Chronic Toxic Effects of Tramadol on the Brains of Adult Albino-Rats and possible Ameliorative Effects of Melatonin

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Running title: Melatonin Effects on Tramadol neurotoxicity

ABSTRACT

Background: Tramadol chronic use as an analgesic represents a noteworthy public health concern due to its neurotoxicity. Melatonin has potent antioxidant and anti-apoptotic properties, therefore mitigating oxidative damage to the brain. **Aim of work:** this experimental research purposed to assess the chronic toxic impacts of tramadol on the brains of adult albino rats of both sexes (males and females) using physical, behavioral, biochemical, histopathological, and immuno-histochemical parameters, and to investigate the possible ameliorative effects of melatonin. **Material and Methods:** This work was performed on 56 adult albino rats (28 males and 28 females) weighing between 180-200 gm. Rats were randomly separated into seven groups of eight rats each: three control groups [negative, solvent and melatonin-treated (10mg/kg/day) group]. The four treated groups were tramadol treated male group, tramadol treated female group (50mg/kg body weight), tramadol-melatonin treated male group, and tramadol-melatonin treated female group. The rats were treated once daily for six months. **Results:** Tramadol administration significantly affected the rats' brains evidenced by reduction in body weight and relative brain weight (RBW%), behavioral alterations, increased levels of ubiquitin-c-terminal hydrolase-1 (UCH-L1), serotonin and noradrenaline and increased the oxidative stress indices, plus the alteration in the brain histology and increased casepase3 immunohistochemical expression that could be ameliorated by melatonin administration. All resulted showed insignificant differences between the corresponding male and female studied groups. **Conclusion**: Daily exposure of adult albino rats of different sexes to (50 mg/kg) tramadol for 6 months resulted in toxic brain effects that could be improved by(10mg/kg/day) melatonin administration.
Keywords: Tramadol, Melatonin, UCH-L1, Oxidative stress, and Brains.

1. INTRODUCTION

Tramadol (TR) hydrochloride is a synthetic opioid that acts centrally and has been demonstrated to be highly efficient in the treatment of pain. Its analgesic efficacy is intermediate among that of a weak opioid and that of morphine ***(Mohamed and Mohamed, 2021).***

Tramadol is used as a worldwide analgesic. It is more tolerable and has lesser adverse reactions than traditional opioids or oral nonsteroidal anti-inflammatory drugs (NSAIDs) ***(Edinoff et al., 2021).***

Since 1995, the Food and Drug Administration (FDA) has authorized TR for the treatment of pain ***(Manouchehri et al., 2022).***

TR has been categorized as a controlled substance in numerous countries due to an elevated prevalence of overdoses and fatalities associated with the drug ***(Nakhaee et al., 2021).***

Tramadol is a multi-receptor drug that functions as a mu-opioid receptor agonist, monoamine and serotonin reuptake inhibitor, and inhibitor of ligand-gated ion channels and certain special protein-coupled receptors and it also has a receptor binding antitussive activity ***(Rahimi et al., 2014).***

The main side impacts of TR treatment are nausea, vomiting, constipation, tachycardia, headache, ataxia, dizziness, drowsiness, somnolence, and loss of consciousness. One of the most susceptible systems to TR is the central nervous system. The structure and function of the brain may be significantly altered by long-term tramadol abuse. Tramadol abuse results in the deterioration of nervous tissues, involving the cerebral cortex, and the symptoms of tramadol abuse are caused by neurodegeneration in this region ***(Aghajanpour et al., 2020).***

Melatonin (N-acetyl-5-methoxytryptamine; MT) is a naturally occurring indole amine that is primarily secreted at night and is essential for the regulation of sleep. Furthermore, it plays a role in a variety of physiological functions, such as the prevention of aging, the strengthening of the immune system, the prevention of apoptosis, the inhibition of cancer cell growth and, the impacts of antioxidants ***(Koohsari et al., 2020).***

Abuse of TR is becoming a more concerning issue within the Egyptian community. It is believed that tramadol is widely utilized and common among Egyptian youth, particularly for the purpose of treating premature ejaculation, extending orgasms, and increasing sexual pleasure. Tramadol was discovered to be utilized by between twenty and forty percent of adults and eighty-three percent of adolescents in Egypt who have substance abuse disorders. Since tramadol is a strongly addictive substance, the Egyptian Ministry of Health has reclassified it from schedule 3 to 1. In 2014, the United Nations Office for Drugs and Crime (UNODC) discovered that five billion pills were available for utilization in Cairo, where tramadol was a highly popular street drug ***(Ahmed et al., 2018).***

2. AIM OF THE WORK

The purpose of the research was to assess the chronic toxic impacts of tramadol on the brains of adult albino rats of both sexes (males and females) using physical, behavioral, oxidative stress markers, histopathological and immune-histochemical reaction for caspase -3, as well as to investigate the possible ameliorative effects of melatonin.

