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ORIGINAL ARTICLE

Prenatal and postnatal development of the cerebellar granule cells following adjuvant administration of thyme and tramadol

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ABSTRACT

Introduction: Tramadol abuse is increasing among male and female teenagers with a history of substance abuse and anxiety. Many adolescent addicts take tramadol as substitute for other narcotics. Tramadol uptake is associated with many side effects. Thyme has been presumed to have a neuroprotective effect against brain damage.

Aim of the work: To assess the harmful effects of tramadol on the pre and postnatal development of the rat cerebellar granule cells and to evaluate the possible ameliorative effect of thyme if being administered with tramadol simultaneously.

Material & Methods: Three sets of forty-eight mature female albino rats were randomly organized into three equal groups; control (G1), tramadol treated (G2) and tramadol+thyme treated (G3). After pregnancy, the pregnant rats of each group were categorized into a group designed to be sacrificed at the gestational ages 13th, 16th and 19th for the prenatal study. In the other pregnant rat group, their offspring were further subdivided according to their ages into 3 subgroups (newborn, 10th and 20th postnatal day) for postnatal evaluation. G1 was not given any treatment. Tramadol HCL (40 mg/Kg/day) dissolved in tab water was given orally to G2. Tramadol (40 mg/Kg/day) and thyme extract (500 mg/kg/day) were given orally to G3. A light, electron microscopic examination and morphometric analysis were performed in the study.

Results: By light and electron microscopic examination, there were degenerative and apoptotic changes in the cerebellar cortex of G2. G3 showed improvement in histological changes. Morphometric results showed a decrease in the cerebellar cortex thickness between the G1 and G2 which was significant and an increase in the cerebellar cortex thickness G3 when compared with that of G2.

Conclusion: The study assigns that tramadol administration induces adverse effects on the prenatal and postnatal development of the rat cerebellar cortex. It also highlights an improving role of thyme in tramadol-induced cerebellar cortex injury of the pre and postnatal development.

Key Words: Prenatal; Postnatal; Cerebellar granule cells; Tramadol; Thyme

INTRODUCTION

Drug addiction is a relapsing chronic brain condition characterized by a need to seek and consume drugs, a loss of inhibition in the ability to control the amount of intake, and the development of a negative hedonic state when drug access is restricted (1) (2) . Addiction also includes a desire for the substances and, in some situations, participation in potentially fatal dangerous actions (3).Because the brain is one of the most metabolically active tissues, it is highly vulnerable to toxicity. In addition to other mechanisms, the neurotoxic effects of drugs abuse are frequently linked to oxidative stress, mitochondrial malfunction, apoptosis, and suppression of neurogenesis (4)(5).

Tramadol is a pain reliever that also has analgesic qualities. Because they act on the central nervous system (CNS) to relieve pain in osteoarthritis, cancer, toothaches, and other chronic conditions, tramadol and other opioid pain medications are classified as narcotics (6) (7).

Tramadol abuse is increasing among male and female teenagers with a history of substance abuse

and anxiety. Many adolescent addicts take tramadol as substitute for other narcotics (8). Tramadol uptake is associated with many side effects. Its main adverse reactions include nausea, dizziness, sedation, dry mouth, sweating and seizures. Respiratory depression has been linked to the drug uptake. Physical dependency and withdrawal syndrome have been linked to long-term usage of high dosages of tramadol. Tramadol causes both common and uncommon opiate withdrawal symptoms, such as convulsions (9)(10). Studies on animals have shown that at extremely high doses, tramadol has a negative impact on organ development, bone growth, and mortality rates (11).

Tramadol should normally be avoided during gestational period because it can induce reversible withdrawal symptoms in the newborn. In the offspring's cerebrum and cerebellum, tramadol usage during gestational and lactation periods lowers neuronal survival, synapse formation, and neuronal development (12). In the cerebellum progeny, tramadol produces oxidative stress. This was demonstrated by a rise in lipid peroxidation and a decrease in the activity of antioxidant enzymes in cerebellar neurons' mitochondria, leading in the generation of reactive oxygen species that harm cells (13) (14).

The cerebellum has the greatest amounts of nitric oxide (NO). NO is a neurotransmitter implicated in brain ageing (15). Tramadol administration results in increased synthesis of NO. This increase in No has a unique role in neurotransmission and vasodilation process at low doses but is neurotoxic at higher doses (16).

Herbal plants have long been utilized as a foundation for traditional cures throughout human history, and they are also used as sources of modern medications. The World Health Organization (WHO) reported that more than 75% of communities in resource-limited nations rely on medicinal plants for their primary health care requirements (17). Thyme is a natural herbal plant that it is widely used as a spice, a tea, and a medicinal herb. As it enhances blood circulation, it functions as an exciting stimulant for the entire circulatory system. One of its newly uptake is using it as an effective adjuvant psychotherapy for depression and other mood disturbances (18). Thymol has been shown in studies to enhance healthy fats and increases omega-3 content in the cell membranes of kidney, heart, and brain cells. Through its contents of vitamins, and minerals, as well as rosmarinic and ursolic acids, may help to prevent cancer (19).

Thyme has a number of health benefits. It contains antiseptic, antibacterial, antibactericidal, antihelmintic, and antioxidant activities in this regard. It has also recently been advocated as a natural alternative to synthetic antioxidants. It has the potential to trap free radicals, which is a key antioxidant mechanism that prevents oxidative damage from occurring. (20). It has been demonstrated to protect the brain against harmful metabolites, and its neuroprotective processes may be connected to the inhibition of neuroactive enzymes (21) (22).

MATERIAL AND METHODS

1-Drugs:

Contramal (tramadol HCl) is provided as 100 mg tablets (Grunenthal, Italy). Thyme aqueous extract was made with dry thyme leaves purchased from a local store. Through the use of an electrical chopper, the leaves of the thyme were crushed into a fine powder. The powder was then extracted for 30 minutes in a covered flask with 200 ml of boiling distilled water (DW). The extract was cooled and filtered to eliminate any particle matter, and the filtrate was then vacuum-dried. Before delivery, the appropriate dosages were weighed and reconstituted in 5 mL of DW (23).

II-Experimental Animals:

A total of 48 mature female albino rats and 12 adult male albino rats weighing 180-200 grams were used in this work. All the animals were provided from Assiut University's Animal House. They have the same environmental circumstances. The animals were housed in separate cages in an appropriately ventilated room with an average temperature (22-24 C) and humidity and were given unrestricted access to food and water on a regular 12h light/12h dark cycle (16).

III-The Experimental Design:

The pregnancy of rats was confirmed by the presence of a vaginal plug. After that, the pregnant female rats were randomly allocated into three main groups; control, tramadol treated and tramadol + thyme treated (13) (20). Some of each of the aforementioned main pregnant group was sacrificed at 13^{th}, 16^{th} and 19^{th} of pregnancy for prenatal study. The other pregnant rat groups (control, tramadol treated and tramadol + thyme treated) were further subdivided into 3 subgroups as follow:

1-Control group (G1): The pregnant female rats from day one of pregnancy till weaning were not received any medications for 8 weeks.

2-Tramadol group (G2): The pregnant female rats were given an oral dosage of tramadol HCL (40 mg/kg/day) suspended in tab water through an oro-gastric tube from 1^{st} day of pregnancy till weaning.

