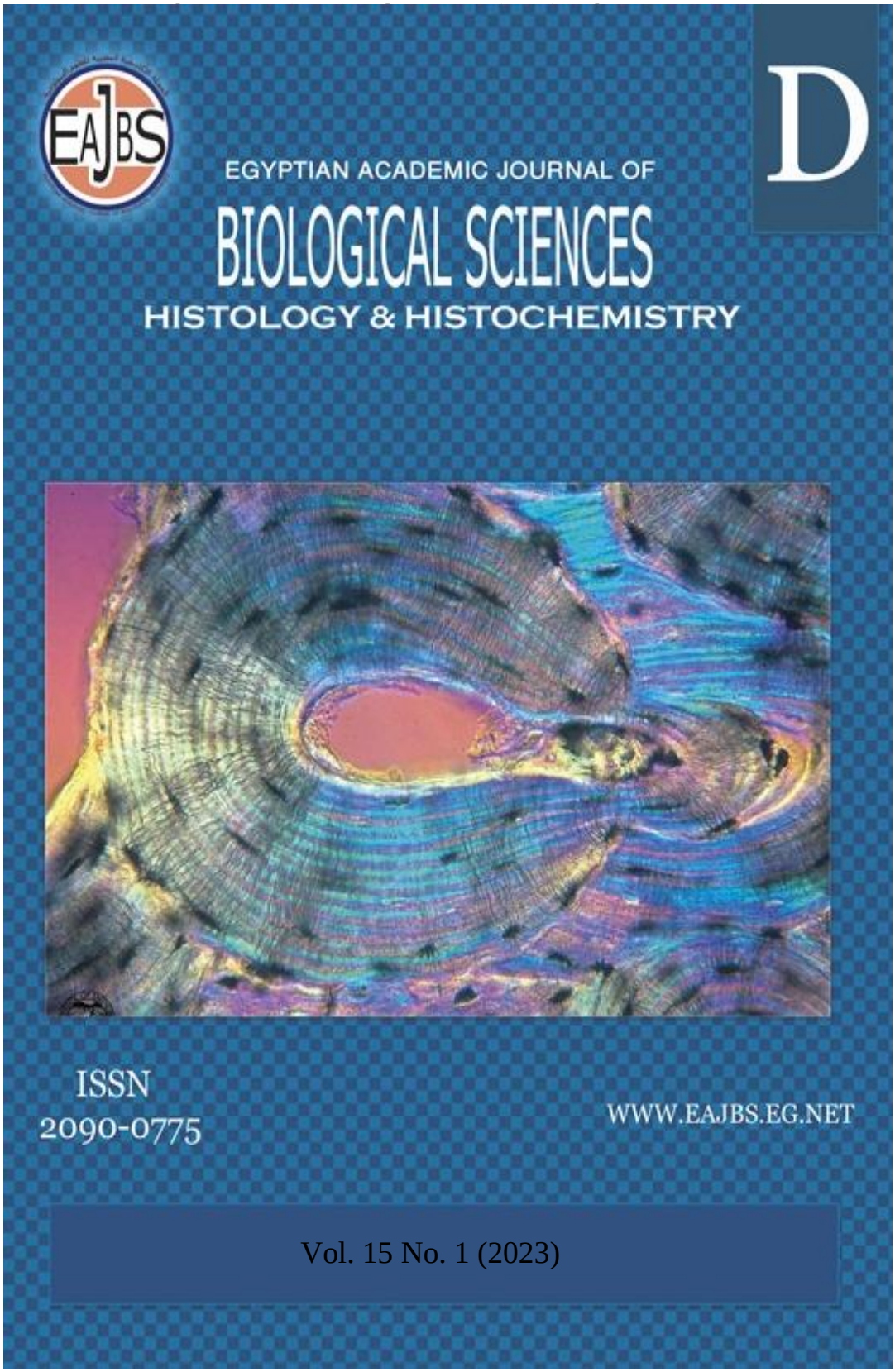

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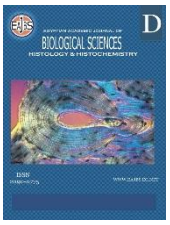

Tramadol Induces Histological Aberrations, Downregulation of Bcl-2 and Upregulations of GFAP, P53 Immunoexpression in the Cerebellum of the Male Albino Rat

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ABSTRACT

Tramadol is a synthetic opioid that exerts its analgesic influence by acting centrally via inhibiting norepinephrine and serotonin reuptake. Also, it blocks the propagation of nociceptive signals by activating the μ-opioid receptors. It is a drug of choice in the treatment and prevention of moderate to severe pains. However, various investigations reported that its chronic use is linked to physical and psychological addiction. Tramadol addiction is a serious global health problem. Tramadol has been extensively prescribed, but its negative consequences are still a cause for concern. Therefore, the main aim of this work was to investigate the probable effects of tramadol administration on the cerebellum of male albino rats histologically and morphometrically. Thirty adult male albino rats were allocated randomly into two groups each having 15 rats. The control group (I), was injected intraperitoneally with 1 ml normal saline 0.9%/day for 4 weeks. The second group (II), was injected intraperitoneally with 50 mg/kg/day of tramadol for 4 weeks. At the end of the experiment, the cerebellum was excised and fixed. Five *μm* serial sections were cut and prepared for the histological (H&E, silver nitrate) and immunohistochemical staining (GFAP, P53 and Bcl-2). In sections stained with Hx&E ; the following histomorphometric measurements were investigated: (1) The cerebellar cortical layers thickness (2) Purkinje cells' number. Additionally, quantitative immunohistochemistry was performed. Obtained data were subjected to statistical analysis. In Hx&E-stained sections, group (II) displayed thinning of Purkinje cells and granular layers of the cerebellar cortex. The cerebellar folia were separated by wide and deep fissures containing markedly congested blood vessels. The molecular layer exhibited numerous areas of marked degeneration. In the Purkinje cell layer, multiple shrunken and degenerated Purkinje cells with pyknotic nuclei were displayed also associated with numerous areas devoid of Purkinje cells. The granular layer showed less densely packed and some pale granule cells. Silver-stained sections of group (II) displayed marked degeneration of Purkinje cells, multiple vacuolations and sparsely arranged, pale-stained, degenerated granule cells. Group (II) displayed marked GFAP and moderate P53 immune-stained cells in both the cerebellar cortex and white matter. On the other hand, group (II) demonstrated a marked decrease in the immunostaining for Bcl-2 in all layers of the cerebellar cortex and its white matter. In group (II), we detected a statistically non-significant increase in molecular layer thickness and a statistically significant decrease in Purkinje cell and granular layer thickness as compared to group (I). Moreover, the number of Purkinje cells showed a statistically significant decrease in group (II). The surface area percentages of GFAP and P53 immuno-expression in group (II) exhibited an extremely statistically significant rise. However, an extremely statistically significant decrease in the surface area percentage of Bcl-2 immuno-expression was demonstrated in group (II) in comparison with group (I). Tramadol can induce deleterious effects on the cerebellar neurons through upregulation of the GFAP, P53, and, downregulation of Bcl-2. These proteins exert their impacts by inducing inflammation, oxidative stress, and release of several mediators, ER stress and apoptosis.

