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Effects of Benzodiazepine Treatment (Xanax) on Cerebellar Cortex of Male Mice: Histopathological and Ultrastructure Studies

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Abstract:

The present study aims to illustrate the effects of Xanax on the histology and ultrastructure of the cerebellum tissue on male mice. Thirty male mice were randomly assigned into two groups (15 mice/each) according to their approximately equal mean body weight. Mice that received orally 0.5 ml saline solution of 0.9 % NaCl by gavage were considered as control mice. Other experimental mice were daily administered 0.5 ml of Xanax at a dose of 1.5 mg/kg b. w. orally by gavage for 8 weeks. Light microscopic examination of the cerebellar tissues of Xanax treated mice revealed histopathological alterations and showed wide spread neuronal affection specifically of the Purkinje cell layer which was reflected on the other two layers. A prominent hemorrhage in the vicinity of the Purkinje and the granular cell layers was also noticed. More conspicuous pathological changes were observed in the Purkinje cells, where they revealed disturbed normal linear organization with marked disarrangement. Prominent ultrastructural alterations of the Purkinje cells were obvious, where most of them appeared deformed and shrunken losing their characteristic pyriform shape, The Bergmann astrocytes revealed as swollen cells with pale nuclei and clear cytoplasm contained increased number of organelles specially mitochondria, filaments and autophagosomes. Ultrastructure of cerebellar tissues of Xanax treated mice confirmed the light microscopic observations. In conclusion, mice administered of 1.5 ml of Xanax revealed different symptoms of toxicity in cerebellar tissues.

Keywords: *Histopathological, Cerebellar Cortex, Xanax, Ultrastructure, Mice.*

Introduction:

Benzodiazepines (BZDs) comprise a large group of psychoactive drugs, that are massively used in human pharmacotherapy for their anxiolytic, hypnotic, myorelaxant, amnesic and anticonvulsive properties [1]. Benzodiazepines with their proven efficacy in panic disorder exerted through control of the central nervous system (CNS) excitability by a selective and potent enhancement of inhibitory gamma amino butyric acid (GABA) mediated neurotransmission [2]. Cloos and Ferreira [3]; D'Hulst *et al.*[4] in their works as ligand-gated channel for Cl^- ions. Binding of BZDs to gamma amino butyric acid receptor A ($GABA_A$) promotes the effects of GABA on $GABA_A$

receptor and consequently increases the conductance of Cl^- across the neuronal cell membrane, increases membrane potential and inhibits neuronal firing [5].

Xanax (Kalma) is a triazolobenzodiazepine used in panic disorder and other anxiety states. Xanax is a newer benzodiazepine that is being used more commonly in overdose [6]. Xanax is the most commonly used anti-anxiety drug because they are believed to be fairly safe and it rapidly reduces the symptoms of anxiety. Moreover, Xanax shows some peculiar neurochemical effects which have not been reported for typical benzodiazepines. Acute[7] and chronic[8] Xanax treatment produces an opposite effect on brain concentrations of corticotropin-releasing hormone (CRH)and on peripheral levels of adrenocorticotropic hormone (ACTH) to that seen after stress and also reduces cate-cholamine concentrations during stress[9]. Stimuli like mild uncontrollable stressors activate central dopamine activity, and this effect is preventedby the administration of anxiolytic agents [10]. Moreover, the mesolimbic dopamine system plays a key role in stress responses and adaptation also in view of the development of depressive symptoms [11]. Anxiolytic drugs are among the most frequently prescribed substances, used regularly by upwards of 10 % of the population in developing countries [12]. Drugs that relieve anxiety generally cause a degree of sedation and drowsiness, which is one of the main drawbacks in the clinical use of anxiolytic drugs. In high doses all these drugs cause unconsciousness and eventually death from respiratory and cardiovascular depression.