3-Tramadol + Thyme group (G3): From the 1^{st} day of pregnancy until weaning, pregnant female rats were given tramadol HCL (40 mg/kg/day) and thyme extract (500 mg/kg/day) orally via an oro-gastric tube. The offsprings of each of G1, G2 and

G3 were sacrificed at the ages of newborn (G1-A), postnatal 10th day (G 1-B) and postnatal 20th day (G1-C**)** for the study evaluation**.**

IV- Methods of study:

A- **Qualitative or Histological study**:

In the prenatal study, the pregnant rats were decapitated at 13th, 16th and 19th gestational age and their embryos were extracted. The embryos cerebella were fixed in Bouin's fluid and then prepared for light microscopic examination. Serial sagittal sections about 5µm in thickness were prepared and stained with gallocyanin – chrom alum stain. Steps were done according to (24). In the postnatal groups, the rats' offsprings of the nine subgroups (3 newborn groups, 3 groups of each the 10th day and the 20th postnatal day) were prepared for semithin and ultrathin examination. The rats' offsprings at the planed age from each group was anesthetized with ether inhalation and perfused by normal saline. After decapitation, the cerebella were removed cautiously from the rat skull. The cerebellum specimens were fixed in 3% buffered glutaraldehyde, washed in phosphate buffer, and fixed in 1% osmium tetroxide, and then dehydrated in alcohol and embedded in epoxy resins. Semithin sections were cut into 1 µm thickness, stained with toluidine blue stain, and examined under a light microscope for morphometric analysis. Ultrathin sections (60–80 nm) were cut, stained with uranyl acetate and lead citrate (25). Finally, the sections were examined by JEOL-JEM-100 SX transmission electron microscope in Electron Microscopy unit, Assuit University, Egypt.

B- Quantitative or Morphometric study:

The morphometric study was performed in the newborn, 10th and 20th postnatal days. For each aforementioned postnatal age group, the three designed experimental groups were studied for the thickness of the external granular layer (EGT), thickness of the molecular layer (MLT), thickness of the Purkinje layer (PLT) and thickness of the internal granular layer (IGT). Thicknesses of each layer was modeled as the length of the perpendicular streamlines between the two boundaries of each laminar structure of interest and calculated (26). From the obtained sets of semi thin sections, photographs of the same area were taken from the sampled 2 consecutive sections. It was measured at a magnification of 100/slide. The measurements were performed in 10 fields in each of five different sections taken from five different rats of each group. Leica Qwin 500 (Leica Ltd) image analyzer computer system was used to analyze all the images(16).

Statistical analysis

The mean values of the data obtained from the image analyzer were analyzed using statistical software (SPSS V23, Inc., IL, USA). For statistical comparison between different groups; one way analysis of variance (ANOVA) test followed by post-hoc Tukey test were used for comparing quantitative parametric data while Kruskal-Wallis test followed by Dunn's test were used for comparing quantitative non-parametric data. Quantitative parametric data were represented as mean± SE, while quantitative non-parametric data were presented in median and interquartile range (IQR). $P<0.05$ was considered statistically significant (27).

RESULTS

A- Light microscopic results:

Prenatal 13th day: In the control group (G1), the cerebellum was formed of two cerebellar primordia covered by the ventricular zone that providing the precursors of the Purkinje neurons (PN) and the deep cerebellar nuclei. The cerebellar primordium was separated from the anterior part of the hind brain by the cavity of the 4th ventricle. The germinal triagone (GT) could be seen (Plate1, figs A-1& B-1).In tramadol treated group (G2), there was discontinuity of the ventricular zone with scanty appearance of the PN precursors. The GT was not found. The 4th ventricle was disorganized and its cavity was dilated (Plate 1, figs A-2 & B-2). In tramadol and thyme treated group (G3), the cerebellar primordium was normal with well-preserved ventricular zone. The 4th ventricle was well organized (Plate 1, figs A-3& B-3).

Prenatal 16th day

In G1, LM examination revealed the presence of a choroid plexus, and the cerebellum was formed of GT that provides external granular layer (EGL) cells which spread nearly the whole surface of the cerebellum and Purkinje cell plate (PCP) that appeared well organized. The foliation of the cerebellar surface could not be noted. A well-organized GT could be observed. The EGL and the PCP were not separated from the cerebellar medulla (Plate2, figs A-1& B-1). In G2, the EGL was apparently reduced in thickness and disrupted. Ill-defined disrupted PCP, dilatation of the 4th ventricle and reduction of arborization of the choroid plexus villi were observed. The EGL was covered by thick non differentiated fibrous layer (Plate2, figs A-2& B-2). In G3, the cerebellum contained the choroid plexus, moderately organized EGL and PCL. There was reduction of thickness of the superficial fibrous layer (Plate2, figs A-3& B-3)

Prenatal 19th day

In G1, well-developed choroid plexus, reduction of length of the GT and the EGL covering the all surface of the cerebellum could be seen. The EGL and PCL were well organized. The foliation of the cerebellar surface was observed. There was a stratification of the EGL and the PCL into multiple

rows (Plate3, figs A-1& B-1). In G2, the EGL was apparently reduced in thickness and not continuous along the surface of the cerebellum. The PCL was ill-defined and disorganized. The foliated pattern of the cerebellar surface was less developed in comparison with the control. The PCL showed disappearance of the stratification pattern (Plate3, figs A-2& B-2). In G3, the EGL was still less continuous along the cerebellar surface but the PCL was of an organized pattern. The surface of the cerebellar surface was more foliated in comparison with G2. The EGL and the PCL were arranged into rows (Plate3, figs A-3& B-3).

ELECTRON MICROSCOPIC RESULTS:

In group 1- A (Neonatal control group**),** the electron microscopic examination (EM) showed that the cerebellar granule cell (CGC) having a large nucleus. CGC contained a euchromatic chromatin and surrounded by a regular nuclear membrane. The mitochondria within the cytoplasm, many rough endoplasmic reticulum cisternae (rER) and free ribosomes (r) could be seen (Fig.1). In group 2-A (Neonatal Tramadol treated group), EM examination revealed that CGC had a condensed nuclear chromatin and an irregularity of the nuclear membrane. The cytoplasm was rarified and contained many large vacuoles, vacuolated mitochondria and dilated rER (Fig. 2).

In group 3-A (Neonatal Tramadol +Thyme treated group), EM examination showed that CGC had a large nucleus with a euchromatic chromatin and surrounded by a regular nuclear membrane. The cytoplasmic organelles are relatively normal with presence of few degenerated mitochondria (Fig. 3).

For group 1- B (Control group of postnatal 10^{th} day), EM examination showed that CGC had large nucleus with a condensed chromatin. The nucleus is surrounded by a thin rim of cytoplasm. Rosette can be seen containing many round mitochondria, synaptic vesicles and rER (Fig. 4).

In group 2- B (Tramadol treated group of postnatal 10^{th} day)**,** the nucleus of CGC had an irregular nuclear membrane. The rosette was disrupted with a rarified cytoplasm and contained dilated rER and degenerated mitochondria (Fig. 5). In group 3-B (Tramadol +Thyme treated group of postnatal 10^{th} day), CGC had a nucleus with nearly normal condensed chromatin and surrounded by a regular nuclear membrane. Some rosettes contained many mitochondria and rER. Other rosettes contained one mitochondrion with destroyed cristae (Fig. 6).

Group 1- C (Control group of Postnatal 20^{th} day) showed that CGC had a large nucleus (N) with condensed chromatin and thin rim of the cytoplasm. A rosette could be seen containing round mitochondria, synaptic vesicles and synaptic cleft. Well preserved myelin sheath could be observed (Fig. 7).Group 2- C (Tramadol treated group of Postnatal 20^{th} day) revealed that some CGC having nucleus more condensation of the chromatin and irregularity of the nuclear membrane. Rarefaction of the cytoplasm could be seen in some CGC. rER and swollen mitochondria with destroyed cristae could be observed. Some other CGC showed destroyed mitochondria, shrunken nucleus and marked rarefaction of the cytoplasm (Fig. 8).In group 3- C (tramadol + thyme treated group of Postnatal 20^{th} day, examinations of CGC showed few mitochondria with less degenerative changes. Less condensation of chromatin, minimal rarefaction of the cytoplasm and a preserved myelin sheath in some CGC could be seen (Fig. 9).