INTRODUCTION

Tramadol is a synthetic opioid that exerts its analgesic influence by acting centrally via inhibiting norepinephrine and serotonin reuptake (Adekomi *et al.*, 2019). Also, at the level of the axon terminal, it blocks the propagation of nociceptive signals by activating the μ-opioid receptors (Awadalla and Salah-Eldin, 2016). This dual action fosters augmented analgesia with fewer side effects (Barbosa *et al.*, 2016). Currently, tramadol has become one of the most commonly prescribed drugs in the world (Zhuo *et al.*, 2012). It is a drug of choice in the treatment and prevention of moderate to severe pains particularly during and after operative procedures. Its dose is modified according to the pain severity and sensitivity of the patient (Elkhateeb *et al.*, 2015). Additionally, tramadol alleviates the symptoms of phobias, depression and anxiety by modulating noradrenergic and serotonergic systems (McKeon *et al.*, 2011) and is used as an opiate alternative in treating opioid withdrawal (Threlkeld *et al.,* 2006). It is also prescribed to manage premature ejaculation (Babalonis *et al.*, 2013).

Though tramadol is structurally and functionally like morphine and codeine, it is believed to be safer and devoid of several severe side effects induced by other conventional opioids. However, various investigations reported that its chronic use is linked to physical and psychological addiction (Ghoneim *et al.*, 2014). Withdrawal symptoms are analogous to opioids such as anxiety, agitation, restlessness, insomnia, tremor, hyperkinesia, paresthesias, sweating and gastrointestinal (Duke *et al.*, 2011).

Tramadol intoxication results in nausea, vomiting, tachycardia, shock, seizures, central nervous system depression, coma and respiratory depression up to respiratory failure and death. High doses are related to the aggravation of symptoms of depression, anxiety and obsessive-compulsive disorders (El-Hadidy and Helaly, 2015).

Tramadol addiction/poisoning is increasing in a trendy way and becoming one of the commonest serious global health problems (El-Hadidy and Helaly, 2015). Moreover, tramadol administration with other drugs such as serotonergic agents, propranolol, ethanol, benzodiazepines and barbiturates may induce serotonin syndrome and fatal toxicity (Sanaei-Zadeh, 2012; Barbera *et al.*, 2013).

Increasing the incidence of tramadol overdose-induced death occurs mostly due to cardiorespiratory depression, resistant shock, (Randall and Crane, 2014), nephrotoxicity and liver failure (Elkhateeb *et al.*, 2015).

Experimentally, chronic tramadol administration resulted in rats' brain weight loss (Zhuo *et al.*, 2012), inhibiting neurogenesis (Cunha-Oliveira *et al.*, 2010), increasing apoptosis, and impairing memory functions by activation of μ-opioid receptors (Hosseini-Sharifabad *et al.*, 2016). These structural and functional alterations could be attributed to oxidative stress, and mitochondrial and endoplasmic reticulum dysfunction (Awadalla and Salah-Eldin, 2016).

Bcl-2 genes act as a stress marker, as it plays a fundamental role in the cell cycle and apoptosis pathways (Ghatei *et al.*, 2017). Three pathways are involved in the process of apoptosis: extrinsic, intrinsic, and perforin-granzyme pathways (Elmore, 2007). Any disturbance in these pathways may lead to variations in the cell number homeostasis and consequently cancer development (MacFarlane and Williams, 2004). Typically, P53 and Bcl-2 genes are involved in modifications of the previous pathways (Elmore, 2007). Bcl-2 regulates apoptosis by preserving the outer membrane of the mitochondria and hence preventing the release of cytochrome-c and initiation of the intrinsic pathway of apoptosis (Nagata and Tanaka, 2017). The anti-apoptotic effect of Bcl-2 promotes cell survival and

subsequently increases the probability of malignancy (Gross, 2016).

Astrocytes are the most prevalent glial cells in the central nervous system (CNS), particularly the cerebellum. They are involved in various functions in the CNS including cellular architectures, movements, ions exchange and maintenance of blood-brain barrier integrity (Suzuki *et al.,* 2003). Throughout brain injury, they play a crucial role in neuronal retrieval via the expression and release of trophic factors and surface molecules including GFAP (Blackburn *et al.,* 2009).

GFAP is a cytoskeletal protein released by astrocytes and is considered a specific marker for its maturation. It assists in altering the shape and mobility of astrocytes by boosting the structural stability of its processes (Lumpkins *et al.,* 2008). Therefore, GFAP is the marker of choice in reflecting the immunohistochemical activities of astrocytes post-tramadol exposure (Nassar *et al.,* 2021).

The P53 protein is a dynamic transcription factor working as a signaling hub (Kamada *et al.,* 2016) and plays a crucial role in regulating cell growth, differentiation, deoxyribonucleic acid (DNA) repair, and apoptosis in several stressful situations. Therefore, P53 exerts a tumor suppressor impact (Brož and Attardi, 2010). When DNA damage is detected, the P53 gene arrests the cell cycle at the end of the G1 phase and delays cell division to allow for DNA repair (Siganaki *et al.,* 2010). In the meantime, P53 declines if DNA is repaired, and the cycle is then completed. If the attempt at repair fails, P53 triggers the cell to undergo apoptosis. As a result, if the P53 gene is disrupted, there will be more DNA damage overall, which will raise the likelihood of tumor genesis (Hoda and Hoda, 2020).

Tramadol has been extensively prescribed for its improved tolerability and better compliance, but its negative consequences are still a cause for concern and are not fully understood.

Therefore, the main aim of this work was to investigate the probable effects of tramadol administration on the cerebellum of male albino rats histologically and morphometrically.

MATERIALS AND METHODS

Animals:

The present work was performed on thirty adults male Sprague Dawley rats weighing 170-210 gm. Animals were obtained from Helwan Farm, Cairo, Egypt. All experimental procedures were approved by FMASU, REC (Faculty of Medicine, Ain Shams University, Research Ethics Committee, Cairo, Egypt) that comply with the Guide for the Care and Use of Laboratory Animals released by the United States National Institute of Health (Code FMASU R 205/2022). For acclimatization, rats were prepared and kept in the Medical Ain Shams Research Institute (MASRI), Faculty of Medicine, Ain Shams University. All animals were housed separately in pathogen-free cages under well-controlled conditions of temperature, humidity, and illumination (12 hours day/ night), with free access to food and water *ad libitum.*

Experimental Design:

Rats were allocated randomly into two groups each having 15 rats. The first group served as the control group (I) and was injected intraperitoneally with 1 ml of normal saline 0.9 %/day for 4 weeks. The second group (II) was injected intraperitoneally with 50 mg/kg/day of tramadol for 4 weeks (Ghoneim *et al.,* 2014).

Drugs and Preparation:

Commercially available Tramadol HCl, 50 mg capsules (Tramal) were purchased from Mina-Pharm, Egypt. The Drug was dissolved in a normal saline solution and administered to the rats (Ragab and Mohamed, 2017; Baghishani *et al.,* 2018).

Specimens' Acquisition and Processing:

At the end of 4 weeks-experiment and 24-h after the last dose of tramadol, the animals were euthanized using an overdose of sodium pentobarbital (100

mg/kg). After scarification, the skull vaults were opened using brain forceps then the whole brain was dissected. The cerebellum was excised and fixed then processed for sectioning and staining. Cerebellar pieces were fixed in 10% neutral buffered formalin for one day, dehydrated in ascending grades of alcohol, cleared in xylene and embedded in paraffin wax (Sarhan and Taalab, 2018). Five µm serial sections were cut and prepared for the histological (H&E, silver nitrate) and immunohistochemical staining (GFAP, P53 and Bcl-2).