They are usually classified by their elimination half-life rather than by differences in efficacy. Long-half-life benzodiazepines, such as diazepam, clonazepam and chlordiazepoxide, accumulate with repeated doses and clearance is significantly increased with age. For this reason, short-half-life benzodiazepines are usually recommended for the elderly because they do not accumulate and presser it greater flexibility dosage [13,14]. Xanax is intermediate-half-life compound (approximately 14 h) whose clearance is somewhat delayed by the aging process [15]. The usual starting dose of Xanax varies between 0.5 and 1 mg per day. In exceptional cases, a peak dose of 10 mg/day was necessary [16]. Benzodiazepines produce dose-dependent adverse effects typical of sedative-hypnotics, although they possess a higher therapeutic-toxic ratio than many other drugs [16,17]. When benzodiazepines are discontinued a well-described discontinuation syndrome may appear [18], typically include restlessness, agitation, anxiety, poor sleep the opposite of

therapeutic benzodiazepines effects. It is likely that many patients continue benzodiazepine use to avoid the appearance of these discontinuation symptoms. 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 [19]. 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 [20]. Acetylcholin esterase (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. This enzyme activates muscles in the peripheral nervous system [21], 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 [22]. This study was aimed to investigate neurotoxic effects of Xanax on the histology and ultrastructure of cerebellar tissues on male mice.

Materials and methods:

Chemicals:

Xanax (Kalma) was purchased from the local pharmacy, and produced by Amoun Pharmaceutical Company, Egypt, in the form of tablets. Each tablet contains 0.5 mg Xanax, and it was dissolved in saline solution (0.9 % NaCl).

Experimental animals:

Thirty male albino mice, weighing approximately 30±3 g each. Animals were maintained at the animal care facility in stainless steel cages (5 mice/cage). All mice were adapted to the controlled environmental conditions at room temperature of 25 ± 2 °C, relative humidity 60-70 %, and normal photoperiod 12 h/d. Also, they were allowed free access of food and drinking water *adlibitum.* Mice were acclimatized to the laboratory environment for two weeks prior to the starting of the experiment. Mice were randomly allotted into two groups (15 mice/each) according to their approximately equal mean body weight. The animals belonging to the first group served as control and were received orally by gavage 0.5 ml saline

solution of 0.9 % NaCl. Other experimental mice were daily administered orally by gavage with 0.5 ml of Xanax at a dose of 1.5 mg/kg b. w. for 8 weeks [23,24]. On completion of the experimental period, animals from each group were sacrificed under ether anesthesia and cerebellar tissues were removed and fixed in the suitable fixative for histological and ultrastructural studies.

Histological studies:

Cerebellar tissues specimens from all groups were fixed in formalin and embedded in paraffin. Sections of 5µm thickness were stained with hematoxylin and eosin using standard procedures [25]. The stained sections were examined under light microscope.

Ultrastructural studies:

Tissues samples were further processed for ultrastructural evaluation by transmission electron microscopy (TEM); the brain samples were cut into small pieces of about 1 mm^3 and fixed in 2.5 % glutaraldehyde for 48hr. The specimens were then washed in 0.1 M phosphate buffer (pH7.4) 3-4 times for 20 min. every time and post-fixed in a buffered solution of 1 % osmium tetroxide at 4ºC for 2 hr. Ultrathin sections (60-70 nm) were cut with a diamond knife using an ultra-microtome (MT6000-X L RMC, Inc.), mounted on copper grids and double stained with uranyl acetate and lead citrate [26]. Grids were viewed and photographed using a transmission electron microscope (JEOLJEM–1200 EX 11, Japan) operated at 60-70 kV.

Results:

Light microscopic findings of the cerebellar tissues:

Sections of cerebellum of the control mice is characterized by complex convoluted folding of cerebellar cortex generating pattern of folia. These folia are formed of an external gray matter and an internal white matter. The gray matter is prevalent at the surface of the cerebellum, forming the cerebellar cortex, whereas the white matter is present in more central regions (Fig. 1). The white matter showed no distinctive histological features. The cerebellar cortex is seen to consist of three different layers. The molecular layer is the outermost layer of the cerebellar cortex (Fig. 2). This layer contains glial cells, few neurons, those of stellate and few scattered nuclei of basket cells. The Purkinji cells have large flask-shaped appearance, and they are arranged typically in a single row at the junction of the molecular layer with the granular layer. Each of these cells has a conspicuous cell body and an

extensive fan-like dendrite tree. Also, they displayed characteristic centrally located vesicular nuclei with prominent nucleoli. The granular layer where it meets the molecular layer will reveal a group of nuclei that are large than the nuclei of granule cells (Fig. 3).

