolated (thick arrow and outer molecular one (ML) with increased microglial nuclei (thin arrow). (c) Showing displacement of Purkinje cells (P) between degenerated granular layer (GL) with vacuolated granule cell (arrow) and vacuolated molecular one (ML). (d) Showing Purkinje cell (P) with pyknotic nucleus or absent one leaving empty space (*) and degenerated granular layer (GL)

contains granule cells with dark cytoplasm surrounded by hales of empty space (arrows) (X 400). Examination of sections of cerebellum of animals treated with GA3 and NAC showed restoration of the normal architecture of three layers of the cerebellum. A relatively monolayer arrangement of Purkinje cell was seen and most of these cells regained their flask shape with central open-face nuclei. Otherwise few cells still showed mild disorganization surrounded with vacuolated neuropil. Both molecular and granular layers showed normal architecture that appeared nearly similar to control ones (Fig. 4a &b).

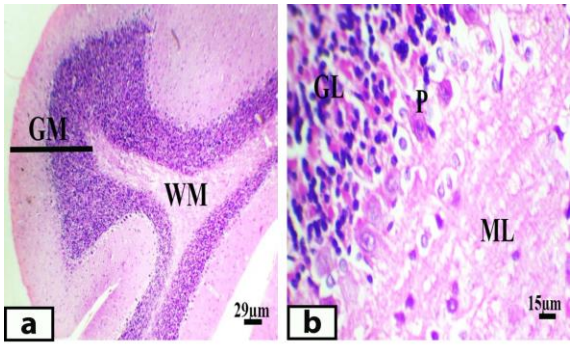

Fig. 4. photomicrographs of sections of cerebellar cortex of the rats treated with GA3 and NAC (a) showing normal white matter (WM) and grey matter (GM), (X 200) and (b) showing normal line appearance of Purkinje cell (P) with flask shape and nearly normal granular layer (GL) and molecular layer (ML), (X 400).

Ultrastructure observations

Electron microscopic examination of the cerebellar cortexes of animals administered NAC did not show clear differences compared with the control group. The Purkinje cell layer appeared to contain irregular shape Purkinje cells with large spherical central euchromatic nuclei and prominent nucleoli. The cytoplasm of these cells contains free ribosomes, lysosomes, rough endoplasmic reticulum and well-defined mitochondria. The Purkinje cells and their nuclei contained layer blocks of condensed chromatin distribution on the inner side of the nuclear envelop which showed slight invagination (Fig. 5a). The granular cells layer showed normal granule cell, the nuclei of these cells exhibited clumped heterochromatin surrounded by little cytoplasm containing rough endoplasmic reticulum, mitochondria and lysosomes (Fig. 5b). The molecular layer is formed of an extension of axons and dendrites of the cells of the same molecular layer and dendrites of the Purkinje cells. These nerve fibers appeared with regular compact myelin sheath formed of regular lamellae. The axoplasm of these nerve fibers contained regularly arranged microtubules, neurofilaments and mitochondria (Fig. 5c).

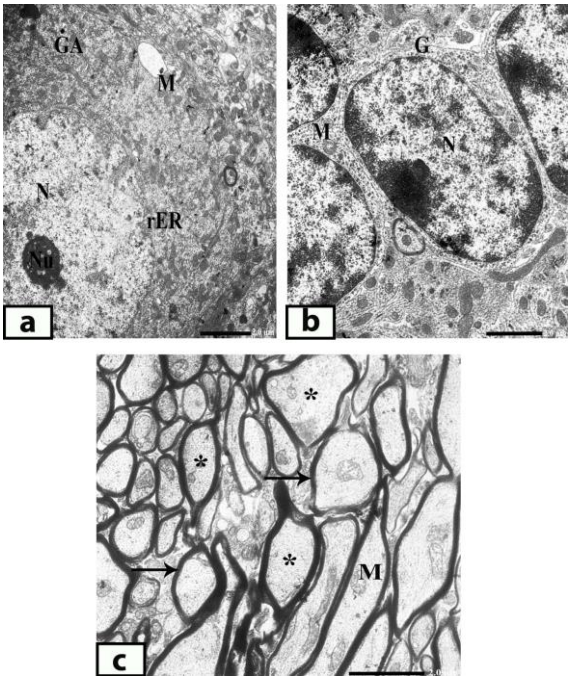

Fig. 5. Electron micrographs of cerebellar cortex of control rats showing (a): Purkinje cell (P) with euchromatic nucleus (N) with prominent nucleolus (Nu), mitochondria (M) and rough endoplasmic reticulum (rER). (b): showing granule cell (G) with center large oval nucleus (N) and thin rim of cytoplasm contains mitochondria (M). (c): showing molecular layer contains transverse sections of myelinated nerve fibers (*) with compact myelin sheath (arrow) and the axoplasm contains mitochondria (M), (X 3000).

