Biochemical and ultra-structural studies of the effect of alprazolam as an anxiolytic drug on the cerebellum of adult male mice

Azza A. Attia[1], Nessrin A. Kheirallah[2]; Samah A. Khalifa[3]

[1,2]Faculty of Science, Zoology Dep., Alexandria University, Egypt; 3Faculty of Science, Omar Elmoktar University, Libya.

ABSTRACT

In the recent decade, there has been increasing concern on the hazard effect of drugs on different species. Stressful life events contribute to the development of many neurodegenerative and neuropsychiatric disorders including depression and anxiety. Alprazolam (ALP) is commonly used and approved for the medical treatment of panic and anxiety disorders, such as generalized anxiety or social anxiety disorders. Thus, it was of a particular interest to investigate the effect of ALP on the neurons of cerebellar cortex of mice, where mice have genomic similarities to human. So, biochemical, histological and ultrastructural investigations are reported on the cerebellum of adult male mice subjected to three different doses of ALP, for two months. These doses were equivalent to the human therapeutic doses as 0.5, 1 and 1.5 mg. In a dose dependant manner, significant decreases in the levels of both acetylcholine enzyme activities and total glutathione are recorded, indicating that the activity of acetylcholine esterase was inhibited by free radical formation. Little histopathological changes were observed in the cerebellar cortex of mice administered with 0.5 mg ALP. Marked alterations were observed in the Purkinje neurons of cerebellar cortex of mice administered with 1 and 1.5 mg ALP, where unstained haloes are seen around most of these cells. Their nuclei were eccentrically placed, and pyknotic. The intracellular structure of Purkinje cells showed dilatation of both rER and Golgi apparatus. Many small vesicles near the Golgi bodies were accumulated to form clusters, probably indicate disturbance in the vesicular transport between rER and Golgi apparatus. These results reflect the injured effect of high dose ALP on brain activity, performing in the possible ultrastructural abnormalities as well as its oxidative stress.

INTRODUCTION

Benzodiazepines comprise a large group of psychoactive drugs that are massively used in human pharmaco-therapy for their anxiolytic and hypnotic anticonvulsant properties (Mandrioli *et al.,* 2008). Alprazolam (ALP) is a potent short acting drug of the benzodiazepines class. It is believed to be fairly safe and used extensively in the treatment of generalized anxiety, panic attacks with or without agoraphobia and depression (Bandelow *et al.*, 2002). Alprazolam is readily absorbed from the gastrointestinal tract, and found to be deposited in blood, urine and even hair samples (Kintz *et al.*, 2005). Also, it is hydroxylated in the liver to α-hydroxy-alprazolam, where it is also pharmacologically active but much less than the parent compound. Isbister *et al.* (2004) demonstrated that ALP was more toxic than other benzodiazepines (Isbister *et al.*, 2004). Intravenous administration of ALP caused an acute significant decrease in brain activation as well as in

the whole-brain cerebral blood flow of 25% to 30 % (Roy-Byrne et al., 1993). Verster *et al.* (2002) concluded that driving is unsafe after administration of ALP because of it produces adverse effects such as drowsiness and sedation, in addition to its therapeutic effect.

Oxidative stress is discussed as a contributor to the initiation or progression of cellular damage and has been implicated in the pathophsiology of many neurodegenerative diseases by inducing the reactive oxygen species (ROS) that oxidize vital cellular components such as lipids, proteins and DNA which produces potentially harmful effects (Liu *et al*., 1994). The cerebellum plays an important role in motor control, and it is involved in some cognitive functions such as attention and language, and probably in some emotional functions such as regulating fear and pleasure responses (Wolf *et al.,* 2009). The cerebellum does not initiate movement, but it contributes to coordination, precision, and accurate timing. Also, it receives input from sensory systems, from other parts of the brain and spinal cord, & integrates these inputs to fine tune motor activity. The cerebellar

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* *Corresponding Author*
Dr: Azza Ahmed Attia, Zoology Department, Faculty of Science, Alexandria University, Egypt.

cortex is divided into three layers (the molecular layer, the Purkinje cell layer and the granular one). At the top lies the outermost molecular layer, which contains the flattened dendrite trees of Purkinje cells, in addition to two types of inhibitory interneuron, stellate and basket cells (Llinas *et al*., 2004). Both of them form gamma amino butyric acid (GABA) ergic synapses onto Purkinje cell dendrites. GABA is the nervous system's primary inhibitory neurotransmitter, found in the brain and spinal cord. Neurotransmitters enable the brain cells or neurons to transmit impulses from one to another. Among the most important cells in the Purkinje cell layer are the cell bodies. These cells are the major efferent neurons of the cerebellum, where they play a central role in the formation of precise-cerebellar networks and are critical for integration of motor function. These Purkinje cells receive excitatory synapses from extracerebellar locations and inhibitory input from within the cerebellum. Also, these cells use GABA as their neurotransmitter, and therefore exert inhibitory effects on their targets (Llinas *et al*., 2004). At the bottom, lies the thick granular layer densely packed with the smallest neurons in the brain, the granule cells, along with much smaller numbers of inter-neurons, mainly Golgi cells (West, 1995). Also, this layer contains the non-myelinated axons of which pass outwards to the molecular layer where they bifurcate to run parallel to the surface of synapse with the dendrites of Purkinje cells. Sorensen and Freedman (2004) studied the effect of alprazolam on the activity and number of rat cerebellar Purkinje neurons. They suggested that some of the depressant effects of ALP may be mediated by an interaction with nor epinephrine. Ozra *et al*. (2010) made a study on the ultrastructural changes of cerebellum of rats exposed to 3 mT electromagnetic field. They found significant decrease in the number of Purkinje cells, condensation of their nuclei, breakdown of the mitochondria, dilatation in the rough endoplasmic reticulum and vacuolization of cytoplasm. Acetylcholine is a major neurotransmitter found in many organisms including humans. Acetylcholinesterase (AchE) hydrolyzes the neurotransmitter acetylcholine, and it is found at mainly neuromuscular junctions and cholinergic brain synapses, where its activity serves to terminate synaptic transmission. The activity of AChE is higher in motor neurons than in sensory neurons. This enzyme activates muscles in the peripheral nervous system (Platt *et al*., 2011), and it has other effects on neurons, where it might cause a slow depolarization by blocking a tonically-active K^+ current, which increases neuronal excitability. Although acetylcholine induces contraction of skeletal muscle, it acts via a different type of receptor (muscarinic) to inhibit contraction of cardiac muscle fibers (Gulledge *et al.,* 2009). Till now, there is a lack of fundamental knowledge regarding the effect of alprazolam (ALP) on animals and consequently on man. Thus, it was of a particular interest to investigate the effect of alprazolam (as a hypnotic and anxiolytic drug) on the neurons of cerebellar cortex of mice, which is one of the central regions in which ordered organizational patterns are most obvious.