Light microscopic examination of the cerebellar cortex of 1.5 mg/kg Xanax treated mice revealed wide spread neuronal affection specifically of the Purkinji cell layer which was reflected on the other two layers. It was of an interesting to observe blood capillaries lying in the vicinity of the Purkinje and the granular cell layers were dilated and congested (Fig. 4). More obvious histopathological changes were observed in the Purkinje cells, where they revealed disturbed normal linear organization with marked disarrangement. Further, there was a departure between the Purkinje cell layer and the underlying granular one (Fig. 5). Most of the Purkinje cells appeared deformed and shrunken, losing their characteristic pyriform shape, and they were rounded in shape and surrounded by a halo of empty spaces. Moreover, they showed variable degrees of cell loss together with characteristics of the cell distortion (Fig. 6).

Ultrastructural findings of the cerebellar tissues:

Electron microscopic examination of the cerebellar cortex of the cerebellum of control mice showed, that the molecular layer is mostly formed of extensions of the second layer, axons and dendrites of cells of the same molecular layer including basket and stellate cells 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-sized mitochondria and short cisternae of rough endoplasmic reticulum (Fig. 7).The stroma of the granular layer contains the so called cerebellar islands, which were characterized by nerve fiber network, where both myelinated and non-myelinated nerve fibers are discerned. The non-myelinated nerve axons have more electron density than the myelinated ones, where the later revealed only electron density through their myelin. Myelinated fibers could be detected between these cells, containing mitochondria and synaptic vesicles. The mitochondria of these cells are mostly rounded in shape and having light appearance (Figs. 8 and 9).

The electron microscopic explanations of cerebellar cortex of cerebellum of 1.5 mg/kg b. w. Xanax treated mice showed, the cytoplasm of

most of these cells showed dilatation of the endoplasmic reticulum cisternae (Fig. 10). An area of neuropile contains many unmyelinated nerve axons containing many mitochondria (Fig. 11). Further, the area of myelinated nerve axons showed irregular outlines and contained loss of mitochondria (Fig. 12).

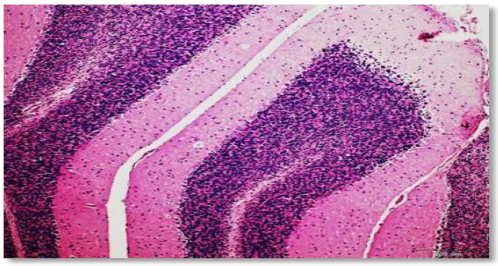

Fig. 1. Photomicrograph of a section of cerebellar cortex of a control mice showing, pattern of folia with an external (gray matter) and internal white matter; [H & E; X100].

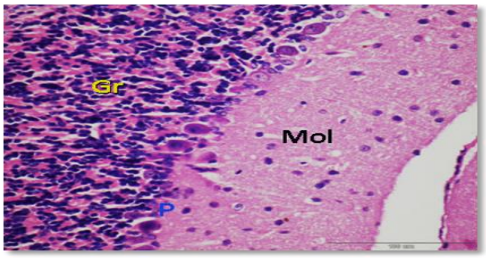

Fig. 2. Photomicrograph of a section of cerebellar cortex of a control mice showing, the three different layers of cerebellar cortex; the outer lightly stained the outer molecular layer (Mol); the inner granular layer (Gr) and the central Purkinje cell layer (P); [H & E; X 200].

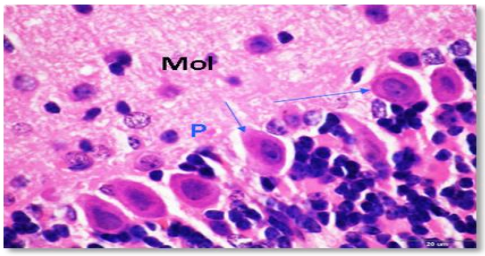

Fig. 3. Photomicrograph of a section of cerebellar cortex of a control mice showing, molecular layer (Mol), Purkinje cells (P) are large flask-shaped arranged in a single row, having and centrally located vesicular nuclei with prominent nucleoli; Purkinji cell (arrows); [H & E; X 400].