Modified Bielschowsky's Silver Staining:

For silver staining, the cerebellar sections of the two groups were deparaffinized and hydrated with distilled water for three minutes. Then, the slides were placed for 15 minutes in a previously warmed silver nitrate (20%) at 40°C. Sections color should turn into light brown. The sections were then submerged for three minutes in distilled water. Until the precipitate that developed was clear, concentrated ammonium hydroxide was gradually added to the silver nitrate solution to create the ammonium silver solution. An excessive amount of ammonium hydroxide could result in a precipitate and poor impregnation of the fibers. Just prior to usage, the solution was made, and it was dumped afterwards. In a 40°C oven, the slides were incubated for 30 minutes in the ammonium silver solution until becoming dark brown. Citric acid 0.5 gm, formaldehyde 20 mL, 100 ml distilled water, and two drops of nitric acid were combined to prepare a developer stock solution. Eight drops of the ammonium hydroxide were added to 50 ml distilled water to create developer working solution. Then we mix the above developer working solution with the developer stock (8 drops) solution. The developer working solution was then applied to the slides for two to five minutes. The slides were examined microscopically to ensure that the staining reaction had occurred. If necessary, the silver reaction could be stopped by dipping the slides in a solution of 1% ammonium hydroxide. For washing, the slides were placed in distilled water (3 minutes) and in sodium thiosulfate 5% (5 minutes) then in distilled water again for 3 minutes. Finally, absolute alcohol, xylene and DPX were used for dehydrating, clearing, and mounting the slides respectively. The slides were examined under a light microscope to detect and analyze any histopathological and morphological changes in various magnification (Ghosh *et al.,* 2021).

Immunohistochemistry Staining Procedures:

***GFAP immunohistochemical staining*:**

Five µm serial cerebellar thickness were placed on positively charged glass slides then deparaffinized and hydrated. The endogenous peroxidase activities were blocked by hydrogen peroxide 10% (10 minutes). For unmasking of the antigenic locations, the slides were impregnated in 0.01 mol/l citrate buffer (pH=6) for 5 minutes in the microwave. To avoid non-specific background staining, the slides were first washed for five minutes in PBS with a pH of 7.4 before being incubated for thirty minutes at 37°C with 1% BSA dissolved in PBS. Two drops of the primary antibody were added to all slides except the negative control and then incubated together for an hour at room temperature. GFAP (Ab-1, clone GA-5 mouse monoclonal antibody, Thermo Scientific, Waltham, USA) was used to treat the slides, then rinsed for 10 minutes with PBS and biotinylated goat-polyvalent secondary antibody few drops. To notice the reaction, the slides were incubated for 15 mins in 3, 3-diaminsobenzidene (DAB) solution then counterstained by Mayer's hematoxylin. Finally dehydration, clearance and DPX mounting of the slides were done to examine it microscopically. (Ali and Sonpol, 2017).

P53 and Bcl-2 immunohistochemical staining:

Xylene and citrate buffer (pH 6) were used for deparaffinization and

retrieval of the antigen at 60 °C respectively. Hydrogen peroxide 3% was used to block the endogenous peroxidase activity. P53 and Bcl-2 primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) then diluted in PBS in dilution 1:1000 for P53 and 1:1000 for Bcl-2. The primary antibodies were incubated overnight with tissue sections at 4°C, then washed. Sections were incubated again for an hour with biotinylated secondary antibodies at room temperature. The immunostaining was identified by using the avidin-biotin complex (ABC) method (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA). Diaminobenzidine (DAB) worked as chromogen. Counterstaining of the slides with hematoxylin, dehydration in graded alcohol, clearance with xylene, and DPX mounting was done (Mousa *et al.,* 2015).

Morphometric Study and Quantitative Immunohistochemistry:

Quantitative immunohistochemistry for GFAP, P53, and Bcl-2 in cerebellar cortex at a magnification of X200 was included in the analysis. The average percentage (%) of the immunoreactivity surface area for each cerebellar section was quantitatively estimated by means of Image J software version 1.50i. In Hx&E-stained sections; two histomorphometric measurements were assessed: (1) Cerebellar cortex layers' thickness was measured using photomicrographs with magnification of X100. (2) Clearly normal Purkinje cells' number was counted in photomicrographs with magnification of X400. The examined cerebellar tissues were taken randomly from 10 fields for each five various sections taken from five various rats of studied groups (El-Bermawy and Salem, 2015).

Statistical Analysis:

Statistical investigations were executed using Graph pad prism version 6.03 (San Diego, CA, U.S.A). Unpaired t-test was chosen for comparison of the spotted histomorphometric and quantitative immunohistochemistry data in both studied groups.

RESULTS

A) Histological Results

1) Hematoxylin & Eosin-Stained Sections:

The control group (I) showed a well-organized cerebellar cortex displaying large folia separated by deep gaps. Three layers of the cerebellar cortex were observed; the outer molecular, the middle Purkinje cell and the inner granular layer (Fig. 1A). The pale outer molecular layer formed mainly of nerve fibers with few basket and stellate cells. The Purkinje cell layer displayed a single row of Purkinje cells. These cells appeared oval or flask-shaped having apical cytoplasmic cones with pale central nuclei. Also, Bergmann cells, displaying pale nuclei, were seen dispersed in between the Purkinje cells. The granular layer displayed multiple tiny rounded densely packed deeply stained granule cells with cerebellar islands consisting of numerous nerve synapses in-between (Fig. 1B).

The experimental group (II) displayed thinning of Purkinje cells and granular cerebellar cortical layers. The cerebellar folia were separated by wide and deep fissures containing markedly congested blood vessels (Fig. 2A). The molecular layer exhibited numerous markedly degenerated areas. In the Purkinje cell layer, multiple degraded and shrunken Purkinje cells showing pyknotic nuclei were displayed also associated with numerous areas devoid of Purkinje cells. The granular layer showed less densely packed and some pale granule cells (Figs. 2B&C).

2) Silver-Stained Sections:

Silver-stained cerebellar sections of group (I) displayed intact Purkinje cells with prominent axons in addition to multiple closely packed rounded deeply stained granule cells (Fig. 3A). However, sections of group (II) displayed marked degeneration of Purkinje cells, multiple vacuolations and sparsely arranged, pale-stained, degenerated granule cells (Fig. 3B).

3) GFAP Immunohistochemistry-Stained Sections:

The control group showed a mild positive response of GFAP immunohistochemistry staining for astrocytes in all cerebellar cortical layers and white matter (Fig. 4A). On the reverse, group (II) displayed evident abundant markedly GFAP immune-stained cells in both the cerebellar cortex and white matter (Fig. 4B).

4) Bcl-2 Immunohistochemistry-Stained Sections:

Group (I) displayed a marked reaction for Bcl-2 immunostaining in all layers of the cerebellar cortex and white matter (Fig. 5A). On the other hand, group (II) displayed a marked decrease in the immunostaining for Bcl-2 in all layers of the cerebellar cortex and its white matter (Fig. 5B).

5) P53 Immunohistochemistry-Stained Sections:

P53 immuno-stained sections of the control group showed a negative reaction (Fig. 6A), while those of group (II) displayed moderate positive reactions in all layers of the cerebellar cortex and white matter (Fig. 6B).