To further substantiate, the biochemical findings, histological and ultrastructural investigations of the cerebellum were undertaken, revealing the prominent ultrastructural changes.

MATERIALS AND METHODS

Experimental animals

Sexually mature, three months old albino male mice, weighing 28 ± 3 g each were obtained from the Medical Research Institute, Alexandria University, Egypt. Animals were maintained at the animal care facility in stainless steel cages (5 mice/cage), which were cleaned daily. All mice were acclimatized to the laboratory environment for two weeks prior to the starting of the experiment, where they were adapted to the controlled environmental conditions at room temperature of 25 ± 2 °C, relative humidity 60-70 %, and at normal photoperiod 12 h/d. The methodology of this work was approved by the Ethics Committee on Animal.

Drug preparation

Alprazolam (Zolam®) was purchased from the local pharmacy, and produced by Amoun Pharmaceutical Company, Egypt, in the form of tablets. Each tablet contains 0.5 mg alprazolam, and it was dissolved in saline solution (0.9 % NaCl). Three different dose levels of alprazolam were prepared according to the equivalent prescribed human doses and according to the severity of symptoms, such as: in state of anxiety (0.5 mg daily) and for depression (1-1.5 mg) (Anwar *et al*., 2011). These doses were calculated to be 0.007, 0.014 and 0.021 mg/kg bw, respectively.

Experiment

Sixty male mice were randomly assigned into four groups (15 mice/each). Mice that received orally by gavage 0.5 ml saline solution of 0.9% NaCl were considered as a normal control. The three experimental groups were daily administered orally by gavage with 0.5 ml of one of the three prepared doses of alprazolam, for two months.

Signs of toxicity

All mice of the experiment were carefully examined daily throughout the duration of experiment in order to depict any apparent behavioural changes and/or signs of toxicity, and percentage of mortality was recorded.

Body and organs weights

Mice of the control and experimental groups were weighed at the beginning of the experiment and at one week intervals during the time experimental period till the end of experiment (two months). The mean body weight and the weight changes were calculated and tabulated**.** The weight change (%) was estimated by dividing the final body weight/ initial body weight ×100.

At the end of the experiment, the control and alprazolam-treated mice were euthanized using diethyl ether and dissected out. The brain tissues were excised out quickly, grossly examined and weighed. The relative weight of brain was calculated according to Matousek (1969**)** by dividing the absolute organ weight (g)/ body weight (g) × 100.

Tissue preparation for the biochemical measurements

Twenty-four hours after the last treatment, the animals were euthanized using diethyl ether. The control brain tissues as well as experimental animals were immediately removed and washed in ice-cold glass slides, homogenized separately in 10 volumes (w/v) of 0.1M phosphate buffer, pH 7.4 using a polytron homogenizer for one minute. The homogenates were centrifuged at 4000 r.p.m. for 20 minutes, and refrigerated at 4 °C. (Pelligrino *et al.*, 1979). The supernatant were used for estimation the quantitative activities of both acetylcholine esterase and the total glutathione transferase.

Estimation of the enzyme acetylcholine esterase enzyme activity

Acetylcholinesterase activity was measured by the method of Ellman *et al.* (1961). This method is based on the hydrolysis of acetylthiocholine iodide (ATChI) as substrate by the enzyme to produce thiocholine and acetic acid. Thiocholine reacts with dithiobis nitrobenzoate (DTNB) to produce the yellow anion of 5-thio-2-nitrobenzoic acid

Estimation of the total glutathione transferase (GST) enzyme activity

Glutathion S transferase enzyme activity was assayed by the method of Habig *et al.* (1974). The biodiagnostic glutathione S- transferase assay kit measures the total GST activity by measuring the conjugation of 1-chloro- 2, 4- dinitrobenzene (CDNB) with reduced glutathione. The conjugation is accompanied by an increase in absorbance at 340 nm. The rate of increase is directly proportional to the GST enzyme activity in the sample.

Light microscopic investigation

Small slices of the cerebellar tissues of the control and ALP-treated mice were fixed overnight at freshly prepared 10% neutral formalin. The fixed specimens were processed by the usual recognized histological methods of dehydration, clearing by xylene and embedding in paraffin wax (Bancroft and Gamble, 2002). Serial sections were cut at 5-6 μm thick, stained with hematoxylin-eosin (H&E), examined and photographed by the light microscopy**.**

The electron microscopic study

Very small slices of the cerebellar tissues of the control and ALP-treated mice were taken out quickly, immediately fixed in 2 % 4F1G, rinsed in 0.1M phosphate buffer, pH = 7.4 at

4˚C for 1 h, then rinsed in 0.1 M phosphate buffer (pH 7.4). This was followed by post-fixation using 2% buffered OsO4 (osmium tetroxide) for 1-2 h at 4˚C. Then, the specimens were washed with phosphate buffer for several times for 30 min, dehydrated in ascending grades of ethanol concentration, and embedded in epon-araldite mixture in labelled beam capsules. Ultrathin sections (50-60 nm) were cut with ultramicrotome using glass knives.

Samples were collected in naked copper mesh-grids and stained with 2% aqueous uranyl acetate for 30 minutes and lead citrate for 20 min (Hayat, 2000). These sections were examined and photographed on a Joel, 100 CX II transmission electron microscopes at the Faculty of Science, Alexandria University, Alexandria, Egypt.

Statistical analysis

For statistical analyses, the SPSS for windows software package version 18.0 (SPSS, Chicago, IL, USA) was used. Data was given in the form of arithmetical mean values and standard deviations. One-way analysis of variance (ANOVA) was performed and variant groups were determined by means of the Duncan test. P value was assumed to be significant at 0.05.

RESULTS

Signs of toxicity

The results revealed more prominent signs of toxicity in mice administered with 1 and 1.5 mg ALP, where most treated mice became progressively less active and showed general weakness.

Also, they had lost their appetite which was noticed after the first time of treatment with the drug, and showed marked loss in their body sizes. Control mice showed no mortality during the experimental period, however, there was a gradual increase in the rate of mortality in a dose dependant manner in ALP-treated mice **(Table 1).**

Body weight change and the relative brain weight

At the end of the experiment, mice treated with 1 and 1.5 mg ALP showed marked significant decreases in the body weight change, comparing to those treated with 0.5 mg ALP and the control. Also, the results revealed statistical significant decrease in the relative brain weight of alprazolam-treated mice, comparing to the control (Table 1).

Table. 1: % of mortality, body weight change and the relative brain weight of mice treated with the different doses of alprazolam (ALP) for two months.