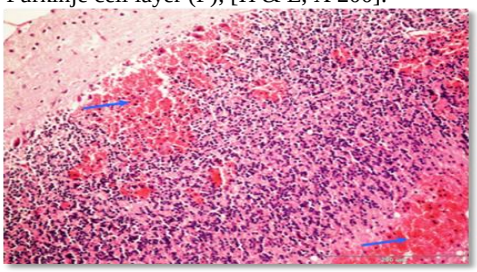

Fig. 4. Photomicrograph of a section of cerebellar cortex of mice treated with 1.5 mg/kg b. w. Xanax showing, dilates and congestion in the Blood (arrows); [H & E; X 100].

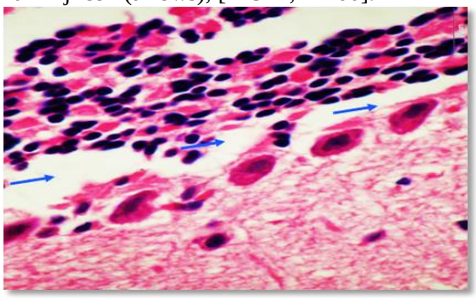

Fig. 5. Photomicrograph of a section of cerebellar cortex of mice treated with 1.5 mg/kg b. w. Xanax showing, marked disruption in one arrangement of Purkinji cell (p) layer; Notice :the separating between the Purkinje cell layer and the underlying granular one (arrows); [H & E; X 400].

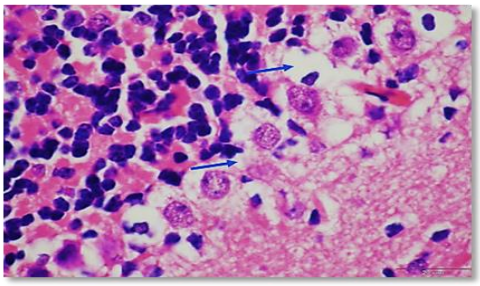

Fig. 6. Photomicrograph of a section of cerebellar cortex of mice treated with 1.5 mg/kg b. w. Xanax showing,, appeared deformed Purkinje cells (P) and shrunken Purkinje cells (P), and rounded in shape and surrounded by a halo of empty spaces (arrows); [H & E; X 400].

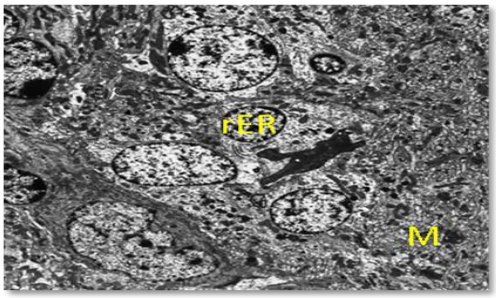

Fig. 7. Transmission electron micrograph of the cerebellar cortex of control mice showing, Basked cells in the molecular layer have pale euchromatic nuclei; The cytoplasm contains dense small sized mitochondria (M) ;short cisternae of (rER); [X 5000].

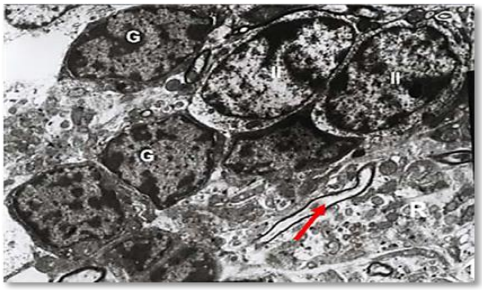

Fig. 8. Transmission electron micrograph of the cerebellar cortex of control mice showing, many granular cells Golgi type II cells (II) appear with larger diameter and lighter nuclei. Mossy rosettes (R) are containing many mitochondria and synaptic vesicles; Notice: the myelinated nerve axon (arrow); [X 5000].

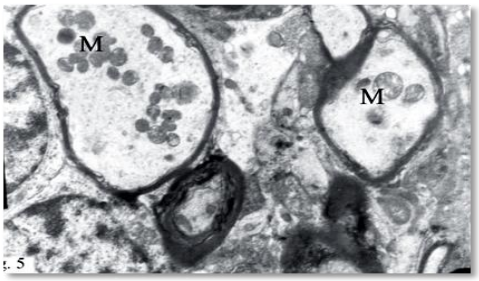

Fig. 9. Transmission electron micrograph of the cerebellar cortex of control mice showing, many myelinated nerve fibers stroma of the granular layer contain gmyelinted nerve axons contains many mitochondria (M); [X 5000].