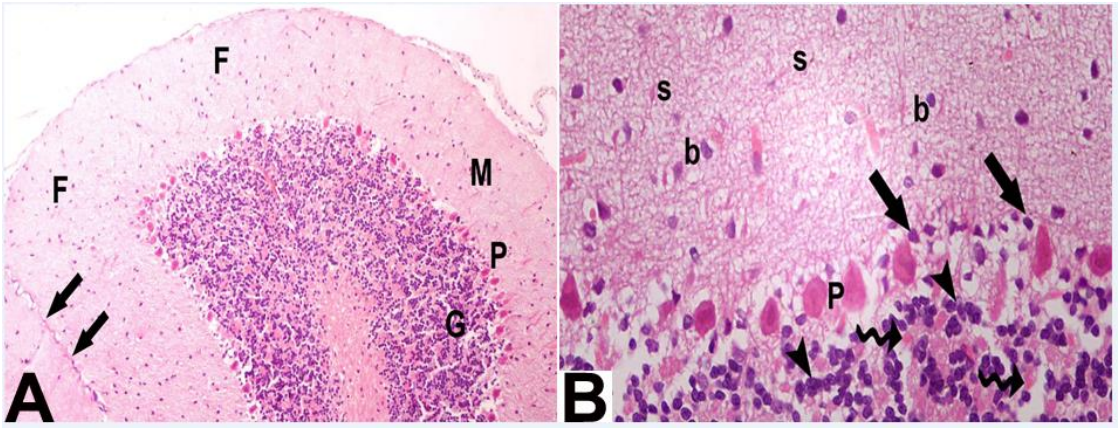

Fig. 1: A photomicrograph of a cerebellar section of group (I) rats; displaying **(A):** a cerebellar folium (F) has a deep fissure (arrow). It also shows well organized cerebellar cortical layers; Molecular layer (M), Purkinje cell layer (P) and granular layer (G), **(B):** apparent faint molecular layer with few stellate (s) and basket cells (b). Purkinje cell layer displayed Purkinje cells (P); flask-shaped, containing central nuclei, disposed in a single row, surrounded by Bergmann cells (arrow). Granular layer comprising multiple closely packed, tiny rounded deeply stained granule cells (arrowhead), have cerebellar islands (wavy arrow) in-between.

(H&E; A X100-B X400)

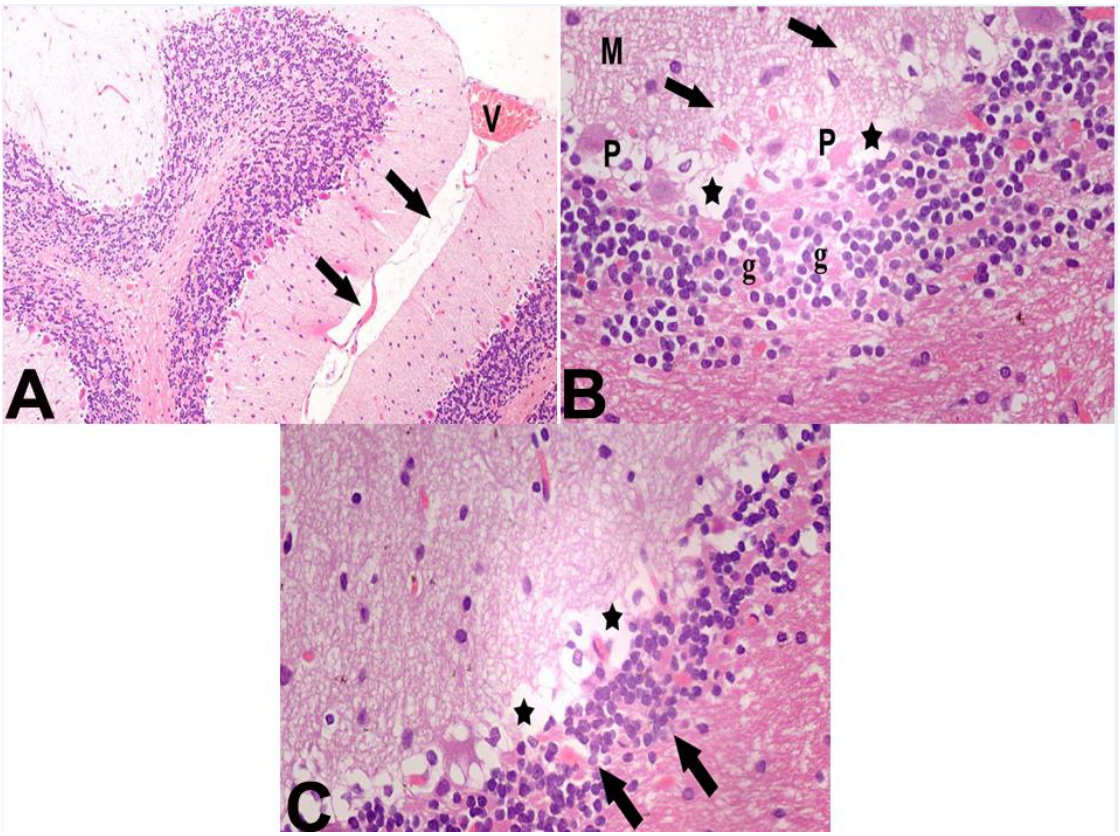

Fig. (2): A photomicrograph of a cerebellar section of group (II) rats; displaying **(A):** thinning of cerebellar cortex with wide deep fissure (arrow) between the cerebellar folia and markedly congested blood vessel (V), **(B)**: noticeable degeneration (arrow) of the molecular layer (M), the Purkinje cell layer exhibited degraded and shrunken Purkinje cells (P) with pyknotic nuclei adjacent to empty areas of Purkinje cells (*) and Granule cells (g) appeared pale and less dense, **(C):** marked areas of Purkinje cell loss (*) and the granular layer showed less density and pale granule cells (arrow).

(H&E; A X100-B&C X400)

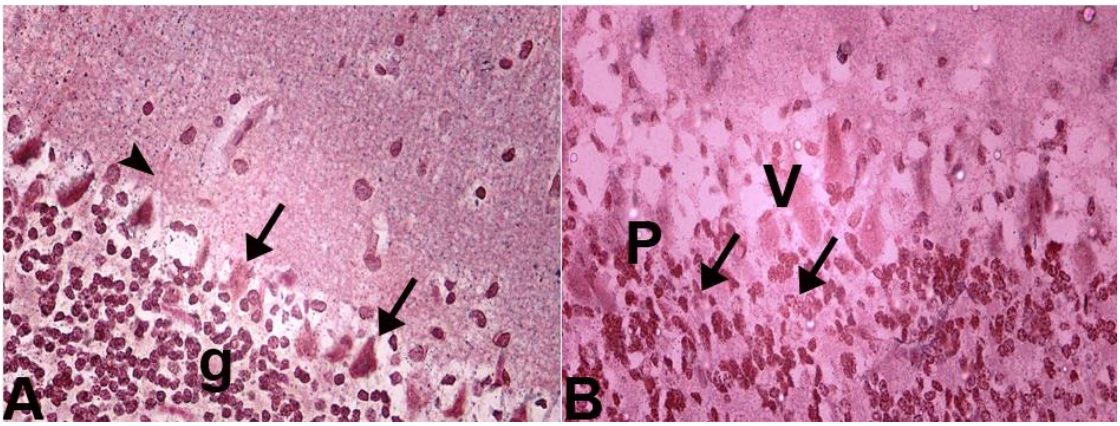

Fig. 3: A photomicrograph of a cerebellar section of rats of **(A):** group (I); displaying silver-stained Purkinje cells (arrow) with prominent axons (arrowhead) and granular layer having multiple rounded intensely stained granule cells (g); **(B):** group (II) displaying degenerated pale Purkinje cells (P), multiple vacuolation (V) and sparsely arranged, pale-stained and degenerated granule cells (arrow). (Silver-staining X400)