II-Morphometrical and Statistical Results:

A-Developmental Results:

EGT wasn't present in the 20 days old rat indicating complete migration of their cells about this age. MLT showed a significant statistical increase from new born to 20 days old age (p value < 0.05). There was a sudden increase in the thickness of this layer between the newborn and 10 days old. PLT showed a significant increase from the new born to 10 days old and significant decrease from 10 to 20 old (p value < 0.05).This increase in PLT denoted that this layer was formed of more than one row organized into a single row at 20days old. IGT revealed a significant increase from the new born to 20 days old (p value < 0.05) indicating massive migration and differentiation of the cells of the external granular which completely disappear at this day to form the internal granular layer (Table 1).

B-Experimental Results

Newborn Group:

BY ANOVA, analysis of EGT at this age revealed a significant statistical difference among G1, G2 and G3 (p value < 0.05).This difference denoted the higher delay in differentiation of the external granular layer of G2 and moderate delay in G3 as compared with G1. MLT analysis revealed an insignificant statistical difference among G1, G2 and G3 (p value > 0.05). This difference indicated that prenatal administration of tramadol had no effect in the MLT of the newborn rat cerebellar cortex. ANOVA analysis of PLT and IGT revealed a significant statistical difference among the three groups (p value < 0.05). This difference indicated that prenatal administration of tramadol had produced a reduction effect in the PLT and IGT of the newborn rat and this effect was partially improved by the combined administration of thyme (Table 2).

10 day's old age

BY ANOVA, analysis of EGT revealed a significant statistical difference among G1, G2 and

G3 (p value < 0.05). This difference denoted the higher delay in differentiation and migration of the cells of the EGL of the G2 and moderate delay in G3 as compared with G1. ANOVA analysis of MLT revealed insignificant statistical difference among the three groups (p value > 0.05). This difference reflected that the prenatal and lactational tramadol administration did not produce effect on the cerebellar MLT development of the 10 days old pups. In PLT and IGT, by ANOVA analysis, again there was a significant statistical difference among the three groups (p value < 0.05) which was a referrer that tramadol administration had a reduction effect in the PLT and IGT of 10 days old pups and this effect was partially alleviated by the combined use of thyme.

20 Days Old Group:

ANOVA analysis of the morphometric parameters revealed that EGT showed significant statistical difference among G1, G2 and G3 (p value < 0.05). This difference denoted the massive delay in differentiation of the cells of G2 and moderate delay in G3. MLT revealed significant statistical difference among the three groups (p value < 0.05). This difference indicated that the prenatal and lactational tramadol administration produced an increase of MLT development of the 20 days old pups as a sign of delay of differentiation of the cells while the combined use of thyme with the tramadol had a reduction effect on the thickness of this layer as a sign of differentiation of the cells. In PLT and IGT, ANOVA analysis showed significant statistical difference among the three groups (p value < 0.05)**.** The difference that was recorded in PLT and IGT among the three groups accounted to the tramadol delirious effects on cerebellar cortex if given in the gestational and lactational periods as a sign of degeneration and this effect is partially enhanced by the combined use of thyme as a sign of regeneration (Table 3).

Table (1): layers thickness of cerebellar cortex among the postnatal control groups of albino rats.

Mean of layer thickness of the cerebellar cortex (um)	Groups	Mean ± SE
EGT	New born	17.88 ± 0.269
	10 days	6.87 ± 0.126*
	20 days	0.00 ± 0.000
MLT	New born	26.37 ± 0.332
	10 days	79.23 ± 0.635*
	20 days	79.81 ± 0.788*
PLT	New born	31.07 ± 0.675
	10 days	37.11 ± 0.806*
	20 days	21.11 ± 0.559. #
IGT	New born	104.17 ± 1.219
	10 days	136.63 ± 1.760*
	20 days	171.06 ± 1.395*

Data are means ± SE from five rats/group..* significantly different (p < 0.05) from newborn. # significantly different (p < 0.05) from 20 days group

Table (2): Means of thickness (um) of the of the different layers of the cerebellar cortex of newborn among the experimental groups

Groups	EGT Mean ± SE	MLT Mean ± SE	PLT Mean ± SE	IGT Mean ± SE
G 1	17.88 ± 0.27(#$)	26.37 ± 0.33	31.07 ± 0.68(#)	104.17 ± 1.22(#$)
G2	27.70 ± 0.92(*$)	25.34 ± 0.44	26.51 ± 0.85(*$)	72.27 ± 4.64 (*$)
G3	22.94 ± 0.89(#*)	25.77± 0.40	3 ± 0.89(#)	94.93 ± 1.68 (#*)

Table (3): Means of thickness (um) of the different layers of the cerebellar cortex of 10th days old among the experimental groups

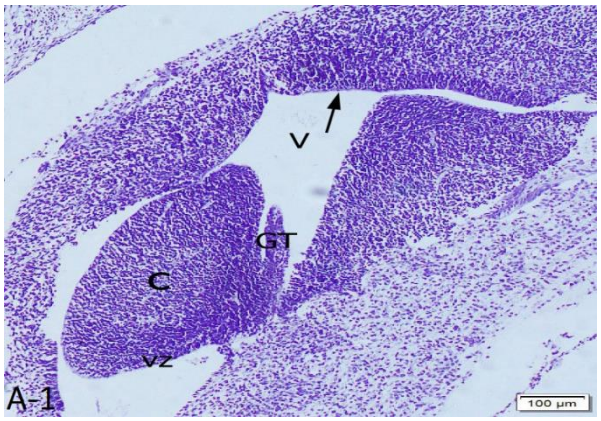
Groups	EGT Mean ± SE	MLT Mean ± SE	PLT Mean ± SE	IGT Mean ± SE
G 1	6.87 ± 0.13(#$)	79.23 ± 0.64(#)	37.11 ± 0.81(#$)	136.63 ± 1.76(#$)
G2	17.47 ± 0.63(*$)	76.73 ± 1.01(*)	26.57 ± 0.97(*$)	86.24 ± 1.37(*$)
G3	12.92 ± 0.62(#*)	78.47 ± 0.57	32.56 ± 1.01(#*)	128.09 ± 2.42(#*)

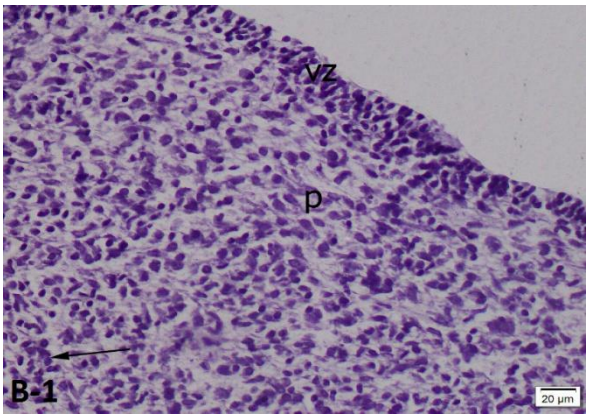
Table (4): Means of thickness (um) of the different layers of the cerebellar cortex of 20th days old among the experimental groups

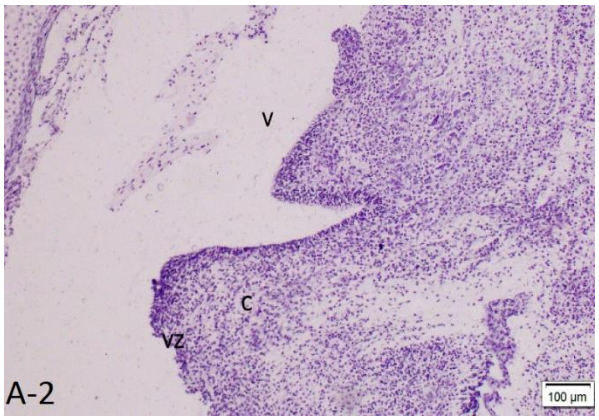
Groups	EGT Mean ± SE	MLT Mean ± SE	PLT Mean ± SE	IGT Mean ± SE
G 1	0.00 ± 0.00(#$)	79.80 ± 0.78(#)	21.11 ± 0.56(#$)	171.72 ± 1.49(#$)
G2	8.12 ± 0.69 (*$)	94.13 ± 0.51(*$)	16.71 ± 0.62(*$)	117.29 ± 7.26(*$)
G3	4.71 ± 0.44 (#*)	85.96 ± 2.06(#)	19.79 ± 0.51(#*)	153.81 ± 2.39(#*)