Experimental groups	No. of mice	No. of dead mice	% of mortality	Mean of body weight (g)		The mean change in body weight	Brain weight	
				Initial	Final		The absolute wt. of brain	The relative Wt. of brain
Control mice	15	0	0.0	30.03±2.98	34.12 ± 3.15 [a]	13.2 ± 1.06[a]	0.44 ± 0.042	1.19 ± 0.106 [a]
0.5 mg/kg bw ALP	15	1	6.6	31.2 ± 3.07	34.3 ± 3.55[a]	9. 94 ± 1.03[b]	0.38 ± 0.036	1.11 ± 0.162 [b]
1 mg/kg bw ALP	15	3	20	28.9 ±2.77	25.2 ± 2.98[b]	-12.8 ± 1.22[c]	0.28 ± 0.025	1.11 ± 0.152 [b]
1.5 mg/kg bw ALP	15	5	33.3	30.5± 2.84	25.4 ± 2 .16[b]	-16.72± 1.68[c]	0.27 ± 0.031	1.06 ± 0.108 [b]

Data are presented as mean ± SD,
The same small letters show no significant differences from the control,
The different small letters indicate that there were significant differences at value of $P \leq 0.05$.

Biochemical assays

Depending on the dose levels of alprazolam, the results revealed significant decreases in both the levels of acetylcholine enzyme activities and the total glutathione transferase in the brain tissues of mice administered with the three doses of ALP, comparing to the control **(Figs. 1 & 2)**.
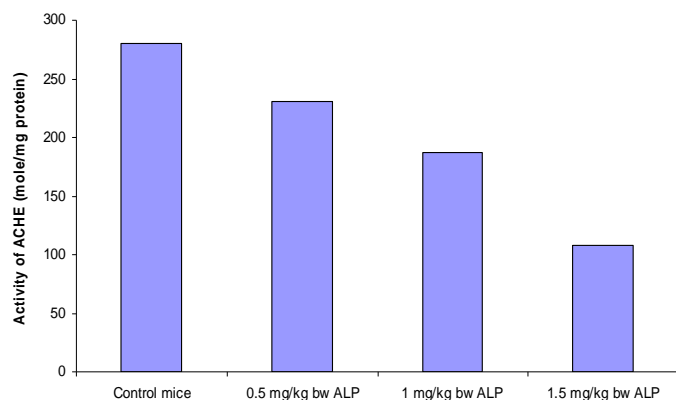

Fig. 1: Activity of acetylcholine esterase enzyme (AChE) in the brain tissues of mice treated with the different doses of alprazolam (ALP) and the control.
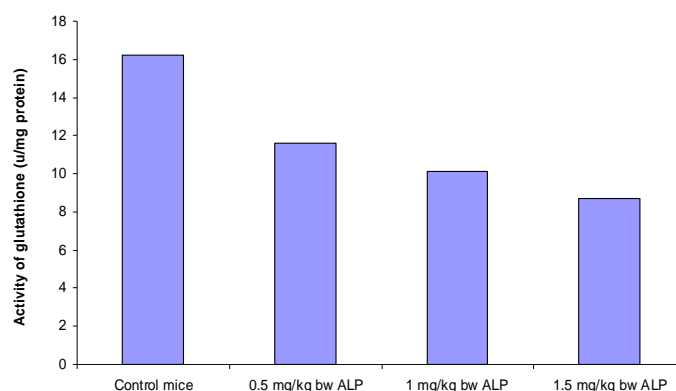

Fig. 2: Activity of the total glutathione transferase in the brain tissues of mice treated with the different doses of alprazolam (ALP) and the control.

Light microscopic findings

The cerebellar cortex of control mice is seen to consist of three different layers: the outermost molecular layer, an inner granular layer and a central Purkinje cell layer. The molecular layer contains glial cells, few neurons, those of stellate and few scattered nuclei of basket cells (Fig. 3a).

The Purkinje cells in the Purkinje cell layer have large flask-shaped appearance, and they are arranged typically in a single row at the junction between the molecular and the granular layers. Each of these cells has a conspicuous cell body and an extensive fan-like dendrite tree. Also, they displayed characteristic centrally located vesicular nuclei and prominent nucleoli (Fig. 3a).

The granular layer is stained intensely with H & E stain, and it is extremely contains numerous small neurons called the granule cells, which have large, rounded prominent nuclei and a small rim of scanty cytoplasm. Also, there are cerebellar islands in between these cells. Further, there were groups of nuclei of the Bergmann astrocytes, scattered in the superficial part of the granular cell layer and in between the Purkinje cells (Figs. 3 a,b).
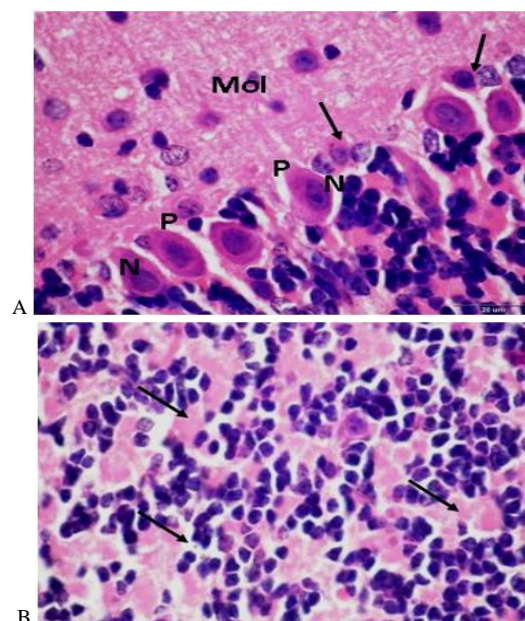

Fig. 3 a, b: Light micrographs of section of cerebellar cortex of cerebellum of control mouse showing: (A): the outer molecular layer (Mol); Purkinje cell layer contains large flask-shaped Purkinje cells (P), having centrally located vesicular nuclei (N) and prominent nucleoli; Note: the dark-stained granular cells (g) in the granular layer; arrows point at the Bergmann astrocytes, X 630. (B): the granular layer contains numerous small dense granule cells and cerebellar islands in between them (arrows), X 630.