3. MATERIALS AND METHODS

With approval number MS 20-2-2022, the experimental design study was approved by the Research Ethics Committee at the Faculty of Medicine, Benha University (REC-FOMBU), Egypt.

3.1 Animals

A 56 healthy adult healthy albino rats of different sexes (28 males and 28 females) were used with an average body weight between 180 and 200 gm. Mice were procured from the Center of Laboratory Animals, Faculty of Veterinary Medicine, Benha University, Egypt.

Prior to the beginning of the investigation, all animals at the animal bread house at Benha Faculty of Veterinary were subjected to a week of passive preliminaries, which involved the consumption of food and water without the administration of any drugs. This was done to assure the physical health of the animals and to weed out any that were ill. All the animals received the same food (Bread, Wheat and Milk). Medication administration for all animals was planned

to start at noon. At the end of the trial, all mice were placed in an induction chamber (open-drop technique) for the conventional inhalation anesthetic protocol; thereby, mice were died under prolonged exposure to a 4% isoflurane. The brain was quickly extracted and rinsed out with ice-cold physiological saline to eradicate clots, and then dissected into different parts. One portion was preserved in formalin (10%) 108 for subsequent histopathological examination.

3.2 Chemicals

Sigma-Aldrich Chemical Co. sourced via EICI and HIMEDIA lab chemicals and biochemicals for all the pharmaceuticals, reagents, and chemicals used in this study. **a. Tramadol**: 99% pure tramadol hydrochloride powder was used. **b. Melatonin** was found as a powder of N-Acetyl-5-methoxytryptamine with a purity level of at least 99%.

3.3 Duration of the study

All groups were treated daily, for 6 months.

3.4 Grouping and experimental design

*The animals were split up into 7 groups, each of which had 8 rats:

1. **Group I (negative control group): (n=8 rats "4 males** and **4 females"):** The basic parameters were measured without intervention, and the animals were permitted to have free access to distilled water and food throughout the duration of the study.
2. **Group II (solvent control group): (n=8 rats "4 males** and **4 females"):** administered a single oral dosage of 0.5 ml of distilled water every day by gavage tube for the whole period of study.
3. **Group III (Melatonin-treated male group): (n=8 rats "4 males** and **4 females"):** treated with melatonin (10mg/kg/day) dispersed in distilled water and administered orally through gastric gavage for a period of six months according to **Adikwu and Bokolo (2017)**.
4. **Group IV (Tramadol treated male group): (n= 8 rats):** treated with a single daily dose 50 mg/kg body weight of tramadol dispersed in distilled water (which represents 1/10 LD50) administered orally through gavage tube for 6 months according to **Ghoneim et al. (2014).**
5. **Group V (Tramadol treated female group): (n= 8 rats):** treated with a single daily dose 50 mg/kg body weight of tramadol dispersed in distilled water (which represents 1/10 LD50) administered orally through gavage tube for 6 months according to **Ghoneim et al. (2014).**
6. **Group VI (Tramadol and melatonin treated male group): (n= 8 rats):** Melatonin was administered as a single daily dosage (10 mg/kg/day), 30 minutes after treatment with a single daily dosage of tramadol (50 mg/kg/day which represents 1/10 LD50) via gastric gavage for 6 months according to **Abdel-Wahhab et al. (2005).**
7. **Group VII (Tramadol and melatonin treated female group): (n= 8 rats):** Melatonin was administered as a single daily dosage (10 mg/kg/day), 30 minutes after treatment with a single daily dosage of tramadol (50 mg/kg/day which represents 1/10 LD50) via gastric gavage for 6 months according to **Abdel-Wahhab et al. (2005)**.

3.5 Parameters of the study:

I. Body weight and ***relative brain weight (RBW%):*** Rats' body weights in all groups were measured and recorded before treatment and every month until the finish of the experiment utilizing a sensitive balance. At the end of the experiment the brains of rats were removed, and cleaned in normal saline and their weights were recorded as regard the following equation:

$$\text{Relative weight (\%)} = \frac{\text{Absolute organ weight (g)}}{\text{Body weiexperiment theifice day (g)}} \times 100$$

(Khalil et al., 2022).