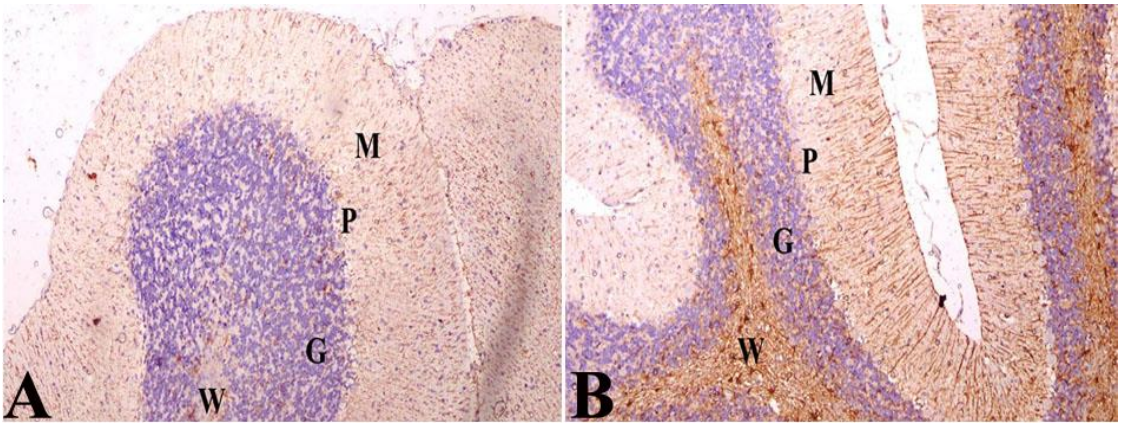

Fig. 4: A photomicrograph of a cerebellar section of rats of **(A)**: group (I); displaying faint positive reaction of GFAP immunohistochemistry staining for astrocytes in cerebellar layers, **(B):** group (II); displaying marked GFAP immunoexpression in all cerebellar layers; molecular (M), Purkinje cell (P), granular (g) and white matter (W). (GFAP-immunostaining X100).

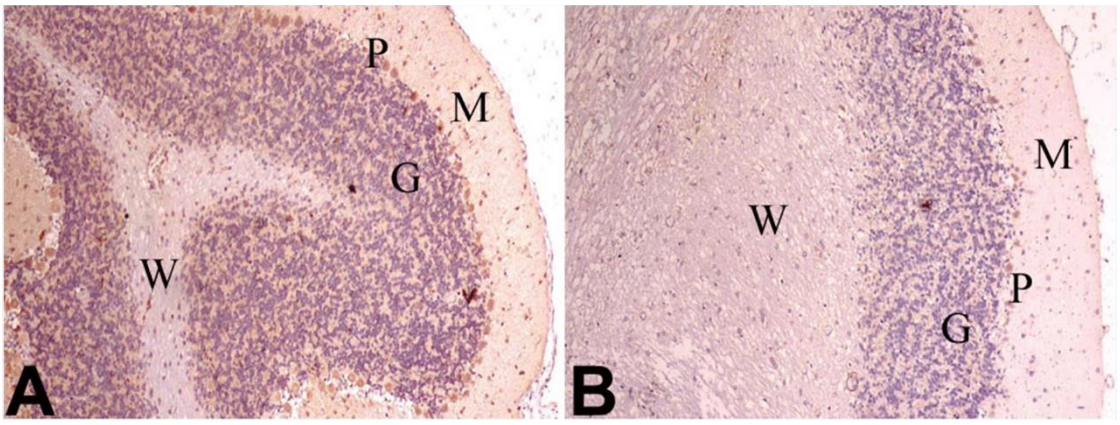

Fig. 5: A photomicrograph of a cerebellar section of rats of **(A):** group (I); displaying marked positive reaction of Bcl-2 immuno-staining in all cerebellar layers; molecular layer (M), Purkinje cell layer (P), granular layer (g) and white matter (W) and **(B):** group (II); showing minimal positive reaction of Bcl-2 immuno-staining in all cerebellar layers; molecular layer (M), Purkinje cell layer (P), granular layer (g) and white matter (W).

(Bcl-2-immunostaining X100)

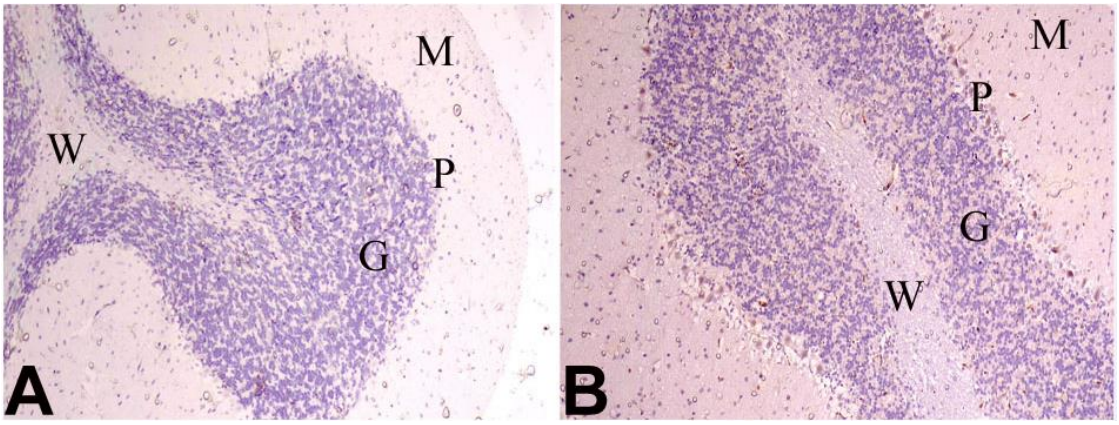

Fig. 6: A photomicrograph of a cerebellar section of rats of **(A):** group (I); displaying negative reaction of P53 immuno-staining in all cerebellar layers, and **(B):** group (II); showing moderate positive reaction of P53 immuno-staining in molecular layer (M), Purkinje cell layer (P), granular layer (g) and white matter (W).
(P53-immunostaining X100)

B) Histomorphometry:

1) Cerebellar Cortex Layers' Thickness:

In group (II), we detected a statistically non-significant increase in molecular layer thickness as compared to group (I). Meanwhile, group (II) displayed a statistically significant decrease in Purkinje cell and granular layers thickness as compared to group (I) (Table 1 & Fig. 7).

2) Purkinje cells number:

Purkinje cells number exhibited a statistically significant decrease in group (II) in comparison to group (I) (Table 1& Fig. 7).

Table 1. Morphometric measurements of the different studied groups.

Parameter	Control	Tramadol-treated	*P*-value
Molecular Layer Thickness (um)	14.99±2.35	17.83±5.71	0.1621
Purkinje cell Layer Thickness (um)	2.94±0.63	1.49±0.34*	<0.0001
Granular Layer Thickness (um)	20.26±4.28	8.23±1.89*	<0.0001
Number of Purkinje cells	8.2+1.62	3.1±1.197*	<0.0001

All results were expressed as mean ± SD. P<0.05 is significant. (*P value is < 0.0001 "extremely statistically significant").

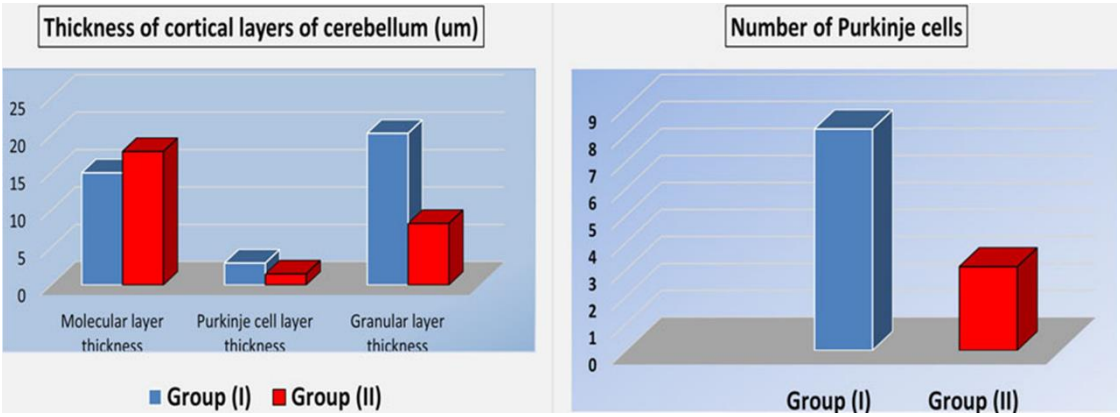

Fig. 7: A histogram showing the thickness of the cerebellar cortical layers and the number of Purkinje cells in the two studied groups.