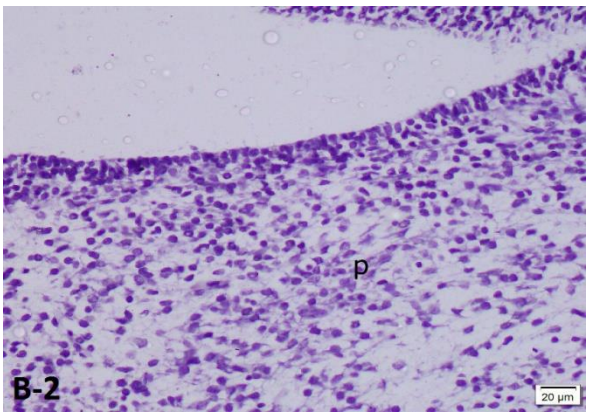
Data are means ± SE from five rats/group, G1 (control group), G2 (tramadol treated group), G3 (thyme+ tramadol treated group).* significantly different ($p < 0.05$) from group I. # significantly different ($p < 0.05$) from group 2. $ significantly different ($p< 0.05$) from group 3.

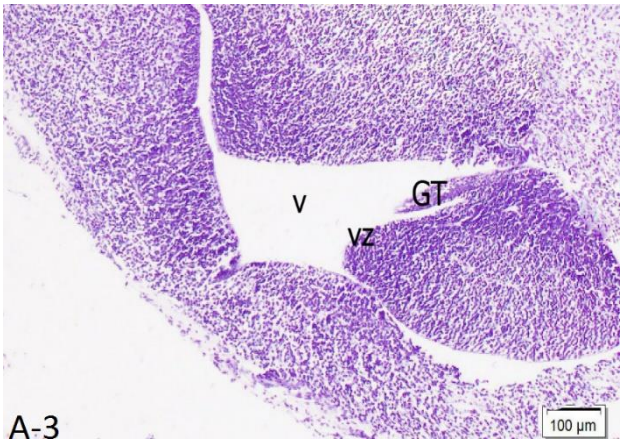
Prenatal and postnatal development of the rat cerebellar granule cells following maternal administration of thyme and tramadol

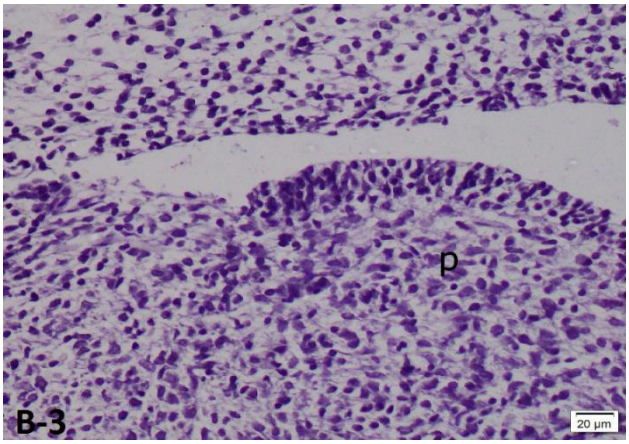






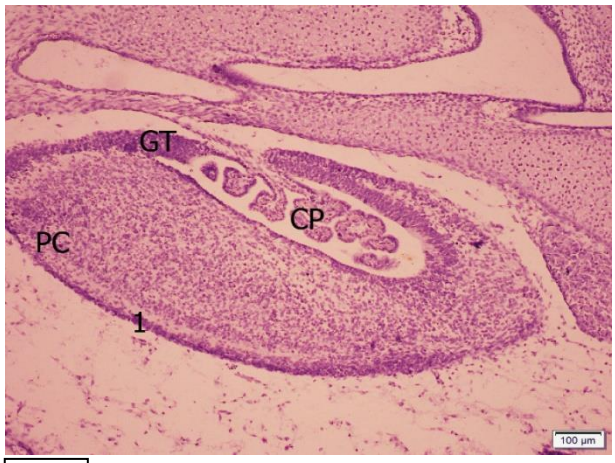




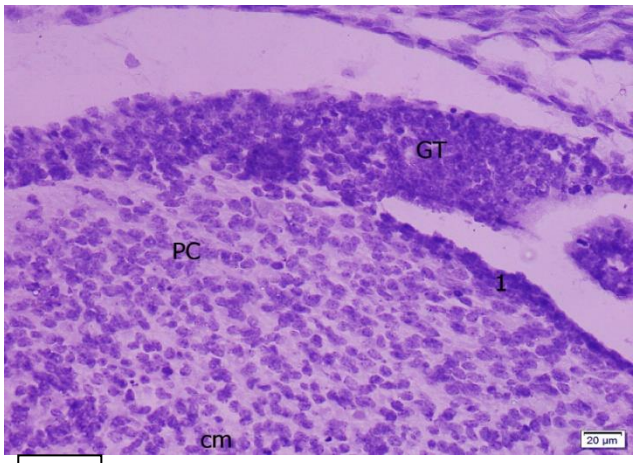


A1-A3 - Gallocyanin chrom-alum stain, × 100 B1-B3- Gallocyanin chrom-alum stain, × 400

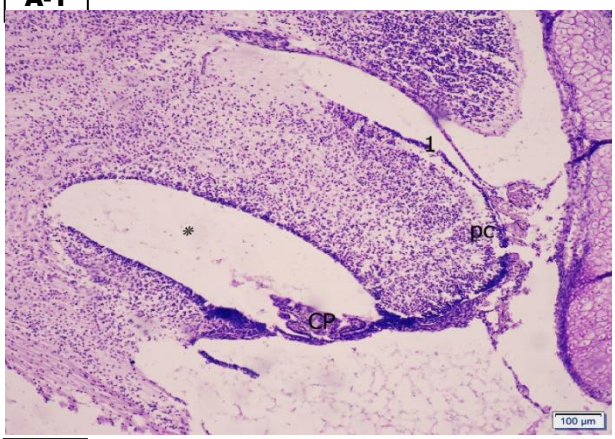
Plate (1): A photomicrograph of sagittal section of the cerebellum at day 13 of the embryonic period of group 1 (Figs. A-1& B-1), group 2 (Figs. A-2& B-2) and group 3 (Figs. A-3&B-3). Figs.A-1& B-1 cerebellar primordium (c) covered by the ventricular zone (vz), germinal trigone (GT), the part of the hind brain (arrow) and cavity of the 4th ventricle (v). Figs.A-2&B-2 showing the cerebellar primordium (c) is present with absence of the germinal triagone. There is discontinuity of the ventricular zone (vz) and scanty appearance of the Purkinje neuronal precursors (p). Figs. A-3 &B-3: showing a germinal triagone (GT), an organized forth ventricle (v). Well preserved ventricular zone (vz) and a nearly normal organization of the Purkinje neuronal precursors (p).