II. Behavioral test (Open-field test):
albino rats were exposed to the open field test to assess the locomotor activity of the rats using an ANY-maze video tracking system in a wooden box which is a square arena that measures (40x40 cm) and wall 35 cm high with the floor divided into 16 sub-squares. The enclosure is divided into 16 squares, a center zone, which is composed of the four central squares, and a periphery zone, which is composed of the twelve adjacent adjoining sides. Number of crossings (number of squares crossed by the animal using all paws) was recorded. The videos were recorded, and scoring was done ***(Aghajanpour et al., 2020).***

***III. Biochemical studies for serum tramadol and Ubiquitin-C-terminal hydrolase-1 (UCH-L1)*:**
A 5 ml syringe was used to draw blood samples from their hearts to estimate (serum tramadol level) via converse phase High-performance liquid chromatography (HPLC) (YMC Co., Ltd. Cape, South Africa) using an ultraviolet (UV) detector set (Johannesburg, South Africa) at 245 nm. Moreover, Ubiquitin-C-terminal hydrolase-1 (UCH-L1) was analyzed utilizing enzyme-linked immunosorbent evaluate (ELISA) kit from Glory Science Co., Ltd. (Del Rio, USA) using ELISA reader (photometer) (Ray Biotech, Canada) as regard the manufacturer's instructions ***(Abdel-Salam et al., 2019).***

IV. Assessment of neurotransmitters in the brain tissues: 100 mg of brain tissue from each rat was homogenized in one milliliter of phosphate buffer saline and stored nightly at -20°C after the brain hemispheres were dissected out. Rat ELISA kits were employed to measure the levels of serotonin and noradrenaline (Life Span BioSciences, LSBio Inc.), USA. Using ELISA reader (photometer) (Ray Biotech, Canada) ***(Baldo, 2021).***

V. Assessment of oxidative stress markers: The commercially available colorimetric methods (diagnostic kits supplied by Bio Diagnostic Company, Egypt) were utilized to investigate superoxide dismutase (SOD), decreased glutathione (GSH), and malondialdehyde (MDA) in brain tissues utilizing Spectro-nanodrop, as per the manufacturer's instructions ***(Hussein et al., 2020)***.

VI. Histopathological study by light microscope: It was done on all animals of each group at Pathology Department, Animal Health Research Institute, Zagazig, Egypt. Immediately after the scarification of rat's brains were soaked in bouin's solution (composed of acetic acid, picric acid, ***and*** formaldehyde in an aqueous solution) that is utilized to stabilize organs that require accurate morphologic analysis. Tissue specimens were stabilized for six-eight hours ***and*** then transferred to seventy percent alcohol before submitting to histology for automated dehydration, paraffin embedding, sectioning ***and*** staining ***(Bancroft and Gamble, 2008).***

VII. Immunohistochemical (IHC) analysis for Caspase-3: The streptavidin/peroxidase method was employed to conduct IHC reactions (Happerfield et al., 1993). Caspase-3 antibodies were employed to localize apoptosis through immunohistochemically staining with a rabbit polyclonal antibody that wasn't specific to any particular species (1:100 diluted; catalog no RP096, Diagnostic Biosystems; USA,). Strict brown nuclear staining of the cell was the only pattern of staining considering being a strong reaction and low density of staining reveals to weak expression. This study used a semi-quantitative method using color intensity [strong (+++), Moderate (++), Mild (+)] ***(Owusu-Afriyie et al., 2018)***.

Statistical analysis:
The data had been gathered, processed, and examined utilizing SPSS [Statistical bundle for social science] version 26. The mean and standard deviation were added for quantitative data. The student's t-test was employed for comparing the mean of two numerical (parametric) groups. The

Mann-Whitney U-test was employed for continuous non-parametric data. The significance threshold for this study was determined at 0.05.

4.RESULTS:

The results of all studied parameters (physical, behavioral, biochemical, histopathological, and immuno-histochemical) in either adult male and female albino rats showed insignificant differences between the corresponding male and female studied groups.

Male Groups

Body weight and RBW% were comparable between all male studied control groups with non-significant difference among them. A statistically greatly significant reduction was found in the body weight and RBW% in tramadol treated group comparing with control groups and insignificant increase in tramadol-melatonin treated group comparing with the TR treated group. Insignificant variance in the tramadol-melatonin treated group was found comparing with the control groups.