Examination of ultrathin sections of the GA3-received group showed deformed Purkinje cell with degenerative features of both nucleus and cytoplasm. Some nuclei appeared irregular, with increased infolding of nuclear enveloped and abnormal distribution of chromatin and others appeared pyknotic characterized with atrophic change and shrinkage.

The cytoplasmic ultrastructural changes include widening rough endoplasmic reticulum exhibiting globular shape associated with detached ribosomes. Moreover, the mitochondria may appear either dark with dense matrix or swollen with partial or completely destroyed cristae and a large number of lysosomes appeared (Fig. 6a-d).

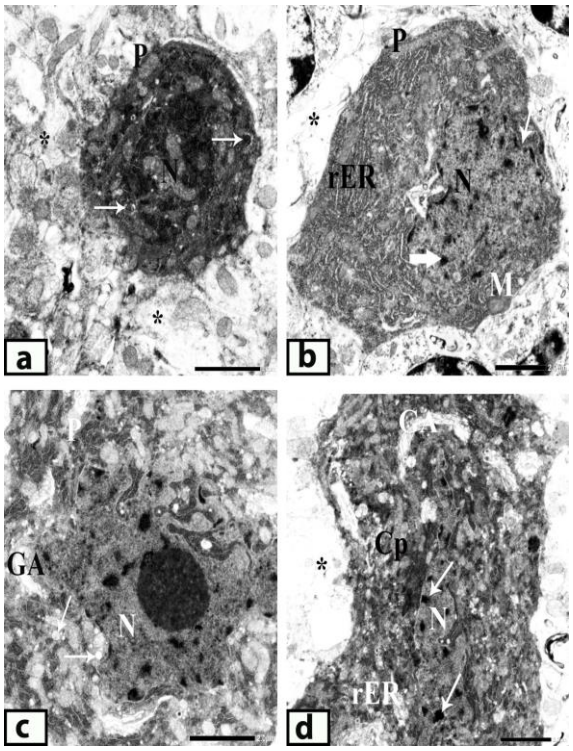

Fig. 6a. Electron micrographs of cerebellar cortex of GA3-treated rats (a): showing Purkinje cell (P) contains degenerated nucleus (N) with higher electron density, the nuclear envelope is indiscernible, lysosomes-like bodies (arrows) in the perikaryon, note the degenerated neuropil (*), (X, 3000). (b): showing Purkinje cell (P) with degenerated nucleus (N) with nuclear envelop (thin arrow), small patches of clumped chromatin (thick arrow), the nuclear envelope was indiscernible, dilated rough endoplasmic reticulum (rER) and disrupted mitochondria (M), (X, 3000). (c): showing Purkinje cell (P) contains irregular nucleus (N), degenerated Golgi apparatus (GA) and some vacuoles (arrows), (X, 4000). (d): showing Purkinje cell (P) contains filiform nucleus (N) with intended nuclear envelop and small patches of clumped chromatin (arrows), dilated rough endoplasmic reticulum (rER) and Golgi apparatus (GA), degenerated cytoplasm (Cp) and neuropil (*), (X, 3000).