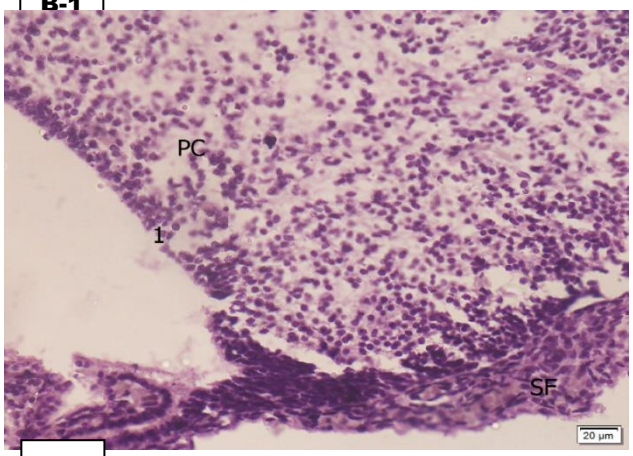
A-1



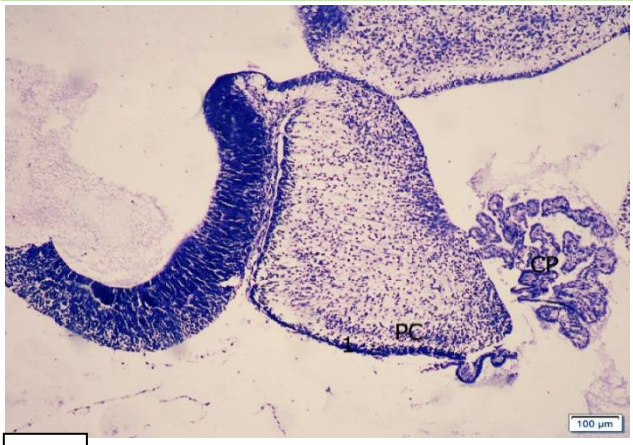
B-1

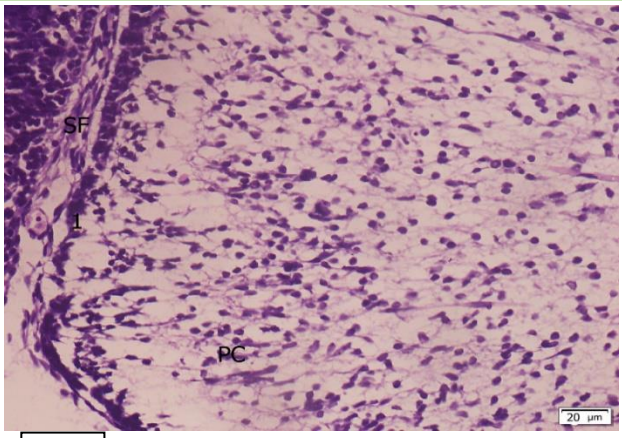


A-2



B-2

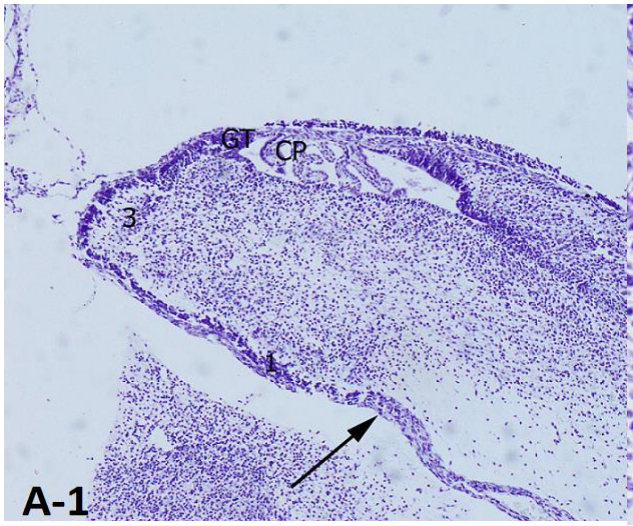


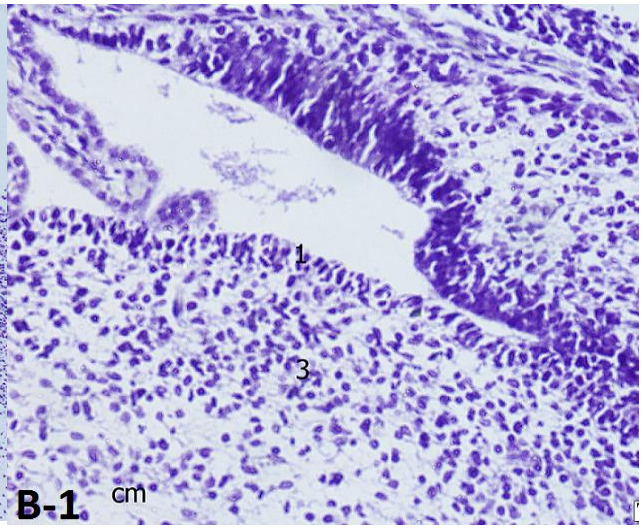


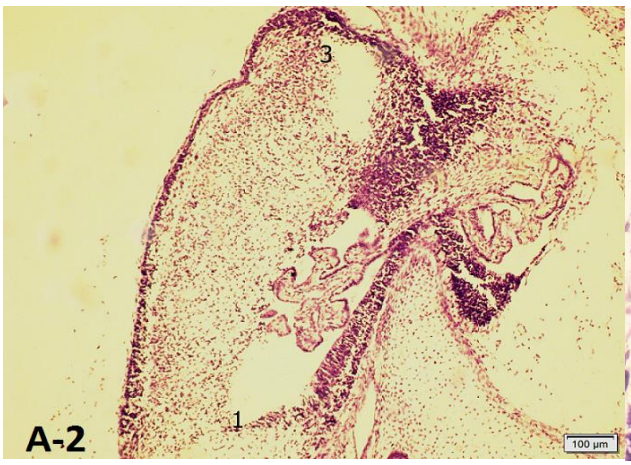
A-3 B-3

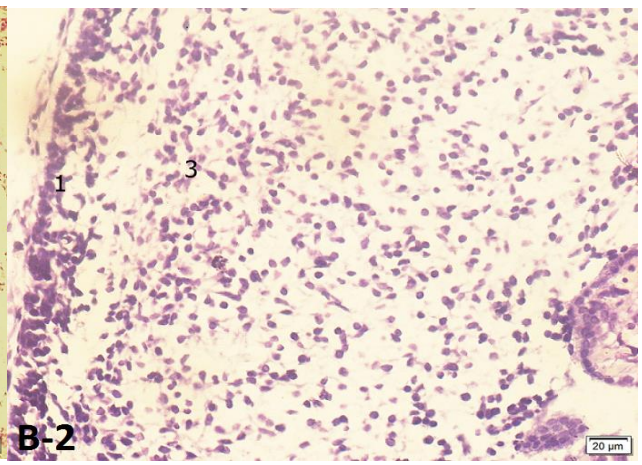
A1-A3 - Gallocyanin chrom-alum stain, × 100 B1-B3- Gallocyanin chrom-alum stain, × 400