Behavioral test parameters were comparable between all studied control groups with non-significant difference among them. A statistically greatly significant reduction in all behavioral test parameters in tramadol treated group was found comparing with control groups. In the tramadol-melatonin treated group, all behavioral test parameters showed a significant increase comparing with TR treated group and there was insignificant statistically variance comparing with control groups, as detected in **table (1)**

Serum tramadol, serum UCH-L1 levels, brain tissue serotonin, NOR, MDA, reduced GSH and SOD levels were comparable between all studied male control groups with non-significant difference among them. Serum tramadol, serum UCH-L1 levels, and brain tissue serotonin, NOR MDA demonstrated a significant rise in the tramadol treated group compared to control groups and demonstrated a significant reduction in tramadol-melatonin treated group comparing with tramadol treated male group, with insignificant difference as compared to control group. Meanwhile brain tissue reduced GSH and SOD levels showed a significant decrease in the tramadol treated group comparing with control groups and showed an insignificant increase in tramadol-melatonin treated group comparing with TR treated group, with insignificant difference as compared to control group, as detected in **table (2).**

Histopathological studies in male groups

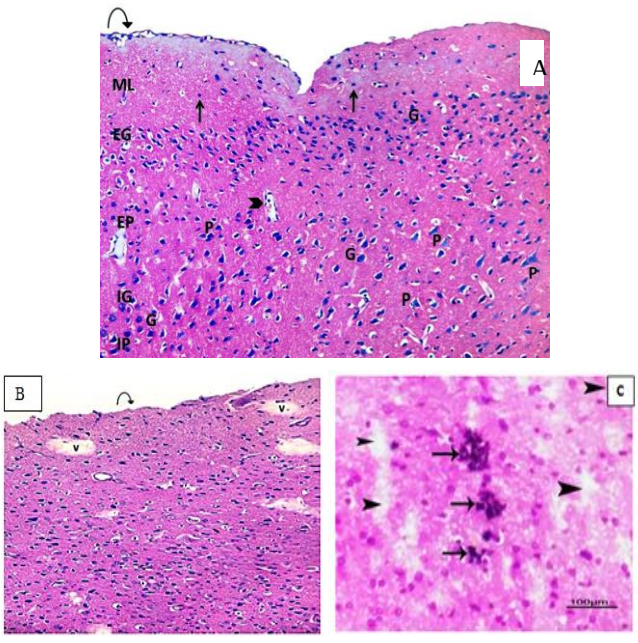

Fig (1) [A]- A photomicrograph of a section in the frontal lobe of cerebral cortex of an adult male control albino rat showing [A well-organized regularly arranged six layers from outer to inner surface: Molecular layer (ML) characterized by presence of glial cells (arrows). External granular (EG), external pyramidal (EP), internal granular (IG), internal pyramidal (IP) and polymorphic layer (PL). External granular and (IG) layers are characterized by rounded granular cells (G). External pyramidal and (IP) layers show pyramidal cells (P) with apical dendrites. A blood vessel can be seen (arrow head). pia mater is seen covering the surface (curved arrow), **(H and E × 200).**

[B]- A photomicrograph of a section in the frontal lobe of cerebral cortex of an adult male albino rat treated with tramadol hydrochloride for six months showing loss of organization of all layers and marked dilated blood vessels with wide perivascular spaces.The molecular layer showed pyknotic neurons surrounded by haloes. The external granular layer showed pyknotic granular cells with deeply stained nuclei. External pyramidal cells appeared pyknotic with deeply stained nuclei and surrounded by haloes. Neuropil appears vacuolated. Internal pyramidal cells were shrunken & showed deeply stained pyknotic nuclei . Some neurons are pyknotic and surrounded by haloes. Acidophilic neuropils.**(H and E × 100).**

[C]- A photomicrograph of a section in the frontal lobe of cerebral cortex of an adult male albino rat treated with tramadol hydrochloride and melatonin for six months showing attenuated cellular apoptosis, focal dark stained cellular and non-cellular deposits (arrows) with foal area of neuropil vacuolation (arrows head) and focal gliosis **(H and E × 100).**