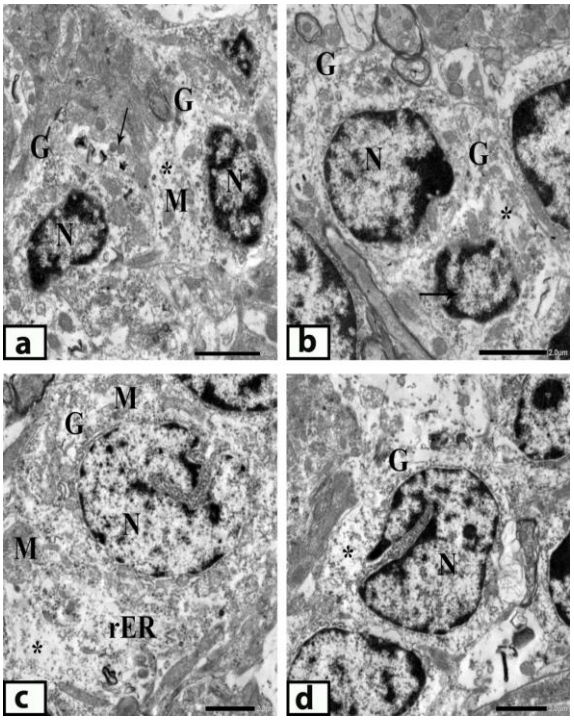

Fig. 7a. Electron micrographs of cerebellar cortex of GA3-treated rats (a): showing degenerated granule cells (G) contain pyknotic nuclei with coarse chromatin (N), rarified cytoplasm (*), lysosomes-like bodies (arrow) and dense mitochondria (M), (X 4000). (b): showing degenerated granule cells (G) contain either karyolised nucleus (arrow) or phagocytic nucleus (N) and rarified cytoplasm (*), (X 4000). (c&d): showing degenerated granule cells (G) contain apoptotic nucleus (N), degenerated mitochondria (M), rough endoplasmic reticulum (rER) and rarified cytoplasm (*), (X 3000).

In regarding to the granular layer, the granular cells appeared variable in size, some nuclei appeared with condensed chromatin and irregular nuclear envelopes while others appeared either pyknotic or apoptotic, fragmented. The neuropil lost its normal appearance and contained degenerated or vacuolated areas (Fig. 7a-d).

The molecular layer of the cerebellum of the same group appeared degenerated with patchy loss; the myelin sheath of the axon of some nerve fibers appeared disrupted, splitting or loss of lamellar layers. Some axons appeared swollen with irregular outlines; rarified axoplasm contains disorganized neurofilaments and swollen mitochondria (Fig. 8 a&b).

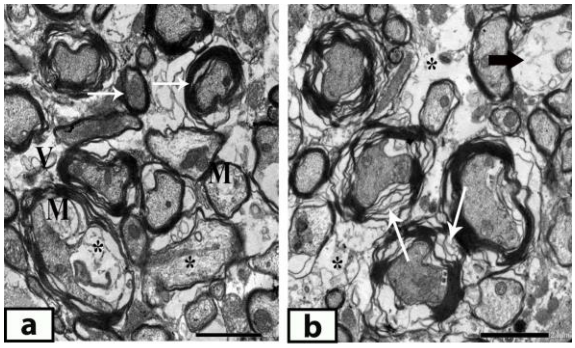

Fig. 8a. Electron micrographs of cerebellar cortex of GA3-treated rat showing transverse sections of thinning and decrease in the number of myelinated nerve axons (arrows) with rarified axoplasm (*), swollen disrupted mitochondria (M) and vacuolization between axons (V), (X 4000). (b): Electron micrograph of cerebellum of GA3-treated rat showing many transverse sections of myelinated nerve fibers with splitting myelin lamellae (thin arrows) rarified axoplasm(thick arrow) and degenerated neuropil (*), (X 4000).