3.Quantitative immunohistochemistry:

GFAP Immuno-Expression:

GFAP immuno-reactivity surface area percentage in group (II) in comparison to group (I) exhibited an extremely statistically significant increase (Table 2 & Fig. 8).

Bcl-2 Immuno-Expression:

Bcl-2 immuno-reactivity surface area percentage displayed an extremely statistically significant decrease in group (II) in comparison to group (I) (Table 2 & Fig. 8).

P53 Immuno-Expression:

Group (II) displayed an extremely statistically significant increase in surface area percentage of P53 immuno-reactivity in comparison to group (I) (Table 2 & Fig. 8).

Table 2. Quantitative immunohistochemistry for the surface area percentages of GFAP, P53, and Bcl-2 immuno-expression in different groups**.**

	Control	Tramadol-treated	P-value
Surface area % of GFAP	5.28±2.08	23.897±9.74*	<0.0001
Surface area % of Bcl-2	27.14±7.09	6.702±5.95*	<0.0001
Surface area % of P53	3.05±2.11	14.46±8.55*	<0.0001

All results were expressed as mean ± SD. P< 0.05 is significant. (*P value is < 0.0001 "extremely statistically significant").

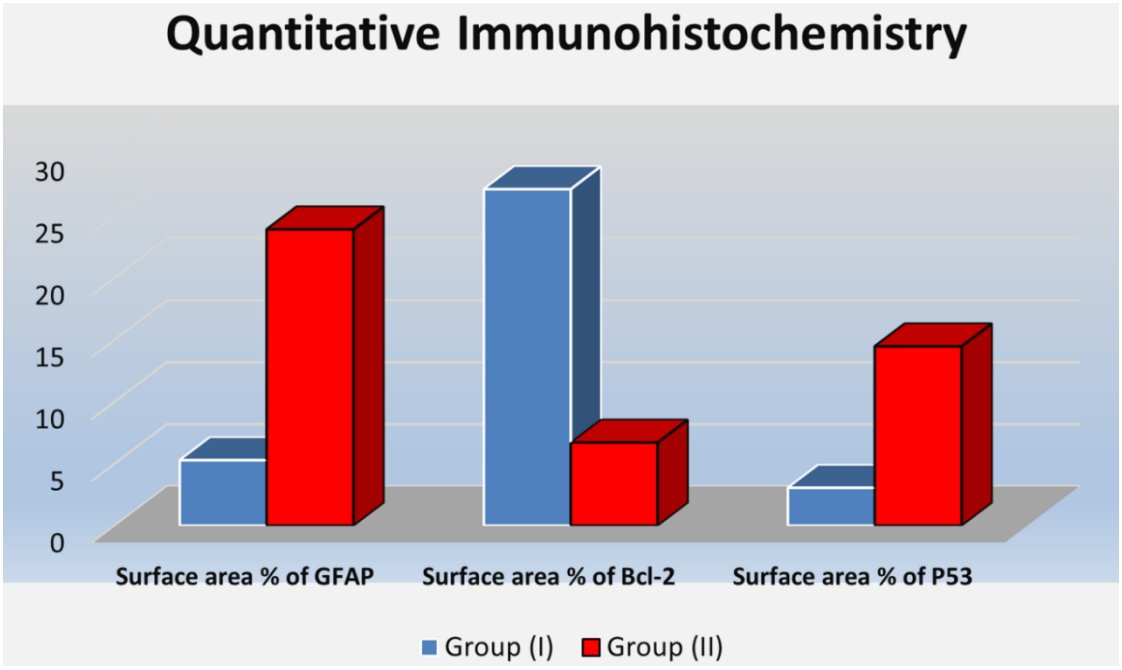

Fig. 8: A histogram showing the quantitative immunohistochemistry of the different immunostains used in the two studied groups.

DISCUSSION

Despite all efforts to avoid and manage it, addiction is a growing social and health issue on a global scale. One of the most widely used pharmaceuticals that are abused is analgesics (Pantelias and Grapsa, 2011). It is commonly known that both naturally occurring opiates (such as morphine and codeine) and synthetic opioids (such as Tramadol, heroin, oxycodone, and buprenorphine) have a high potential for misuse (Moratti *et al.*, 2010; Meyer *et al.*, 2014). Moreover, similar to other opiates, tramadol has been demonstrated to cause physical and psychological addiction (Lanier *et al.*, 2010). Recently, tramadol abuse has greatly increased in Middle Eastern nations and has spread to developed nations, possibly as a result of its illegal widespread availability and affordable cost (Fawzi, 2011). Moreover, its rapid absorption and easy penetration into the blood-brain barrier (Grond and

Sablotzki, 2004) allow it to have a variety of effects on the central nervous system (Hosseini-Sharifabad *et al.*, 2016). Due to this, it has been linked to violent acts, conflicts, vehicle collisions and several side effects, including confusion, seizures, and respiratory distress (Clarot *et al.*, 2003).

In the present study, the experimental group (II) displayed thinning of the Purkinje cell and granular layers of the cerebellar cortex. The cerebellar cortical molecular layer exhibited numerous regions of marked degeneration. In the layer of Purkinje cells, multiple degraded and shrunken Purkinje cells having pyknotic nuclei were displayed also associated with numerous areas of loss. The granular layer showed less densely packed and some pale granule cells.

A histomorphological quantitative study demonstrated a statistically non-significant increase of the molecular layer thickness associated with a significant decrease in the thickness of Purkinje cell and granular layers as compared to the control group. Moreover, the number of Purkinje cells showed a statistically significant decrease in the experimental group.

These results go in agreement with (Liu *et al.*, 2013; Ezi *et al.*, 2021) who demonstrated that tramadol induces obvious inflammatory, apoptotic changes, cytoplasmic contraction, decreased chromatin density, diminished cell volume and neuronal degeneration via altering their cytoskeleton in the rat motor cerebellar cortex. Moreover, Omar and El-Hawwary, (2014); El-Bermawy and Salem, (2015) and Sarhan and Taalab, (2018) noticed marked damage in Purkinje cells associated with the vacuolation of the neuropil and cerebellar islets and ascribed this due to swelling in the neural processes and the presynaptic nerve endings.

The detrimental effects of tramadol on the cerebellum are expected given the wealth of information describing the function of opioid (μ) receptors in the cerebellum (Thomasy et al. 2007). Tramadol has been found to disrupt many crucial enzymes in the rat cerebellum (Ezzeldin *et al.*, 2014).

In the liver, tramadol is mainly metabolized by the cytochrome P450 isozyme into five distinct metabolites. One of these metabolites, O-desmethyl tramadol, has a longer half-life and a 200-fold higher μ-affinity than tramadol (Raffa, 2006). It has been proposed that tramadol consumption can have a possible dose-dependent neurotoxic impact on the cerebellum in an ascending manner (El-Bermawy and Salem, 2015).

Former experiments reported that tramadol induces neurotoxicity in the cerebral cortex (Nafea *et al.*, 2016; Baghishani *et al.*, 2018 and Aghajanpour *et al.*, 2020). Yet, it is unclear how it affects the cerebellar cortex.

The precise underlying neurotoxicity mechanism of tramadol is not well recognized; however, several mechanisms are postulated to explain tramadol-induced neurotoxicities such as oxidative stress, mitochondrial dysfunction, endoplasmic reticulum stress, apoptosis, and inhibition of neurogenesis (Awadalla and Salah-Eldin, 2016; Sarhan and Taalab, 2018).