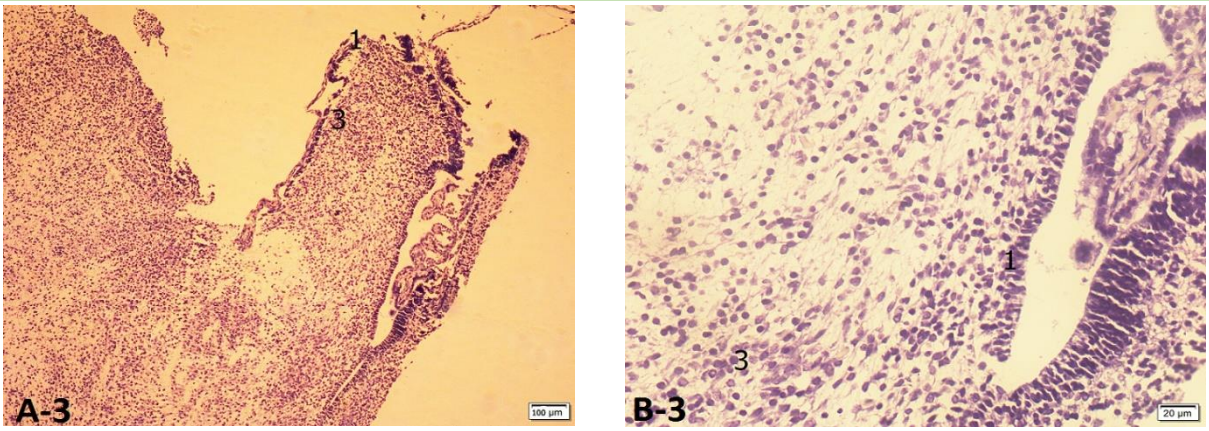
Plate (2): A photomicrograph of sagittal section of the cerebellum at day 16 of the embryonic period of group 1 (Figs. A-1& B-1), group 2 (Figs. A-2& B-2) and group 3 (Figs. A-3&B-3). Figs.A-1& B-1 showing a choroid plexus (CP), a germinal triagone (GT), external granular layer (1) .The Purkinje cell plate (PC) is not separated from the cerebellar medulla (cm). Figs.A-2&B-2 showing the external granular layer (1) is apparently reduced in thickness and disrupted. Ill-defined Purkinje cell plate (PC) can be observed. Dilatation of the 4th ventricle (*) and reduction of arborization of the choroid plexus (CP) could be observed. Thick fibrous layer (SF) adjacent to the external granular layer can be noted. Figs. A-3 &B-3: showing an apparent normal choroid plexus (CP), a preserved organization of external granular layer (1) and a Purkinje cell plate (PC). An apparent reduced thickness of the superficial fibrous layer (SF) can be noted.











A1-A3 - Gallocyanin chrom-alum stain, × 100 B1-B3- Gallocyanin chrom-alum stain, × 400
Plate (3): A photomicrograph of sagittal section of the cerebellum at day 19 of the embryonic period of group I (Figs. A-1& B-1), group 2 (Figs. A-2& B-2) and group 3 (Figs. A-3&B-3). Figs.A-1& B-1 showing choroid plexus (CP), reduction of length of the germinal triagone (GT) stratification of EGL (1), organized PL (3) and cerebellar foliation (arrow). Figs.A-2&B-2 showing disturbance of the continuity of the EGL (1) and ill-defined disorganized PL (3). Figs. A-3 &B-3: showing more or less continuous EGL (1) and recovery of PL organization (3). The surface of the cerebellar surface is more foliated.

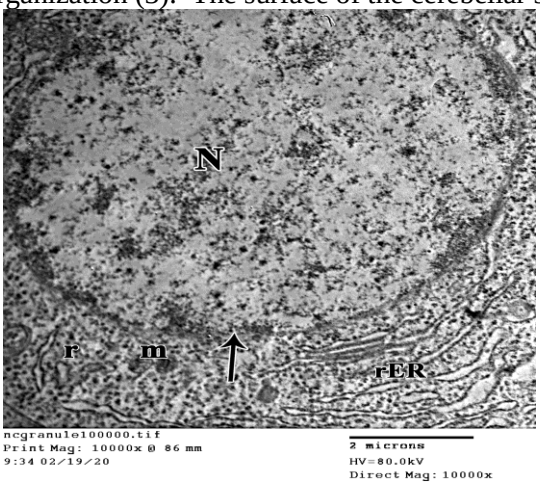


Fig. (1). An electron micrograph of a cerebellar granule cell of control group from the newborn rat showing that the internal granular neuron has a large nucleus (N) containing an euchromatic chromatin and surrounded by a regular nuclear membrane (black arrow). The cytoplasm contains mitochondria (m), rough endoplasmic reticulum cisternae (rER) and free ribosomes(r).TEM, × 1000

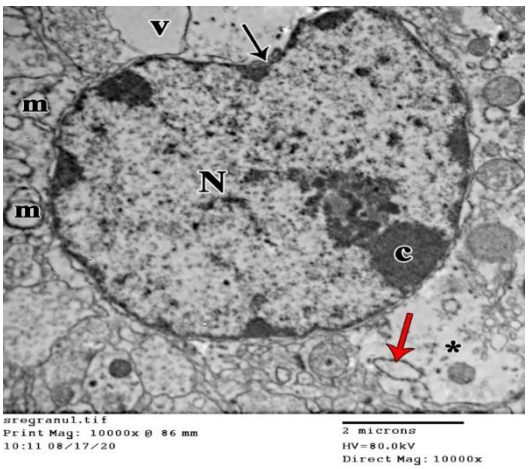


Fig. (2). An electron micrograph of a cerebellar granule cell of tramadol group from the newborn rat showing a large nucleus (N) containing condensed chromatin (c) and surrounded by an irregular nuclear membrane (black arrow). The cytoplasm is rarified (asterisks) and contains large vacuole (v),vacuolated mitochondria (m) and dilated rough endoplasmic reticulum cisternae (red arrow). TEM, × 10000

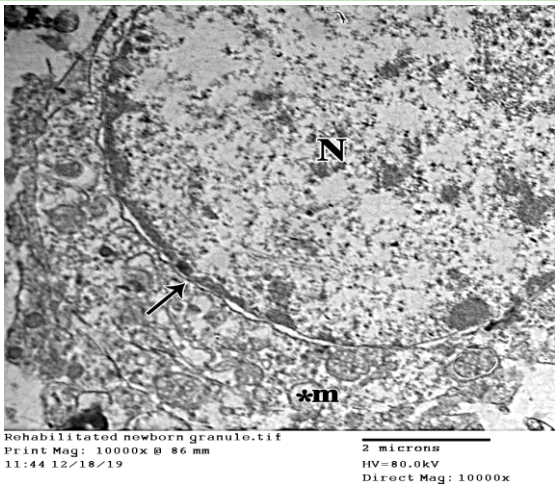


Fig.(3). An electron micrograph of a cerebellar granule cell of tramadol + thyme group from the newborn rat showing a large nucleus (N) with an euchromatic chromatin and surrounded by a regular nuclear membrane (black arrow). The cytoplasmic oranganells are relatively normal with the presence of few degenerated mitochondria (*m). TEM, × 10000

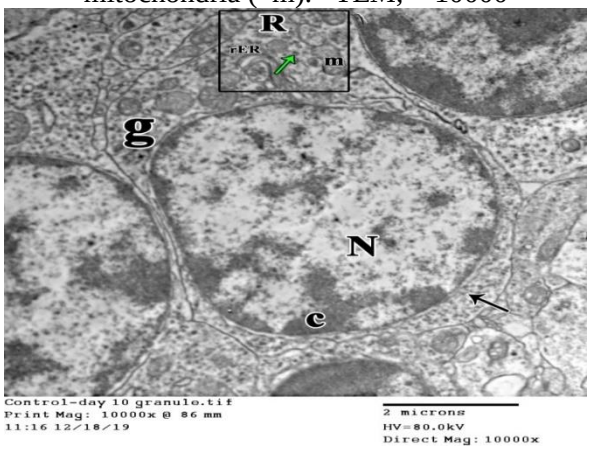


Fig.(4): An electron micrograph of cerebellar granule cells of control group from the rat of postnatal 10th day old showing the structure of the granular cells (g) containing large nuclei (N) with condensed chromatin (c). The nucleus is surrounded by a thin rim of the cytoplasm (black arrow). A rosette (R) can be seen containing many round mitochondria (m), synaptic vesicles (green arrow) and rough endoplasmic reticulum (rER) TEM, × 10000