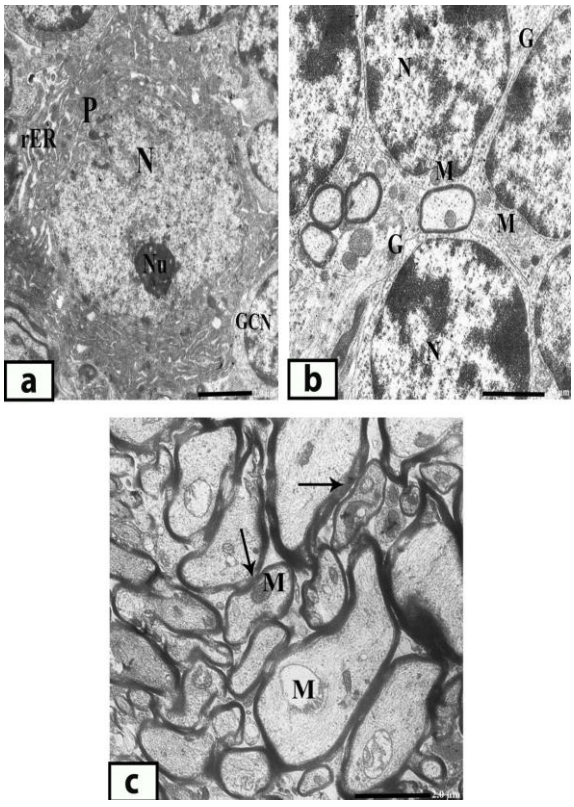

Fig. 9a. Electron micrograph of cerebellum of GA3 and NAC-treated rat showing Purkinje cell (P) with normal nucleus (N), prominent nucleolus (Nu), rough endoplasmic reticulum (rER), note granular cell nucleus (GCN), (X 3000). (b): showing normal granule cell (G) with round nucleus (N) and mitochondria (M) and surrounded by normal neuropil, (X 3000). (c): showing molecular layer contains normal transverse sections of myelinated nerve fibers with axon of myelinated nerve fibers with compact lamellae (arrow) and mitochondria (M) within their homogenous axoplasm.

Concerning the animals treated with NAC showed an obvious improvement of the cerebellar cortex that appeared similar to control one except for few degenerative changes still appeared.

Most of the Purkinje cells appeared with slight invagination of the cell membrane, the nuclei showed normal distribution of chromatin and the cytoplasm appeared to contain normal organelles although few vacuoles and some dilated rough endoplasmic reticulum still found (Fig. 9a).

The granule cell appeared rounded with clumped heterochromatin as well as active lysosomes, normal mitochondria and rough endoplasmic reticulum within the thin rim of cytoplasm (Fig. 9b).

The molecular layer appears mostly normal without vacuoles and normal myelinated fibers with compact lamellae and homogenous axoplasm contain normal mitochondria were seen (Fig. 9c).

Immunohistochemical results for Bcl-2 and Bax proteins

Immunohistochemical analysis for Bcl-2 protein showed uniform staining of the cytoplasm and nuclear membrane of the large number of control as well as NAC-treated group.

Cerebellar cortex examination of animals treated with GA3 showed a marked increase of Bcl-2 positive staining cells.

In addition, the treatment with GA3 and NAC together showed marked restoration of normal Bcl-2 expression (Fig. 10a-d)

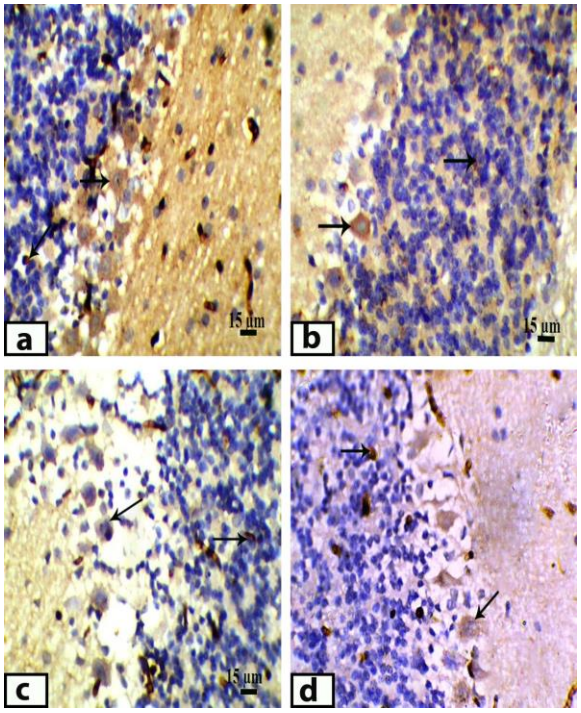

Fig. 10. photomicrographs of cerebellar cortex (a) of the control rat showing positive Bcl-2 expression in the cytoplasm of most granule and Purkinje cells (arrows). (b) Of NAC-treated rat showing normal Bcl-2 expression in the cytoplasm of most granular and Purkinje cells (arrows). (c) of GA3-treated rat showing positive Bcl-2 expression in the cytoplasm of few granular and Purkinje cells (arrows). (d) of rat treated with GA3 and NAC showing positive Bcl-2 expression in the cytoplasm of most granule and Purkinje cells (arrows), (X 400).