Tramadol exposure causes signaling cascades to be dysregulated, which are mostly involved in neurodegenerative illnesses such as Huntington's disease, Parkinson's disease, Alzheimer's disease, prion disease and amyotrophic lateral sclerosis. It implies that tramadol toxicity and neurodegeneration share similar underlying mechanisms. One of these cascades is oxidative phosphorylation deregulation, and subsequent redox dyshomeostasis, which plays a crucial role in neurodegenerative proteinopathies. Correspondingly, RNA degradation disruption can negatively influence neuronal RNA homeostasis (Briston and Hicks, 2018).

Ezi *et al.*, (2021) proposed that necroptosis, an alternative process that mimics necrosis and apoptosis, causes neuronal loss in the Purkinje layer. In addition, necroptosis is regulated by

RIPK3 protein and mixed lineage domain-like pseudokinase and this was confirmed by increased immunohistochemical expression in the tramadol-treated group (Dhuriya and Sharma, 2018). Iba-1, the microglia marker, levels also increase dramatically in the cerebellum and serve as a signal of inflammation. The increased brain levels of pro-inflammatory mediators are related to microglial activation. Fully activated microglia can cause neurotoxic consequences (M1 phenotype) (Block *et al.,* 2007). However, others have suggested that activated microglia could play a role in the generation (M2 phenotype) (Simard and Rivest, 2007).

The M1 microglia immune response is prompted by the TLRs activation through pathogen-associated molecular patterns (PAMPs), intracellular proteins produced by damaged neurons., adenosine triphosphate and complement 1q produced by astrocytes in reaction to neuronal injuries (Tang and Le, 2016). The activated M1 microglia secrete pro-inflammatory mediators such as IL-1β, IL-6, and TNF-α (Moehle and West, 2015; Tang and Le, 2016). M2-activated microglia, induced by apoptotic cell signaling, are linked to the repair, regeneration, and releasing anti-inflammatory mediators such as IL-10 and TGF-β (Hoogland *et al.*, 2015; Moehle and West, 2015 and Tang and Le, 2016).

Inflammatory indicators including TNF- α and IL-1, apoptotic biomarker (BAX), and autophagy genes (MAP1LC3B and LAMP2) showed dysregulated gene expression, indicating the important roles that apoptosis, inflammation, and autophagy play a role in tramadol-induced cell death in the cerebellum.

Moreover, due to oxidative stress, chronic tramadol usage in rats causes an increase in apoptosis in the cerebral cortex (Ghoneim *et al.*, 2014). These neurotoxic impacts could be attributed to inhibiting the antioxidants as glutathione peroxidase and/or overproduction of reactive oxygen species (ROS) as nitric oxide (Atici *et al.*, 2005; Mohamed, Ghaffar and El Husseiny, 2015 and Bameri *et al.*, 2018).

ROS could activate several reactive mediators leading to the destruction of the cellular components and subsequent production of secondary toxic substances. One of these mediators is the nuclear factor-kappa B (NF-κB) which is an oxidative stress-sensitive transcription factor. It triggers the transcription of inflammatory genes and the subsequent creation of numerous inflammatory mediators (Bonizzi and Karin, 2004). This overproduction of inflammatory mediators may be linked to microglial growth in the cerebellar cortex (Liu *et al.*, 2013; Mohamed and Mahmoud, 2019).

In the experimental group, the cerebellar folia were separated by wide and deep fissures containing markedly congested blood vessels which could be explained by the overproduction of vasodilator nitric oxide induced by tramadol (Sarhan and Taalab, 2018). In rat cerebral and cerebellar cortex, similar results were formerly reported following chronic use of tramadol (Ahmed and Kurkar, 2014; Omar and El-Hawwary, 2014; El-Bermawy and Salem, 2015; Ragab and Mohamed, 2017).

In the present work, the experimental group (II) displayed evident abundant markedly GFAP immune-stained cells in both the cerebellar cortex and white matter. These results were confirmed by quantitative immunohistochemistry that displayed an extremely statistically significant increase in GFAP immuno-reactivity surface area percentage in group (II) in comparison to group (I).

These results go consistent with other previous experiments which identified a significant increase in the GFAP immunoexpression in all layers of the hippocampus (Hussein, Abdel Mola and Rateb, 2020) and striatum (Marie-Claire *et al.*, 2004). Additionally, marked swelling of the processes and body of the astrocytes was observed together with

rough endoplasmic reticulum (rER) strands swelling and mitochondrial damage.The astrocytic proliferation and swelling including its processes with a noticeable increase in GFAP content were defined as astrogliosis (Sofroniew and Vinters, 2010).

This astrogliosis plays a significant role in various processes in the normal brain, including the control of ion homeostasis, the production and release of trophic and inflammatory agents, cell and tissue repair and regeneration, and blood-brain barrier maintenance (Ojo *et al.*, 2015)

Nevertheless, prolonged drug abuse induces astrogliosis as an innate immune response to neurotoxicity and brain injury that may lead to alterations in synaptogenesis and neurogenesis (Hama *et al.*, 2004; Barker and Ullian, 2008). Furthermore, astrogliosis can stimulate apoptosis and/or necrosis via increasing Ca^{2+} and consequently carbonyl oxidation production (Hauser *et al.*, 1998). According to earlier research, the activation of glial cells, particularly astrocytes, was revealed to be caused by opioid signaling and information processing in the brain, namely by the direct stimulation of μ-receptors in astrocyte membranes (Beardsley and Hauser, 2014) and (Koyama, 2015). Moreover, opioids may exert their effects by activating aquaprotein-4. This protein, near the astrocytic end feet, wraps the blood vessels and plays as a water channel for adjusting water and amino acid in the brain (Fan *et al.*, 2005).

Group (I) displayed a marked reaction for Bcl-2 immunostaining in all layers of the cerebellar cortex and white matter. On the other hand, group (II) displayed a marked decrease in the immunostaining for Bcl-2 in all layers of the cerebellar cortex and its white matter. By quantitative immunohistochemical study, an extremely statistically significant decrease in Bcl-2 immuno-reactivity surface area percentage was demonstrated in group (II) in comparison to group (I). In experimental ischemic and traumatic rodent models, Bcl-2 modulates neuronal apoptosis. Bcl-2 mRNA expression and Bcl-2 protein are upregulated in survived neurons and deficient in apoptotic cells. Bcl-2 is a proto-oncogene first detected in follicular lymphoma and hence its name. The anti-apoptotic Bcl-2 gene and its 26 kDa protein play a key role in cellular protection against ischemic and traumatic injuries that induce apoptosis. Intracellularly, it is detected near the free radical-producing sites such as the endoplasmic reticulum, nuclear membranes and mitochondria to inhibit the peroxidation injury (Deng *et al.*, 2020). Moreover, it increases cellular longevity via maintaining calcium homeostasis, heterodimerization with Bax, and hindering pro-apoptotic proteins, endonuclease G and cytochrome c release from the mitochondria (Li *et al.*, 2001; Gross, 2016).

The anti-apoptotic Bcl-2 protein regulates membrane permeability by inhibiting the pore formation and release of apoptotic proteins from the mitochondria after traumatic brain injury in rats. In experimental models of nervous system injury, Bcl-2 mRNA expression and Bcl-2 protein are found to be increased in surviving neurons (Graham *et al.*, 2000).

P53 immuno-stained sections of group (II) displayed moderate positive reaction in all layers of the cerebellar cortex and white matter, and this was confirmed by quantitative studies that displayed a statistically significant increase in P53 immuno-reactivity surface area percentage in group (II) in comparison to group (I).