In H&E-stained sections of cerebellar cortex of 0.5 mg alprazolam-treated mice, few Purkinje cells were affected in between the normal ones, while the molecular and the granular layers were more or less affected, and they were similar to the control (Figs.4a,b). The Purkinje cells showed no typical arrangement in a layer and revealed cytoplasmic eosinophilic homogenization in association (Fig.4a). Further, the Bergmann astrocytes were increased in number around these cells. In the cerebellar cortex of mice administered with 1 mg alprazolam, the Purkinje cells revealed marked degenerative changes, where the monolayer arrangement of them was disrupted. Further multilayer deposition of these cells was observed in some sections with loss of their normal pyriform shaped appearance, and they were mostly pyknotic (Figs. 5a,b). The molecular layer had clear degenerated areas (Fig.5a). In the granular layer, the granule cells were compact and fused together forming degenerated necrotic areas, and revealing severe signs of injury and they had lost their normal organization (Fig.5b). The histopathological observations of cerebellar cortex of 1.5 mg alprazolam-treated mice showed a prominent hemorrhage in the vicinity of the Purkinje and the granular cell layers (Fig.6a). Most of the Purkinje cells appeared deformed and shrunken, losing their characteristic pyriform shaped, and they acquired a triangular forms (Figs.6a,b). The cell surface of some of them showed spiny protrusions of plasma membrane and they showed haloes spaces around them (Fig.6a). In other sections, these Purkinje cells were displaced upwards in the molecular layer, while others were migrated downwards in the granular one (Fig.6b).
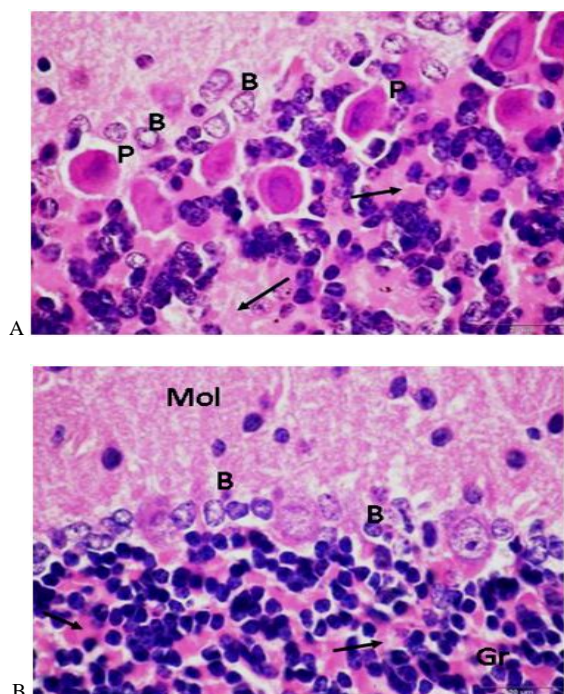
Fig. 4 a, b: Light micrographs of section of cerebellar cortex of cerebellum of mice treated with 0.5 mg/kg bw alprazolam showing: (a) No typical arrangement of Purkinje cells (P) in a layer; the granular cell layer showed normal cerebellar island (arrows); Bergmann astrocytes (B), X 630. (b): The molecular (Mol) and the granular cells (Gr) are more or less similar to the control; Notice: the cerebellar islands (arrows) and the Bergmann astrocytes (B), X 630.
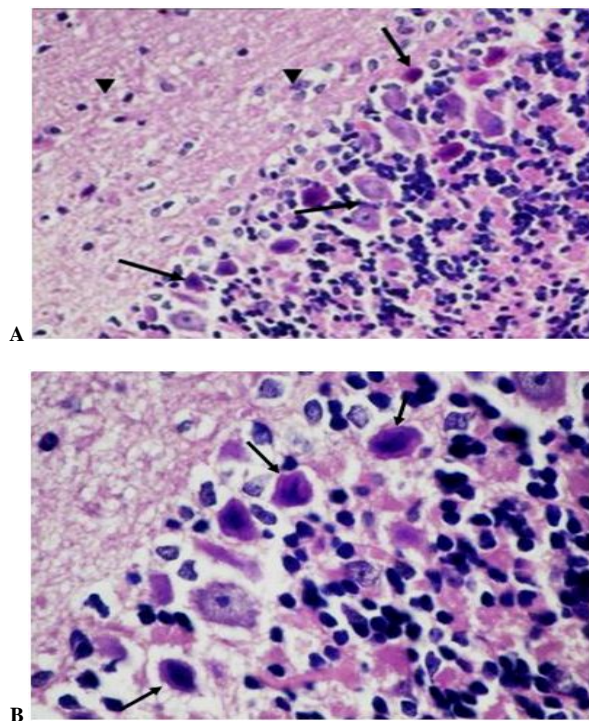

Fig. 5 a,b: Light micrographs of section of cerebellar cortex of cerebellum of mice treated with 1 mg/kg bw alprazolam showing: (a): Multilayer deposition of Purkinje cells (arrows) with marked loss in their normal appearance; degenerated areas of the molecular layer (arrowheads), X 400. (b): Many pyknotic nuclei (arrows) of Purkinje cells; granular cells had lost their normal organization, X 400.

The granular layer showed a generalized decrease in the granule cell population and revealed patchy cell loss or depletion leaving islet- like bold spots **(Fig. 6a)**. Further, marked loss in the Bergmann astrocytes was observed in most sections **(Figs. 6a,b)**.
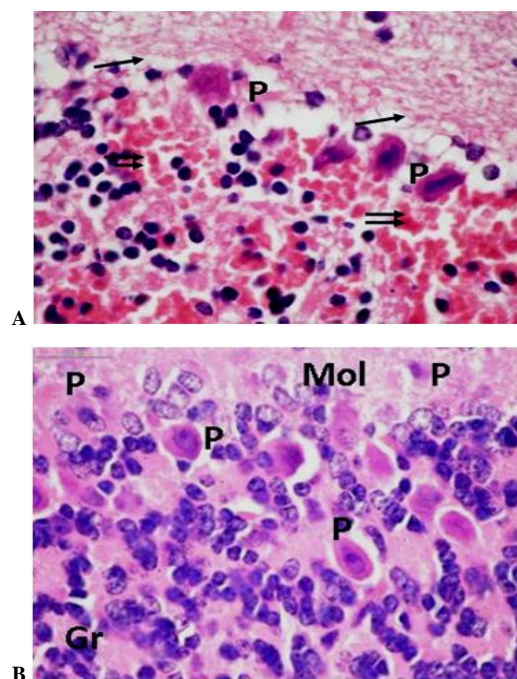

Fig. 6 a, b: Light micrographs of section of cerebellar cortex of cerebellum of mice treated with 1.5 mg/kg bw alprazolam showing: (a): Prominent hemorrhage in the vicinity of the granular layer (double arrows); Note: the disorganized and shrunken Purkinje cells (P) with condensed nuclei; arrows point at the halo empty spaces around the Purkinje cells, X 630. (b): Displacement of Purkinje cells (P) downwards in the granular cell layer (Gr) and upward in the molecular layer (Mol), X 630.