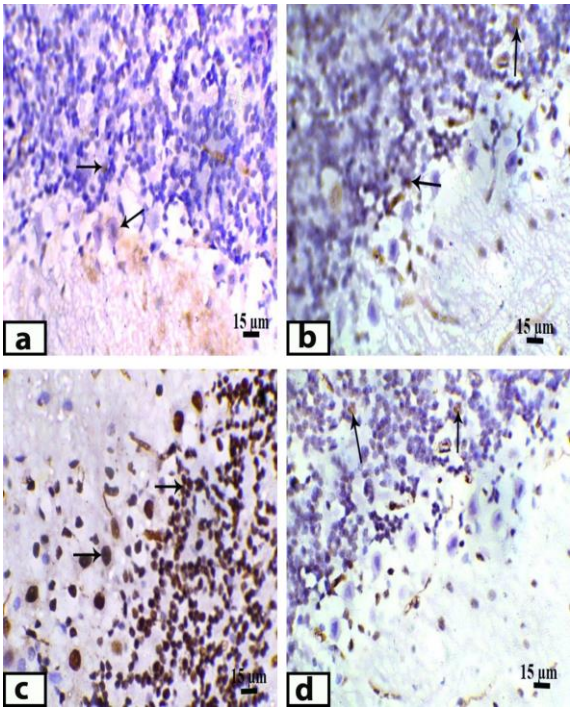

Fig. 11. photomicrographs of cerebellar cortex (a) of the control rat showing positive Bax expression in few granule and Purkinje cells (arrows). (b) of NAC-treated rat showing normal Bax expression in the granule and Purkinje cells (arrows). (c) of GA3-treated rat showing positive Bax expression in the most of granular and Purkinje cells (arrows). (d) of rat treated with GA3 and NAC showing Bax positive expression in few of granule and Purkinje cells (arrows), (X 400).

Immunohistochemical analysis for Bax protein showed diffuse cell membrane and cytoplasmic staining, randomly distributed in the large number of Purkinje and granule cells of GA3 treated rats. Moreover, Bax staining was nearly absent in cerebellar cortex tissues of both normal and NAC-treated groups.

Moreover, animals treated with both GA3 and NAC showed few numbers of Purkinje and granule cells with positive immunohistochemical expression (Fig. 11a-d). Fig. 12 showed Bcl-2 and Bax-labeling index in different groups after six weeks of different treatments. This index is significant at P≤ 0.01 in animals given GA3 compared with those treated with GA3 and NAC.

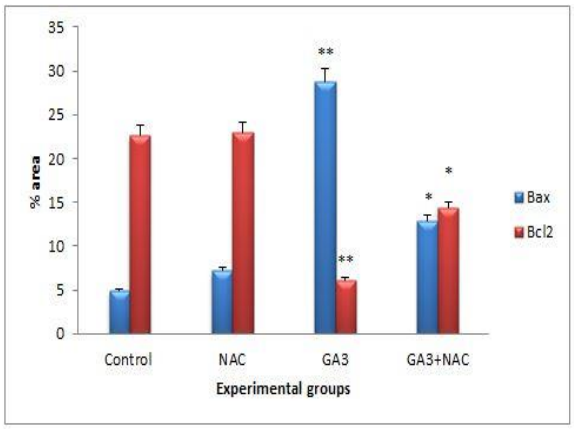

Fig. 12. The percentage of area of Bcl-2 and Bax expression in different expremental groups.

The values are expressed as mean ± SD, n= 10 sections

(**) Significant comparing with control (P≤ 0.001)

(*) Significant comparing with GA3 (P≤ 0.01)

The modulation effect of N-acetyl cysteine on GA3-induced changes in the oxidative stress markers of the brain

The treatment of the NAC recorded non-significant change in the level of lipid peroxidation that represented by MDA level (1.42±0.492) SOD and GSH activities (27.16±7.60 and 29.41±5.25, respectively) when compared with the control group (1.61±0.641, 28.72±5.47 and 26.55±4.87 respectively). A significant increase in the level of MDA (4.24±0.782) and a significant decrease in the SOD activity and GSH levels (14.01±3.09 and 13.32±4.46respectively) were recorded in the GA3 group when compared with the control ones. While animals treated with GA3 and NAC recorded a significant decrease in the level of MDA (2.47±1.007) and a significant increase in the activity of SOD and GSH level (23.31±4.54 and 22.90±7.86 respectively) when compared with the GA3 group (Fig. 13& 14).