Normally in post-mitotic cells, the p53 protein concentration is below the required level needed to be detected by the immunohistochemical techniques. In these cells, the endoplasmic reticulum (ER) is responsible for the production and folding of variable proteins such as p53 that enter the secretory/tertiary pathway. In ER, various post-translational

modifications occur to ensure the protein functions (Luo *et al.*, 2006).

Several physiological alterations interfere with the processes of protein folding in the ER lumen, leading to the accumulation of unfolded or misfolded proteins (ER stress). This stress initiates and activates unfolded protein response (UPR) which is an adaptive reaction (UPR) (Winnay and Kahn, 2011).

In aging subjects, ER stress was reported to be the first step of P53 reaction in the cytoplasm of the cerebellar nerve cells. The second step was the P53 accumulation in the nuclei of neurons undergoing degeneration. Earlier studies have demonstrated that P53 is involved in the neurodegenerative activities caused by ischemia or other neuronal injuries (McGahan *et al.*, 1998; Cenini *et al.*, 2008). Post-transcriptional alteration can modify P53 tertiary structure preventing its binding to particular DNA structures (Uberti *et al.*, 2006). Increased P53 levels in cases of DNA damage, and cellular distress signals, may provoke cell cycle arrest and apoptosis via regulation of various genes transcription (Maślińska *et al.*, 2017).

In cases of DNA damage, the p53 gene causes cell cycle arrest in the late G1 phase giving time for DNA repair. In cases of successful DNA repair, the levels of P53 decrease and restoration of the cell cycle occurs. However, in cases of unsuccessful DNA repair, the levels of P53 increase and fosters apoptosis (Siganaki *et al.*, 2010). Therefore, impairment in P53 gene will raise the risk of tumor genesis due to the increased DNA damage (Elmore, 2007). Nuclear P53 combines with the DNA and activates the expression of pro-apoptotic genes. On the other hand, the cytoplasmic P53 triggers apoptosis by direct interaction with the Bcl-2 family (Chipuk *et al.*, 2005).

Conclusion:

In conclusion, all results of this study document that tramadol can induce deleterious effects on the cerebellar neurons through upregulation of the GFAP, P53 and, downregulation of Bcl-2. These proteins exert their impacts by inducing inflammation, oxidative stress, the release of several mediators, ER stress and apoptosis. Therefore, measures and restrictions to tramadol abuse must be taken by the concerned authorities.

Ethical consideration: All experimental procedures were approved by FMASU, REC (Faculty of Medicine, Ain Shams University, Research Ethics Committee, Cairo, Egypt) that comply with the Guide for the Care and Use of Laboratory Animals released by the United States National Institute of Health (Code FMASU R 205/2022).

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ARABIC SUMMARY

الترامادول يسبب التغيرات النسيجية مع تقليل مستوى التعبير المناعي ل Bcl-2 وزيادة مستوى التعبير المناعي ل P53, GFAP في مخيخ ذكر الجرذ الأبيض

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الترامادول هو مادة أفيونية اصطناعية تمارس تأثيرها المسكن من خلال العمل بشكل مركزي عن طريق تثبيط امتصاص النوربينفرين والسيروتونين. كما أنه يمنع انتشار الاشارات المسببة للألم عن طريق تنشيط مستقبلات الأفيون.

إنه الدواء المفضل في العلاج والوقاية من الآلام المتوسطة والشديدة. وبالرغم من ذلك أفادت العديد من الدراسات أن استخدامه المزمن مرتبط بالإدمان الجسدي والنفسي. إن إدمان الترامادول مشكلة صحية عالمية خطيرة. يوصف ترامادول طبيا على نطاق واسع، لكن عواقبه السلبية لا تزال مدعاة للقلق، لذلك كان الهدف الرئيسي من هذا العمل هو دراسة الآثار المحتملة لإعطاء عقار الترامادول على مخيخ ذكور الجرذان البيضاء من الناحية النسيجية والشكلية.

تم تقسيم ثلاثين من ذكور الجرذان البيضاء بشكل عشوائي إلى مجموعتين تحتوي كل منهما على خمسة عشر جرذًا. المجموعة الضابطة (I) ، حقنت داخل الصفاق بواحد ملليترمن المحلول الملحي العادي 0.9٪ يوميا لمدة أربعة أسابيع. المجموعة الثانية (II) ، حقنت داخل الصفاق بـ 50 مجم / كجم / يوم من الترامادول لمدة أربعة أسابيع.

في نهاية التجربة، تم استئصال المخيخ وتثبيته. ثم تقطعيه الى مقاطع تسلسلية وإعدادها للصباغة النسيجية (الهيماتوكسيلين والأيوسين ، نترات الفضة) وكذلك الصبغات الكيميائية المناعية (GFAP ، P53، Bcl-2).

في المقاطع المصبوغة بصبغة الهيماتوكسيلين والأيوسين؛ تم تقييم المعلمات النسيجية التالية: (1) سمك القشرة المخيخية (2) عدد خلايا بركنجي الطبيعية، بالإضافة إلى ذلك تم عمل القياسات الكيميائية المناعية الكمية، والبيانات التي تم الحصول عليها خضعت للتحليل الإحصائي.

في المقاطع المصبوغة بالهيماتوكسيلين والأيوسين ، أظهرت المجموعة (II) ترققًا لطبقات خلايا بركنجى والطبقة الحبيبية للقشرة المخيخية. وكذلك الورقات المخيخية تم فصلها بشقوق واسعة وعميقة تحتوي على أوعية دموية محتقنة بشكل ملحوظ. أظهرت الطبقة الجزيئية مناطق عديدة من التنكس الملحوظ. أما طبقة خلية بركنجي فقد أظهرت العديد من خلايا بركنجي منكمشة ومتحللة ذات نوى متحللة بالاضافة الى مناطق عديدة خالية من خلايا بركنجى، أما الطبقة الحبيبية فقد ظهرت أقل كثافة مع شحوب فى بعض الخلايا الحبيبية. أظهرت المقاطع المصبوغة بالفضة من المجموعة (II) تنكسا ملحوظًا لخلايا بركنجي ، وفجوات متعددة وكذا خلايا حبيبية شاحبة اللون ومتحللة.

المجموعة (II) أظهرت تصبغا مناعيا ملحوظا لل GFAPومعتدلا لل P53 في كل من القشرة المخيخية والمادة البيضاء. وعلى صعيد اخر وجد انخفاضًا ملحوظًا في التصبغ المناعي لـ Bcl-2 في جميع طبقات القشرة المخيخية والمادة البيضاء فى المجموعة الثانية. في المجموعة (II) ، اكتشفنا زيادة غير ذات دلالة إحصائية في سماكة الطبقة الجزيئية وانخفاض معتد به إحصائيًا في سمك الطبقة الحبيبية ووطبقة خلايا بركنجي مقارنةً بالمجموعة (I) علاوة على ذلك انخفضت أعداد خلايا بركنجي انخفاضًا ذا دلالة إحصائية في المجموعة(II)

أظهرت النسب المئوية لمساحة السطح للتعبير المناعى ل GFAP و P53في المجموعة (II) زيادة ذات دلالة إحصائية. ومع ذلك، فقد ظهر انخفاض معتد به إحصائيًا في النسبة المئوية لمساحة السطح للتعبير المناعي ل Bcl-2في المجموعة (II) مقارنة بالمجموعة(I) .

نستخلص مما سبق أن الترامادول يمكن أن يتسبب في حدوث تأثيرات ضارة على الخلايا العصبية المخيخية من خلال زيادة ال GFAP و53P وتقليل ال Bcl-2. تلك البروتينات تحدث تأثيرها عن طريق استحداث الالتهاب والإجهاد التأكسدي وإطلاق العديد من المواد الوسيطة وإجهاد الشبكة الاندوبلازمية وكذلك موت الخلايا المبرمج.