Ultrastructural findings

Ultrastructural examination of the cerebellar cortex of control mice revealed that the molecular layer is mostly formed of an extension of axons and dendrites of cells of the same molecular layer including basket and stellate cells, and of the second Purkinje cell layer, as well as climbing and mossy fibers derived from the deeper structure of the cerebellum. The basket cells are widely distant from each other and contain a small amount of cytoplasm surrounding pale euchromatic nuclei. The cytoplasm of these cells contains few dense small mitochondria (Fig. 7a), short cisternae of rough endoplasmic reticulum, free ribosomes, and electron dense bodies. The Purkinje cells contain large eccentric euchromatic nuclei and well defined nucleoli. Their contours were irregular and their nuclei were bounded by rough endoplasmic reticulum organized into aggregates of parallel cisternae (Fig. 7b). The granular layer contains many dark small granule cells, having slightly darker spherical nuclei with their characteristic condensed chromatin surrounded by very little cytoplasm (Fig. 7c). The Bergmann astrocytes are observed ensheathing the Purkinje cells, and their nuclei contain large blocks of condensed chromatin distributed on the inner side of the nuclear envelope, and surrounded the mossy rosettes containing many mitochondria and synaptic vesicles (Fig. 7d).
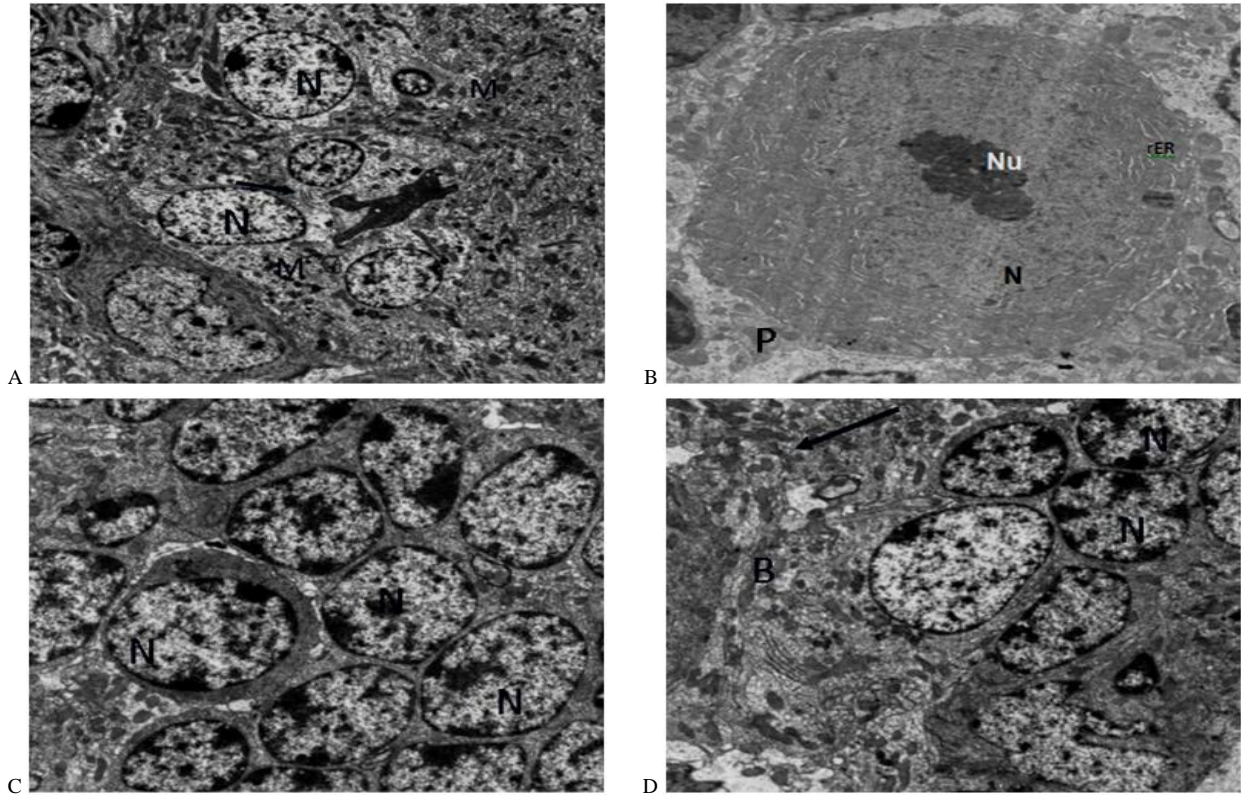