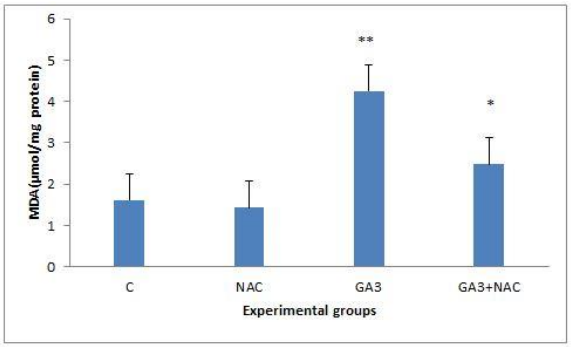

Fig. 13. Effects of GA3 and NAC and their combination on the level of MDA in cerebellar cortex of different experimental groups.

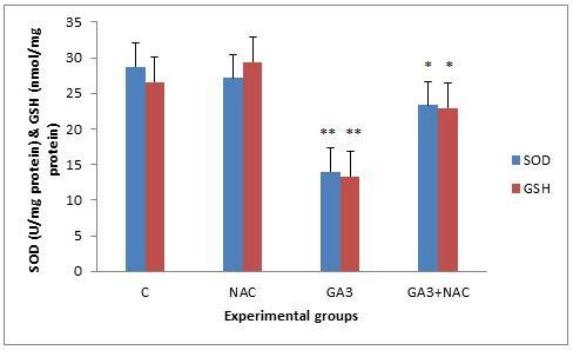

Fig. 14. Effects of GA3 and NAC and their combination on the level of GSH and SOD activity in cerebellar cortex of different experimental groups.

The values are expressed as mean ± SD, n=8

(**) Significant comparing with control (P≤ 0.001)

(*) Significant comparing with GA3 (P≤ 0.01)

Discussion

Clarification of the mechanisms associated with PGRs neurotoxicity is important to find out the effective complementary therapy. Different mechanisms have been proposed; however, oxidative injury and inflammation seem to be the basic mechanisms in the PGRs-neurotoxicity.

In the present study, GA3-treatment induced a significant decrease in the bodyweight of treating rats. Similarly, El-Okazy (2008) observed loss in the bodyweight of mice given GA3 and attributed that to the reduction of food consumption of the treated animals**.**

The present findings confirmed that the oxidative damage induced by GA3 leading to the development of severe pathological alterations in the cerebellar cortex. Most of these alterations were noticed in granule and the Purkinje cells; the Purkinje cells appeared with multilayers, scattered between granular and molecular layers, with pyknotic nuclei, eosinophilic cytoplasm and surrounded by vacuolated neuropil, these results may be attributed to the ability of GA3 to induce inflammation and oxidative damage in the cerebellar cortex. These current results are in accordance with other studies reported by Troudi *et al.,* 2012 and Ali *et al.* 2018 who mentioned that GA3 induced cerebellar morphological alterations because it caused initiation or progression of cellular damage by inducing the reactive oxygen species (ROS) that oxidized vital cellular components such as lipids, proteins and DNA producing potentially harmful effects. Similarly Yilmaz and Celik (2009) reported that the central nervous system is more susceptible to oxidative damage induced by PGRs. Moreover, Abou-zeid and Abd-Ellah (2015) reported that GA3-treatment showed prominent cerebellar pathological features and attributed that to increase ROS production.

The GA3-treatment in the present study showed severe ultrastructural alternations which were more obvious in the Purkinje and granule cells which may be attributed to the inflammatory effect of GA3 that associated with its oxidative stress resulted in tissue damage.

The present results are in agreement with those recorded by Abou-zeid and Abd-Ellah (2015) in the cerebellar tissues of GA3-treated rats. Similarly, Blokhina *et al.* (2003) confirmed the present findings, and they reported that excessive ROS provoke inflammation and the development of tissue necrosis. Also, Ali *et al.* (2018) found that GA3 caused deformed, shrunken Purkinje and granule cells that appeared with variable sizes with condensed nuclear chromatin, pyknosis, with irregular nuclear envelopes, and vacuolated cytoplasm contains disrupted swollen mitochondria and degenerated rER. The same authors suggested that these changes may be due to the elaboration of free radicals induced by GA3 treatment leading to reduced Purkinje cell proliferation, differentiation and maturation with an increase in Purkinje cells' death.