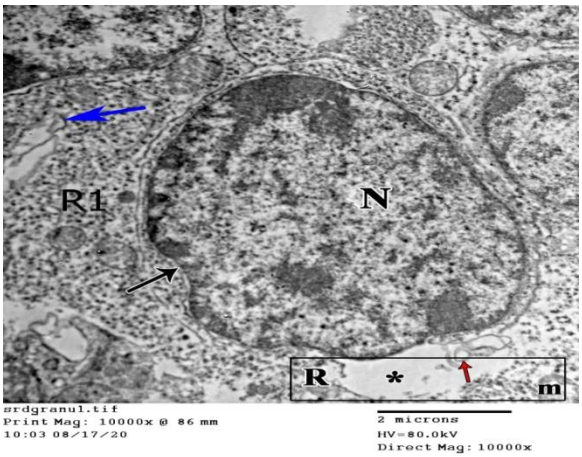


Fig.(5): An electron micrograph of a cerebellar granule cell of tramadol group from the rat of postnatal 10th day old shows the nucleus (N) of a granule cell having an irregular nuclear membrane (black arrow). The rosette (R) is disrupted with a rarified cytoplasm (asterisks) and contains dilated rough endoplasmic reticulum cisternae (red arrow) and degenerated mitochondria (m).The other rosette (R1) shows degenerated mitochondria (blue arrow). TEM, × 10000

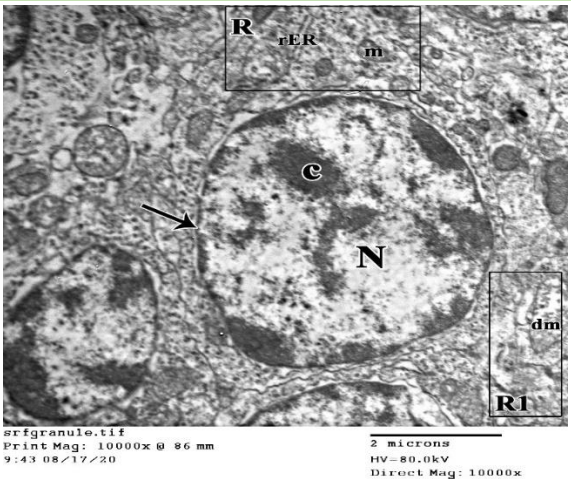


Fig.(6): An electron micrograph of a cerebellar granule cell of tramadol +thyme group from the rat of postnatal 10th day showing a nucleus (N) having a nearly normal condensed chromatin (c) and surrounded by a regular nuclear membrane (black arrow). Some rosettes (R) contain many mitochondria (m) and rER. Other rosettes (R1) contain destroyed mitochondrion cristae (dm) TEM, × 10000

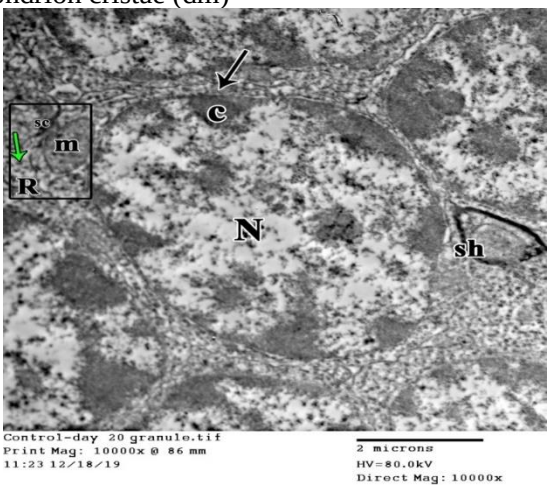


Fig.(7). An electron micrograph of cerebellar granule cells of control group from the rat of postnatal 20th day old showing the structure of granule cells, Each granule cell contains large nucleus (N) with a condensed chromatin (c) and a thin rim of the cytoplasm (black arrow).
A rosette (R) can be seen containing round mitochondria (m), cytoplasmic vesicles(green arrow) and synaptic cleft (sc). Note : sh is intact myelin sheath. TEM, × 10000

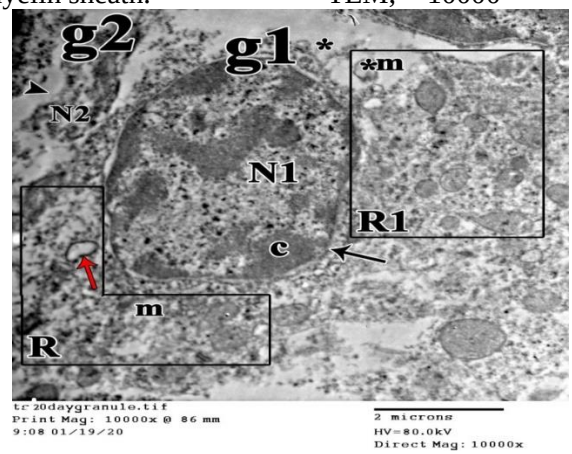


Fig. (8). An electron micrograph of cerebellar granule cells of tramadol group from the rat of postnatal 20th day old. One granule cell (g1) shows a nucleus (N1) with more condensed chromatin (c) and irregular nuclear membrane (black arrow). Rarefaction of the cytoplasm (asterisks) is seen. One rosette (R) shows dilated rER (red arrow) and swollen destroyed mitochondria (m). The other rosette (R1) shows destroyed mitochondria (*m). The other granule cell (g2) shows a shrunken nucleus (N2) and marked rarefaction of the cytoplasm (arrow head). TEM, × 10000

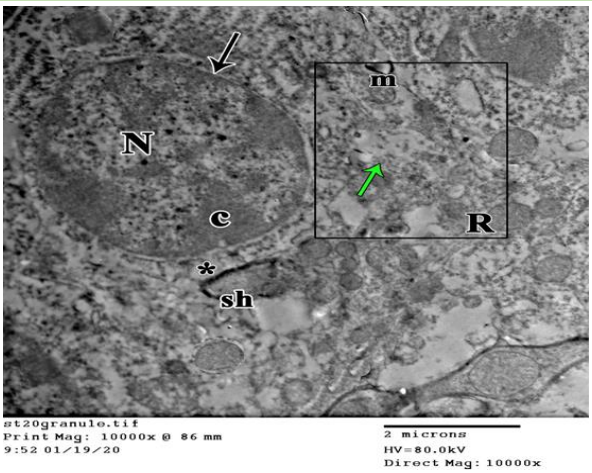


Fig. (9). An electron micrograph of a cerebellar granule cell of tramadol + thyme group from the rat of postnatal 20th day old shows nucleus (N) with nearly normal condensation of the chromatin (c) and having a regular nuclear membrane (black arrow) . The rosette (R) shows intact mitochondria (m) and well preserved synaptic vesicles (green arrow). Minimal rarefaction of the cytoplasm (asterisks) is noted. Myelin sheath (sh) is preserved. TEM, × 10000

Fig. (3). An electron micrograph of a cerebellar granule cell of tramadol + thyme group from the newborn rat showing a large nucleus (N) with an euchromatic chromatin and surrounded by a regular nuclear membrane (black arrow). The cytoplasmic oranganells are relatively normal with the presence of few degenerated mitochondria (*m).

DISCUSSION

The current study aimed to examine the prenatal and postnatal effect of thyme co administration with tramadol on the cerebellar granule cells of albino rats. The cerebellar granule cells were chosen as a target of study because their unique maturation from mitotic granule cell precursors (GCPs) to postmitotic GCs, and their radial migration from EGL along to the formation of the IGL (28).