Fig. 7 (a-d): Electron micrographs of section of cerebellar cortex of cerebellum of control mice showing: (a): The pale euchromatic nuclei (N) of Basket cells in the molecular layer; the cytoplasm contains dense small-sized mitochondria (M), X 3000; (b): An apparently normal Purkinje cell (P) with large ovoid eccentric nucleus (N) and apparent nucleolus (Nu); aggregate cisternae of rough endoplasmic reticulum (rER), X 4000; (c): Many dark small granule cells having slightly darker nuclei (N) and thin rim cytoplasm, X 5000. (d): The granular layer contains many dark nuclei (N) of the granule cells; Bergmann astrocytes (B); arrow points at area of mossy rosettes with many mitochondria and synaptic vesicles, X 4000.
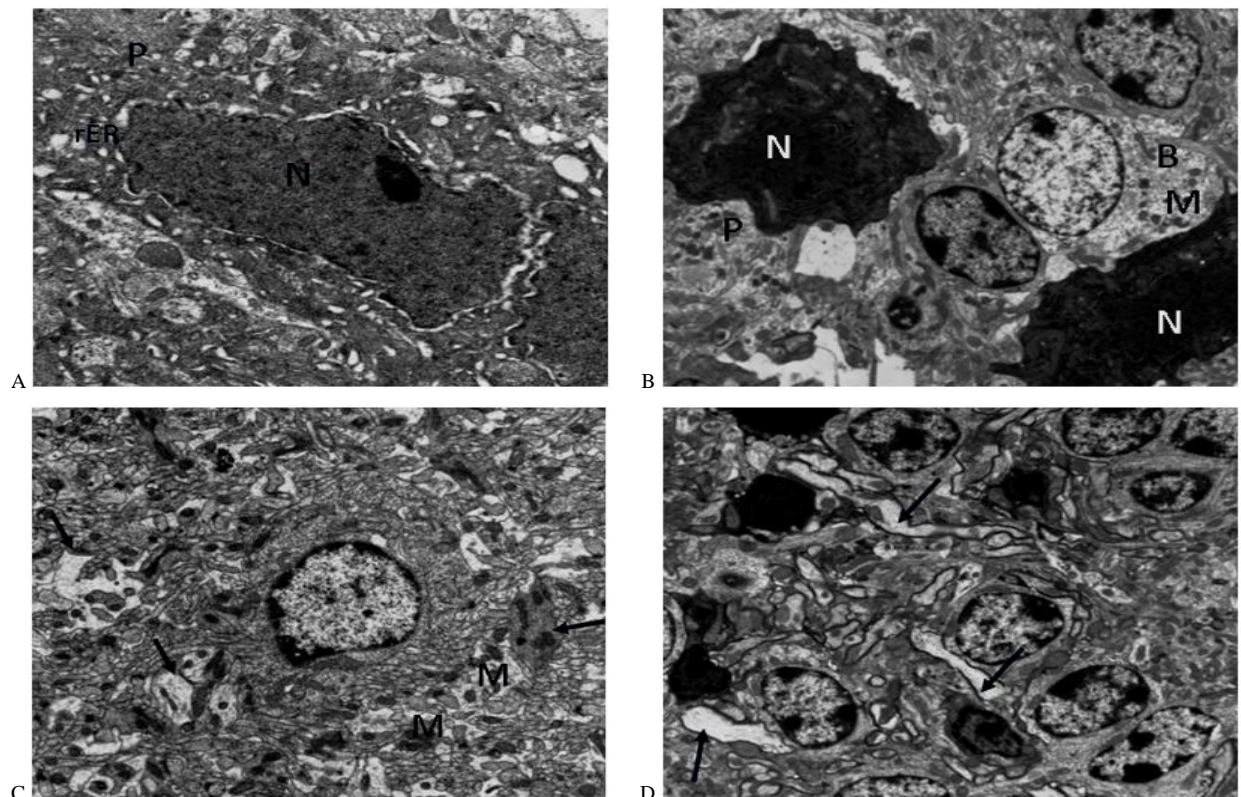
Fig. 8 (a-d): Electron micrograph of section of cerebellar cortex of cerebellum of mice treated with 0.5 mg/kg b.w alprazolam showing: (a): a shrunken Purkinje cell (P) with an irregular-shaped nucleus (N) and vacuolated rER, X 5000. (b): Small sized pyknotic nuclei (N) of the destructed Purkinje cells (P); small dense mitochondria (M) in the cytoplasm of Bergmann astrocyte (B), X 5000. (c): An area of mossy fibers in the molecular layer containing many synaptic vesicles (arrows); the mitochondria (M) contain indistinct cristae, X 5000. (d): Loss of the lamellar compact structure of myelin in the myelinated axons (arrows) of the granular layer, X 5000.
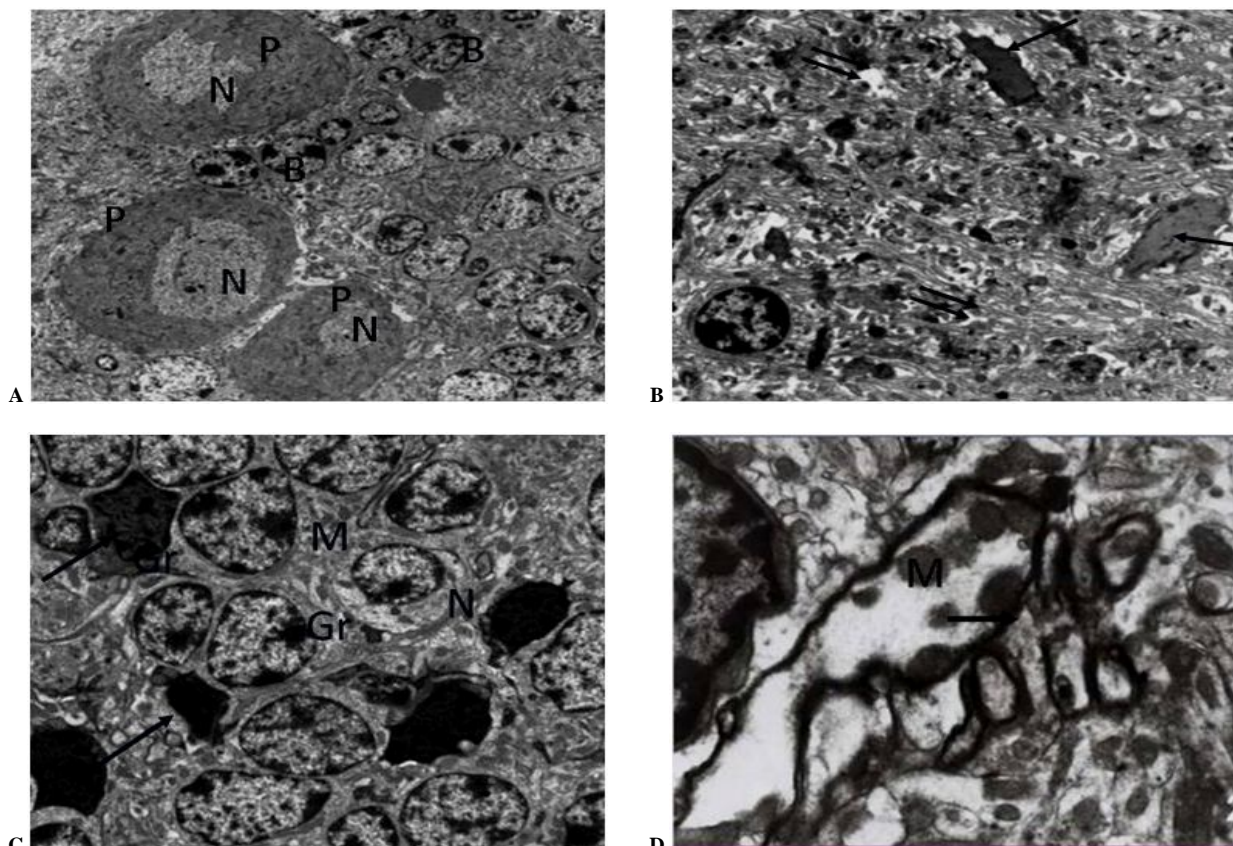
In cerebellar cortex of 0.5 mg alprazolam-treated mice, the Purkinje cells were mostly affected, where they exhibited marked shrinkage of their cell bodies and the cytoplasm contained vacuolated rough endoplasmic reticulum (Fig. 8a). In other sections, these cells contained pyknotic nuclei (Fig. 8b).