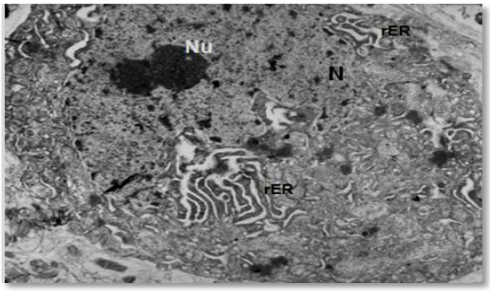

Fig. 10. Transmission electron micrograph of the cerebellar cortex of cerebellum of mice treated with 1.5 mg/kg b. w. Xanax showing, dilated rough endoplasmic reticulum (rRE) in the cytoplasm of Purkinje cell (P); [X 5000].

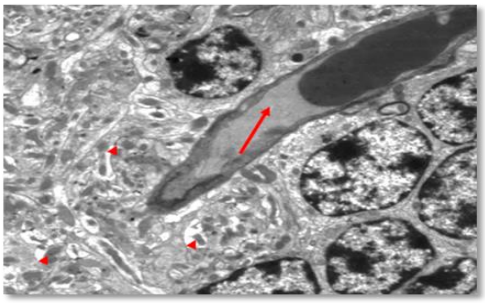

Fig. 11. Transmission electron micrograph of the cerebellar cortex of cerebellum of mice treated with 1.5 mg/kg b. w. Xanax showing, apart granular cells and dilated blood *capillary*(arrow); area of neuropile with many *unmyelinated* nerve axons containing mitochondria (M) (arrows); [X 5000].

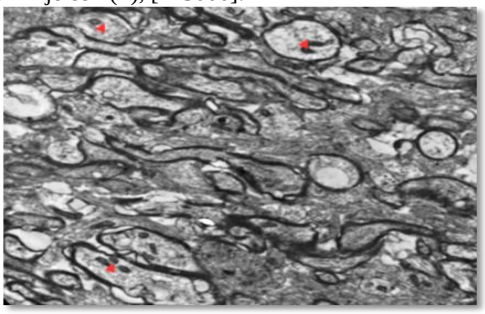

Fig. 12. Transmission electron micrograph of the cerebellar cortex of cerebellum of mice treated with 1.5 mg/kg b. w. Xanax showing, an area of myelinated nerve axons having neruoglial outlines and containing loss of mitochondria (M) (arrows); [X 5000].

Discussion:

The present study found that the effects of a single oral dose of the Xanax on the cerebellar cortex of cerebellum of mice. Our results were disrupted and disorganized and the most remarkable ultrastructural changes are observed in the Purkinje neurons. These cells had lost their specific appearance, reduced in their size, and had lost their cell boundaries. Additionally, 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 [27,24], who studied the effect of sodium fluoride on then euro degenerative changes of mice and rats. Further, many unstained haloes were seen around most of the destructed Purkinje cells in Xanax-treated mice. This was in accordance with the suggestion of [28], who attributed that due to shrinkage of Purkinje cells and withdrawal of their protoplasmic processes, secondary to disintegration of the cytoskeletal elements of these cells. The electron microscopic explanations of cerebellar cortex of cerebellum of 1.5 mg/kg b. w. Xanax treated mice showed, 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 Xanax 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 structure of Purkinje cells, high doses of Xanax-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 endoplasmic reticulum and Golgi apparatus.

Several studies have reported 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 [29]. Gold *et al.*[30] 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.

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 [31]. Also, the brain exhibits distinct variations in cellular as well as regional distribution of antioxidant biochemical defenses [32]. Thus, neural cells and/or brain regions are likely to differentially respond to changes in metabolic rates associated with the generation of ROS [33]. Trivedi *et al.*[34] and Zhang *et al.*[35], 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 [36], it can be said that increase in ROS observed in the present experiment might have inhibited the acetylcholine esterase activity in the cerebellum of Xanax 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.*[37] 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.

In conclusion, this study demonstrates that, mice administered of 1.5 ml of Xanax revealed different symptoms of toxicity in cerebellar tissues. Because this effect remains after adjustment for dose, co-ingested medication and age, this greater toxicity appears due to intrinsic toxicity of Xanax. Xanax overdose should be regarded as more significant than other benzodiazepines.

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