LM examination of the developing cerebellum of the control and thyme groups revealed that in gestational day 13, the cerebellum was formed of a primordium that covered by the ventricular zone of the cerebellar. These findings were in agreement with the study of (29) (30). In accordance with (29), in gestational day 16 the developing cerebellum was formed of germinal trigone (GT) that provides external granular layer (EGL) cells which spread nearly the whole surface of the Purkinje cell layer (PL) that appeared well organized and less aroborized choroid plexus. The foliation of the cerebellar surface couldn't be seen at this age. In gestational day 19, the developing cerebellum showed well developed choroid plexus with arborization of its villi, reduction of the length of the Gt and the EGL. The EGL and PL were well organized. These two layers were formed of multiple strata especially the EGL. The PL was distinct from the cerebellar medulla. The foliation of the cerebellar surface was observed. These results were confirmed by (31). In our work the light microscopic results of the tramadol treated group revealed that all studied ages showed a developmental impairment as in gestational day 13. The gestational day 19 showed less developed foliated pattern of the cerebellar surface. The EGL didn't cover the whole surface of the cerebellum in both gestational days 16 and 19. These findings were documented by (32). Furthermore, (12)

reported that tramadol uptake during pregnancy causes cerebellar atrophy with loss of characteristic pattern of folia and sulci, dilation of the 4th ventricle and changes in choroid plexus villi arborization. In both gestational days 16 and 19, there were continuity of the EGL and the PL, enhancement of the foliated pattern of the cerebellar surface and normal width of the 4th ventricle. These results might be attributed to the antioxidant activity of thyme and indicate its efficient neuroprotective effect against oxidative-stress-mediated tissue injury. In coincide with our results, (21) reported that thyme have neuroprotective effect against hippocampal injury induced by cerebral ischeamia-reperfusion through inhibition of oxidative stress and apoptosis.

For studying the histological changes in the postnatal development of the cerebellar cortex, ultra- examination was confined to the granular cells because its unique migratory pattern and its important role in cerebellar diseases (33) . Although, the pathways involved in cerebellar granule cell death are also present in other neurons and hence the basis for the selectivity of these chemicals for cerebellar granule cells in vivo is not clear, but probably involves a number of factors. These include the small size of cerebellar granule cells, the unique subunit composition of their N-methyl-D-aspartate receptors, their low DNA repair ability, low levels of calcium-binding proteins and vulnerability during postnatal brain development and distribution of glutathione and its conjugating and metabolizing enzymes are all important factors in determining the sensitivity of cerebellar granule cells to toxic compounds (34).

The cell bodies of the granule neurons of the control and thyme pups showed a polygonal shape .The nucleus of the granule cell contained peripheral clumps of condensed chromatin and were surrounded by a thin shell of cytoplasm .The rosette of the granule cell contained free ribosomes, a few short segments of rER, few mitochondria, and synaptic vesicles. This was in keep with (35) (36) (37). Granule cells of the newborn were composed of primitive two cell-layers thick. Each cell layer is composed of large round-shaped cell with prominent centrally located nucleoli. These results were in accordance with the studies of (38) (39).

In the tramadol treated group, there were degenerative changes of neurons appeared in the all different studied ages. The neuronal degenerative changes were in form of irregular nuclear membrane, focal clumping of the chromatin, rarefaction of the perikaryon, and disorganization of the cytoplasmic organelles with presence of multiple vacuolated or destroyed mitochondria, lipofuscin granules, scanty synaptic vesicles and dilated rER. In this regard, many researchers clarified that the chronic use of tramadol in increasing doses caused neuron degeneration in the rat brain, which could contribute toward brain dysfunction (40) (41). In harmony with these results (42) noticed that the histopathologic changes of brain tissues seem to be the results of neural exhaustion by the sustained augmented activity in response to continuous administration of tramadol. This effect could be also explained by previous studies that reported the long term use of opioid causes cell apoptosis with cytoplasmic contraction, reduction in cell volume, chromatin condensation and induces neuronal damage through affection on their cytoskeleton (43). In harmony with these findings (44) (45) stated the cerebellar-damaging effects of opioids including tramadol are reduced cell proliferation, cell differentiation and increased cellular death.

The ultra-structure findings of the granule cells of the cerebellar cortex in the thyme + tramadol group showed degenerative changes were much littler. This was confirmed by nearly normal condensation of the nuclear chromatin, regular nuclear membrane, most of the cytoplasmic and rosettes organelles were with normal morphology and appearance of the myelin sheath in the neuropil. These results were in accordance with the findings of (46) who reported the use of thyme improve the morphology of the brain neurons.

In this study the observed mitochondrial alterations might be related to oxidative and endoplasmic reticulum stress (47). In endoplasmic reticulum (ER)-stressed cells, increased Ca2 + release from ER opens the mitochondrial permeability transition pores with loss of cytochrome C and activates nitric oxide synthytase leading to ROS production. ROS causes more Ca2 +release from ER and creates a vicious cycle that induces mitochondrial dysfunction and induces apoptosis (48) (49).

The dilated ER might be also due to tramadol-induced lipid peroxidation and endoplasmic reticulum stress which could predispose it to oxidative stress during increased load of protein folding (50). The degenerative vacuolations could be attributed to the damaged cell organoid from exposure to free radicals (51) (52). The little number of the synaptic vesicles and degeneration of synaptic spines was caused by that the neurotransmitter system was disrupted after tramadol uptake (53).

Morphometrical and statistical studies

In the developmental age groups of the present work, the EGT showed a significant decrease from new born to 10 days old. This layer wasn't present in the 20th day age. These results were in harmony with (54) who reported that the area and width of

the external granular layer reached a peak after the first postnatal week, then declined, until it disappeared at about three weeks of age. In addition (55) ,in their study, stated that in the 3-week old, the EGT was markedly reduced and the neuronal cells became collapsed. The mean MLT showed a significant increase from new born to 20th day age. These findings were in harmony with the study of (37) who stated that the thin molecular layer was formed between the Purkinje cells row and the external granular layer at day7 and increased in thickness gradually to become maximum at day 28 postnatal. We found that the mean PLT showed a significant increase from new born to 10 days old then it showed significant decrease from 10th days to 20th day old .These results were in accordance with (55) who reported that the Purkinje cell layer composed of primitive two cell-layers thick in the newborn then increased in thickness and became one row with regional difference containing large round shaped cells at the seven day. In contrast, (35) reported that the Purkinje cells at the postnatal 14thday showed an obvious increase in size of their cell bodies that were pyriform or flask shaped and arranged in a single row on the surface of internal granular layer. The examination of G2 in comparison with G1 revealed a significant increase in the mean EGT in both the newborn and the 10th postnatal day. The MLT mean was significantly increased in the postnatal 20th day age. The mean PLT and the IGT were significantly decreased in all different studied postnatal age groups. As proposed by (16), the increase in EGT(in the newborn and the 10th postnatal ages and its presence in the 20th postnatal day) and in the MLT (in the 20th postnatal day) could be attributed to the delay of migration of the cells from the external granular layer to form the cells of the internal granular layer. So, the decrease of the PLT and MLT might be caused by the apoptosis of their cellular components. The morphometric parameters of G3 revealed a significant increase when compared with that of G2. This denotes to the improving role of thyme to tramadol delirious effect. Furthermore, it has been presumed that thyme potentiates the effectiveness of other phenolic compounds in our diet in management of Parkinson's disease via its content of **farnesol , luteolin , baicalein** and **thymol** which are natural anti-aging neuroprotectives (56) (57). The noticed changes in the morphometric parameters could be referred to both a decreased brain contents of malondialdehyde and increased nitric oxide which are linked with brain aging (58).

CONCLUSION & RECOMMENDATIONS

Although we've come a long way in understanding many issues about the effect of thyme as one of the herbal plants on cerebellar cortex, there are still a lot of inquiries. For example, which molecular mechanisms are involved in such effect produced by thyme administration? However, our study concluded that tramadol administration induces adverse effects on the prenatal and postnatal development of the cerebellar cortex. It also highlights an improving role of thyme as one of the herbal medications in tramadol-induced cerebellar cortex injury of the pre and postnatal development. Finally, it is highly recommended to supplement thyme as adjuvant administration in the management of the tramadol neuronal damage if the use of tramadol is necessary.

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