In the molecular layer, few dendrites were affected in between the normal ones and the regular compact lamellar structure of the myelin sheath was preserved around the apparently normal axons and synaptic vesicles (Fig. 8c). In the granular layer, there was marked loss of the lamellar compact structure of the myelinated axons (Fig. 8d). The Bergmann cells were observed with little cytoplasmic area and contained very small dense mitochondria (Fig. 8b).

Further, marked alterations in the Purkinje cells were prominent in the cerebellar cortex of mice administered with 1 mg alprazolam, where many cells were smaller in size, containing pale-staining nuclei and indistinct nucleoli. In addition, prominent infoldings in the nuclear envelope were noticed (Fig. 9a).

The molecular layer revealed the appearance of many necrotic cells, and the axons appeared swollen with irregular outlines and revealed disorganization of their neurofilaments (Fig. 9b). The granular cells are observed with their rounded heterochromatic nuclei and the cytoplasm exhibited few

mitochondria and short strands of rough endoplasmic reticulum. Further, many star-shaped cells with condensed nuclei were detected (Fig. 9c). The myelinated axons revealed areas of degenerative changes in the form of disruption, splitting and loss of the lamellar compact structure of myelin layers (Fig. 9d). Many Bergmann astrocytes were observed en-sheathing Purkinje cells with their processes. These cells contained pale euchromatic nuclei and few cytoplasmic organelles as mitochondria (Fig. 9a). In cerebellar cortex of 1.5 mg alprazolam-treated mice, prominent ultrastructural alterations were obviously observed in the Purkinje cells. Most of these cells appeared deformed and shrunken, losing their characteristic pyriform shaped, and they had increased infoldings of their nuclear envelopes. The cytoplasm showed dilatation of the endoplasmic reticulum cisternae (Fig. 10a). Further, many nuclei of the Purkinje cells were pyknotic (Fig. 10b). The molecular layer revealed more necrotic areas and dilated nerve fibers having loss of mitochondria (Fig. 10c). In the granular layer, the myelinated axons revealed severe areas of degenerative changes in the form of disruption, splitting and loss of the compact lamellar structure of myelinated axons (Fig. 10d). Moreover, the Bergmann astrocytes revealed as swollen cells with pale nuclei and clear cytoplasm. These cells surrounded the mossy rosettes containing many mitochondria and synaptic vesicles (Fig. 10b).

Figs. 9 (a-d): Electron micrographs of section of cerebellar cortex of cerebellum of mice treated with 1 mg/kg b.w alprazolam showing: (a): Many small-sized Purkinje cells (P) having pale-stained nuclei (N) with prominent infoldings in the nuclear envelopes; Notice: the appearance of many Bergmann astrocytes (B) en-sheathing the Purkinje cells, X 3000. (b): Many necrotic cells (arrows) in the molecular layer; doublearrows point at the disorganized appearance of synaptic vesicles, X 3000. (c): The nuclei (N) of the granule cells (Gr); few mitochondria (M); many star-shaped cells (arrows) with condensed nuclei, X 3000. (d): The mitochondria (M) with condensed matrix in the nerve fibers; arrows point at spitting of the myelin sheath in some areas of myelinated fibers, X 5000.
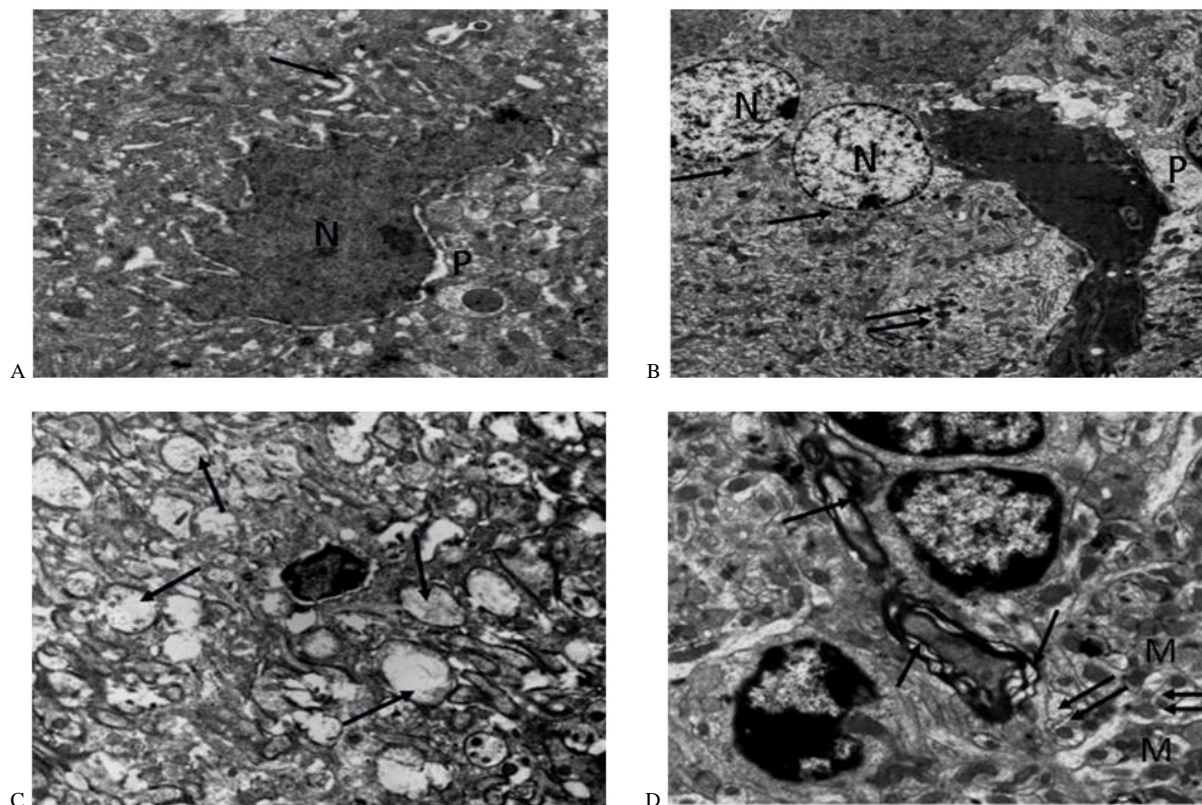
Figs. 10 (a-d): Electron micrographs of cerebellar cortex of cerebellum of mice treated with 1.5 mg/kg b.w. alprazolam showing: (a): a shrunken Purkinje cell (P) having a small deformed-shaped nucleus (N); Note: the increased infolding of the nuclear envelope and the vacuolated rough endoplasmic reticulum (arrow), X 5000. (b): A pyknotic nuclei of Purkinje cell (P); an area of mossy rosettes contains many mitochondria and synaptic vesicles (doublearrow); euchromatic nuclei (N) of Bergmann astrocytes (arrows), X 4000. (c): A necrotic area of the molecular layer; Notice: swollen nerve fibers (arrows) having marked loss in mitochondria, X 4000. (d): Area of the granular layer containing the myelinated axons of degenerative changes in the form of disruption, splitting and loss of the lamellar structure (arrows); many synaptic vesicles (doublearrows) containing mitochondria (M), X 5000.