Sherlock and Dooly (2002) demonstrated that increase the amount of ROS production which might enhance the permeability of cell membranes leading to an increase of intracellular water that led to cytoplasmic swelling and vacuolization. Sobaniec-Lotowska (2001) suggested that the marked changes in the mitochondria of Purkinje cells could be due to inhibition of oxidative phosphorylation. For interpretation of the mechanism of mitochondrial swelling, Jaeschke *et al.* (2002) mentioned that oxidative stress had contributed to the opening of the mitochondrial permeability transition pore (PTP) which led to the formation of a high-conductance channel, in the inner mitochondrial membrane and led to mitochondrial swelling and subsequent release of cytochrome C from the intermembrane space. Upon mitochondrial swelling, when the outer mitochondrial membrane ruptures the proteins involved in the effector phase of apoptosis (such as cytochrome C) released into the cytosol is inevitable (Morganti *et al.,* 2018).

In addition, Johar *et al.* (2004) suggested that apoptosis could be followed by mitochondrial swelling, endoplasmic reticulum dilatation and lysosomal rupture before reduction and termination of the nuclei. In addition, Aliev *et al.* (2010) documented that the electron density of the mitochondrial matrix has been attributed to oxidative stress and the active substance initially in the mitochondria correlated to apoptosis.

Immunolocalization of Bcl-2 in the present study showed a decrease in Bcl-2 expression and an increase in Bax expression in cerebellar tissue of GA3-treated rats. Alsemeh *et al.* (2019) found significantly lower expression of Bcl-2 and high expression of Bax in the GA3-treated rats. Additionally, Lin and Beal (2006) attributed the cells apoptotic morphology; in the neurodegenerative disease may be caused by free radicals that blocked neuronal activity, causing neurons to receive internal signals to commit suicide (apoptosis). Similar results were observed by Amer and Hussien (2010) who found a significant decrease in Bcl -2 expression in the renal cortex of the rats treated with GA3 and the reduction in protein content was attributed partially to the decreased level of protein synthesis and to the hyperactivity of hydrolytic enzymes in the affected renal cells. Examination of sera of animals treated with GA3 in the present study recorded a significant reduction in the content of SOD, GSH and elevation in the MDA level.

The reduction in the antioxidant enzyme activities might be due to its excessive consumption to decrease the induced oxidative stress. Celik *et al.* (2006) reported that GA3 treated rats recorded a significantly decrease in the antioxidant enzyme activities and accumulation of peroxidation product (MDA) in different rat organs as the liver and brain. Orrenius *et al.* (2003) found that the PGRs compounds including GA3 can accelerate lipid peroxidation, up to 65-fold, in different tissues and this was attributed to the formation of superoxide radicals that may react with the lipids, possibly by hydrogen abstraction leading to cell-oxidative damage. The present data were in line with Abd El Azim (2017) who stated that GA3-treated rats recorded, decrease in the activities of antioxidant enzymes (SOD, GSH) and increased MDA level in the brain of the adult male albino rats.

The present study shown that; the NAC had a beneficial effect on neuronal morphology and showed an obvious degree of improvement in recorded pathological features induced by GA3. This improvement may be attributed to the ability of NAC to stimulate GSH-synthesis inside the cell which plays

a critical role as a free radical scavenger. These results were confirmed by Soleimani *et al.* (2015) who observed NAC treatment improved memory impairment and neurotoxicity in the rat hippocampus and suggested that NAC is an excellent source of sulfhydryl groups and converted into metabolites that able to stimulate GSH synthesis and acts directly as a free radical scavenger. It has been reported that NAC protect against the proliferation of cytokines, increase neuronal survival and decrease apoptosis through its anti-inflammatory effect via deactivating necrosis factor-κB and its antioxidant activity by enhancing GSH which consider the important mechanisms behind the reported improvement in the pro-inflammatory cytokines (Chen *et al.* 2008; Samuni *et al.* 2013). Therefore, the crosstalk between antioxidant, anti-inflammatory and anti-apoptotic mechanisms exerted by NAC indicated its potential neuroprotective role (Soliman, *et al.,* 2017). Fallah *et al.* (2018) found that the NAC anti-inflammatory properties play an important role in protecting the injured hippocampal cells. Moreover, NAC could serve as an appropriate and safe complementary therapeutic agent to attenuate the toxicity induced in the brain. The mechanisms by which NAC provides protection are largely dependent on the interplay between its antioxidant and anti-inflammatory activities (Abdel-Wahab and Moussa 2019). Aboubakr *et al.* (2019) reported that NAC ameliorated the hepatic histopathological and biochemical changes attributed that to the homeostasis in the oxidantive/antioxidantive status supplied by NAC that restored the altered oxidative stress markers.