DISCUSSION

The current results revealed significant decreases in the body weights and the relative brain weights of alprazolam-treated mice, depending on the dose levels. This decrease might be either due to the decrease in appetite of mice, or the drug toxicity which might accelerates the water elimination in urine. Also, this might be due to the gastrointestinal toxicity and thereby reduced ingestion of food (Tikoo *et al.*, 2007). Similar observations have been reported by many investigators (Gonz and Evans 1997) who studied the neurotoxic effect of lead on cerebellum of rats. The current results showed that 1.5 mg/kg bw ALP induces significant decreases in the activity of the total glutathione, which was in proportional with the decrease in the activity of acetylcholine esterase in the brain tissues of the treated mice. The brain, compared to liver, lung and other organs, contains relatively low levels of enzymatic and non-enzymatic antioxidants and high amounts of peroxidizable polyunsaturated lipids, rendering it more vulnerable to oxidative stress compared to other tissues (Bondy, 1997). Also, the brain exhibits distinct variations in cellular as well as regional distribution of antioxidant biochemical defenses (Verma and Srivastava, 2001). Thus, neural cells and/or brain regions are likely to differentially respond to changes in metabolic rates associated with the generation of ROS (Hussain *et al.*, 1995).

The cellular glutathione is the most abundant low molecular weight thiol involved in antioxidant defense in animal cells, and it is the major antioxidant compound that acts directly both in removing reactive oxygen species (ROS), and as a substrate for several peroxidases. Therefore, it can be said that increase in ROS production due to exposure to ALP led to the decrease in the level of acetylcholine esterase activity. This is confirmed with the suggestion of Tsakiri et al. (2000) who reported that the activity of acetylcholine esterase was inhibited by free radical formation. Feoli *et al.* (2006) suggested that glutathione deficiency contributes to oxidative stress in many brain disorders, including seizure and stroke, as well as in neurodegenerative diseases such as Alzheimer's and Parkinson's diseases. Stress has been shown to affect several brain activities and promote long-term changes in multiple neural systems (Imbe *et al.*, 2006). Levi and Basuaj (2000) found that stress has been shown to cause a decrease in the level of glutathione which protect the tissues from oxidative damage. Trivedi *et al.* (2007) and Zhang *et al.* (2008) attributed the cause of oxidative stress in the brain tissue after sodium fluoride treatment to be due to decreased mRNA and protein expression levels of neural cell adhesion molecules in neurons, contributing to the neuronal dysfunction and synaptic injury. In accordance with the suggestion of Tsakiri et al. (2000), it can be said that increase in ROS observed in the present experiment

might have inhibited the acetylcholine esterase activity in the cerebellum of alprazolam-treated animals. This could upset the pro-oxidant/antioxidant balance within the brain, which could be one of the reasons for decreased acetylcholine esterase activity. Gilgun-Sherki *et al.* (2001) suggested that ROS attack glial cells and neurons which are post-mitotic cells, and therefore they are particularly sensitive to free radicals, leading to neuronal damage. The histological structure of the cerebellar cortex of cerebellum of mice administered with the different doses of ALP was disrupted and disorganized and the most remarkable ultrastructural changes are observed in the Purkinje neurons. These cells had lost their specific "flask shaped" appearance, reduced in their size, and had lost their cell boundaries. Further, the cell surface of some cells showed spinous protrusions of plasma membrane and they acquired a triangular shape. These results are in consistence with the results of many investigators (Shivarajashankara *et al.*, 2002) who studied the effect of sodium fluoride on the neurodegenerative changes of mice and rats. Further, many unstained haloes were seen around most of the destructed Purkinje cells in ALP-treated mice. This was in accordance with the suggestion of Abou-Elghait *et al.* (2010) who attributed that due to shrinkage of Purkinje cells and withdrawal of their protoplasmic processes, secondary to disintegration of the cytoskelelal elements of these cells. Ultrastructurally, the increase in the enfolding of the nuclear envelope of the Purkinje cells and the appearance of many cytoplasmic changes might reflect the association between alprazolam and oxidative stress. The nuclei of these cells were eccentrically placed nuclei, many of them were pyknotic, and have condensation of chromatin. As regards to the intracellular n structure of Purkinje cells, high doses of ALP-treated mice showed cytoplasmic vacuolation, dilatation of rER and Golgi apparatus. Rough endoplamic cisternae were dilated, and many small vesicles were accumulated to form clusters near the Golgi bodies which was probably an indicator of the disturbance in the vesicular transport between rough endoplamic reticulum and Golgi apparatus.

These findings could be explained by the report of previous investigators who suggested that dying neurons can undergo condensation and dissolution of chromatin (Olivi *et al.*, 1993). The cytoplasm of most of Purkinje cells showed marked decrease and prominent vacuolation in the intracellular organelles. Sobaniec-Lotowska (2001) suggested that the marked changes in mitochondria of Purkinje cells could be interpreted as disorder of intercellular biochemical events such as inhibition of oxidative phosphorylation due to direct toxic effect of the drug valproate or its metabolites. Gold *et al.* (2004) have postulated that the intracellular changes in Purkinje cells can result from oxygen deprivation (anoxia), because neurons require relatively large quantities of oxygen due to their high metabolic rate. In accordance with the results of Brizzee *et al.* (1975), there was a decrease in Nissl's material and rough endoplasmic reticulum resulting in a decrease ability of the neurons to carry out the vital function of protein synthesis. Jacob (2007) postulated that the dilation of the endoplasmic reticulum is possible during necrosis.

In consistence with the suggestion of Ratan *et al.* (1994), the appearance of many dark neurons might reflect a certain phase of apoptosis as they displayed markedly condensed cytoplasm and nucleoplasm. Sobaniec Lotowska (2001) believed that the dark small neurons is usually ischemic due to possible substantial abnormalities in the capillary wall of the cerebellar cortex and with subsequent disorders in the structural elements of the blood-brain barrier to neurons and vice versa Further, the present results revealed that the myelinated axons of cerebellar cortex of ALP-treated mice revealed considerable degenerative structural changes within their axoplasm as well as splitting and disruption. This was similar to that recorded by Sobaniec Lotowska (2002) who made an ultrastructural study on synaptic junctions in the cerebellar cortex in experimental valproate encephalopathy. It was reported that the myelinated nerve fibers seen in the molecular layer of cerebellar cortex were thought to belong to the parallel fibers of granule nerve cells or to the recurrent collaterals of Purkinje cell axons, while those seen in the granular layer were mostly believed to belong to the axons of Purkinje cells. Lehning *et al.* (2002) and Sobaniec-Lotowska and Lotowska (2005) explained that the disruption in myelination was attributed to the changes in myelin basic protein secondary to membrane damage and axonal degeneration after exposure to toxic substance. They added that dysmyelination including folding and splitting at various levels of the myelin lamellae was attributed to increased water content in degenerating nerve causing intramyelinic edema with separation of myelin lamellae. The present study summarizes our current understanding about the mechanism by which alprazolam exert its toxic effect on the neuronal cells of cerebellar cortex of male mice.

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