The current study showed ultrastructural improvement in the three- layer of cerebellum in animals treated with GA3 and NAC and most of affecting Purkinje and granule cells appeared with regular nuclei, most of the rough endoplasmic reticulum returns its regular flattened shape and the mitochondria appeared normal with regular cristae this means the cell showed ground-glass appearance. Similarly, Hussein *et al.* (2011) reported that the ground glass appearance of hepatocytes was confirmed by electron microscopic examination which showed an improvement in the form of reappearance of cell organelles as mitochondria, cisternae of rER and the nuclei appeared euchromatic with prominent nucleoli.

Moreover, the increased rER was explained by Csalaa, *et al.* (2005) who mentioned that rER had a very important role in the synthesis and packaging of proteins that might be used by the cell to synthesize membrane, other cell organelles as mitochondria. Moreover, NAC may also have anti-inflammatory effects, by reducing intracerebral levels of tumor necrosis factor-α, interleukin-1β, and inducible nitrogen oxide synthase (Paintlia *et al.*, 2004). Furthermore, NAC caused marked neuroprotection associated with improvement of the redox state and inhibition of apoptosis after induced hypoxic-ischemic brain injury in neonatal rats (Wang *et al.*, 2007).

In the current work, NAC treatment improved the brain content of SOD, GSH and MDA that retrained to the normal measurements. Moreover, NAC acts as a GSH precursor that can cross the blood brain barrier and transport into cells. Inside the cells, NAC is deacylated to l-cysteine and therefore increases the rate of GSH synthesis, stimulated superoxide dismutase, GSH and improved neuronal morphology in addition to protecting the rat prefrontal cortex and hippocampus from induced-oxidative stress (Samuni *et al.*, 2013). The cells have different mechanisms to alleviate oxidative damage. The primary defense is occurred by enzymatic antioxidants like SOD, CAT and GSH that scavenges the free radicals resulted from the ROS. The antioxidant enzymes act in concert with non-enzymatic antioxidants, including glutathione which considered the second line of defense of the antioxidant enzymes (Celik and Tuluce, 2006).

In the current work, the immunohistochemical results showed an increase in the expression of Bcl-2 and a decrease in Bax expression rats treated with GA3 and NAC. Similarly, Kim *et al.* (2010) reported that NAC significantly improved renal function and decreased the activation of Bax and whereas it increased Bcl-2 via the anti-apoptotic pathway. While, NAC treatment significantly attenuated the apoptotic cells induced by diabetes and it reduced positive apoptosis cells, the

ratio of Bax/Bcl-2, and expression of cleaved-caspase-3 but has a more profound effect on autophagy because it could attenuate autophagy to a greater extent (Wang *et al.,* 2017). Moreover, Abd-El-Aty and Masoud (2016) reported that the presence of phagosomes in the recovery group associated with the presence of many signs of improvement indicates continuous trials of the defense system to auto-regulate and enhance apoptosis to eliminate the affected dead cells. Moreover, NAC prevents DNA adduct formation and spontaneous mutation during DNA repair. It also elevates methylation of hypomethylated DNA, protects repair enzymes and subsequently improves DNA damage (Stephenson *et al.* 2013). Additionally, it influences gene expression, signaling pathway viability, apoptosis and inflammatory responses (Alboni *et al.* 2013).

Rong and Distelhorst (2008) reported that the apoptosis associated with the recovery phase has been contributed to the remodeling of injured cells and facilitation of their return to the normal structural and functional state.

This study concluded that NAC ameliorated the cerebellar injury induced by GA3 by its anti-inflammatory, anti-apoptotic and antioxidant properties as well as normalized its histo-architecture and ultrastructure and retrained cerebellar GSH, SOD and MDA to normal ranges.

Conflict of interest statement

The author has no conflict of interest to report.

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