Immunohistochemical reaction for caspase-3 in male groups

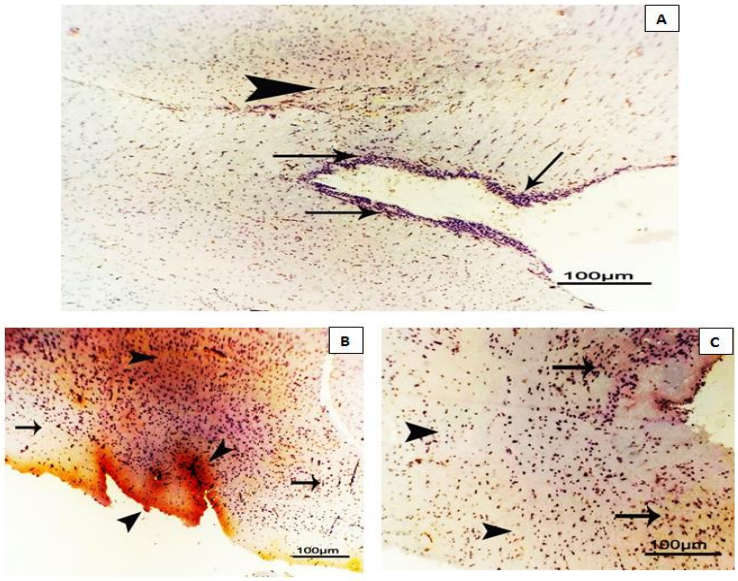

Fig (2) [A]- A Photomicrograph of peroxidase-stained section in the frontal lobe of cerebral cortex of an adult male control albino rat showing diffuse negative reaction (arrows) minimal positive reaction (arrow head) for caspase-3, **(IHC × 100)**.
[B]- A Photomicrograph of peroxidase-stained section in the frontal lobe of cerebral cortex of an adult male albino rat treated with tramadol hydrochloride for six months showing focal severe positive reaction (+++) (arrowhead) to moderate (++) reaction (arrows) for caspase-3, **(IHC × 100).**
[C]- A Photomicrograph of peroxidase-stained section in the frontal lobe of cerebral cortex of an adult male albino rat treated with tramadol hydrochloride and melatonin for six months showing focal mild positive reaction (+) (arrows) to negative (-) reaction (arrows head) for caspase-3, **(IHC × 100)**.

Histopathological studies in different studied female groups

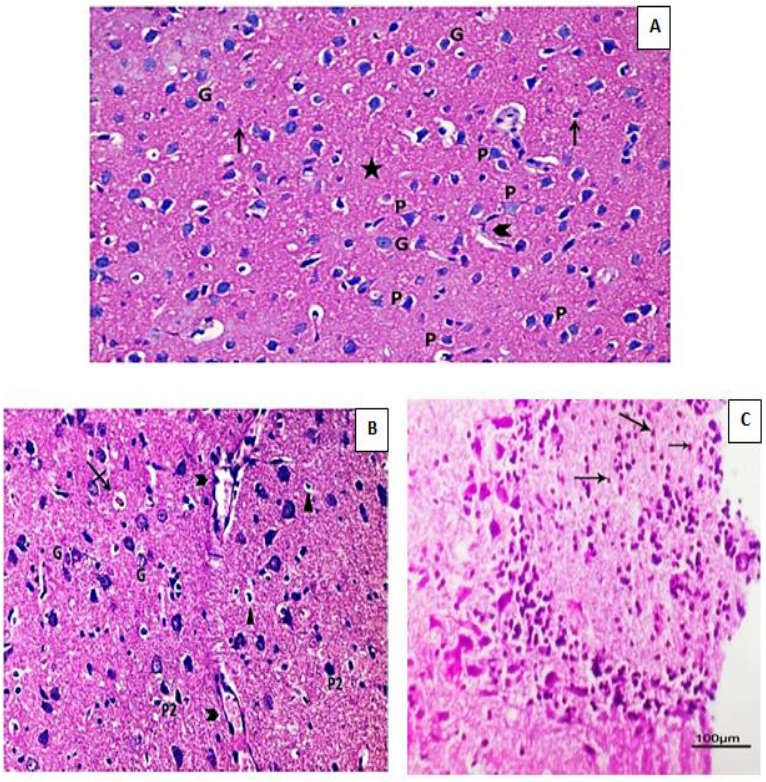

Fig (3) [A]- A photomicrograph of a section in the frontal lobe of cerebral cortex of an adult female control albino rat demonistrating: well organized outer molecular layer, rounded granular cells (G) with open face nuclei in the internal granular layer. The internal pyramidal layer contains large sized pyramidal cells with triangular cell bodies, basophilic cytoplasm, rounded nuclei and long apical dendrites (P). Glial cells can be seen (thin arrows). Normally distributed neurons and neuroglia cells in the neuropil (star). Normal non congested blood vessel can be seen (arrow head) **(H** and **E× 400).**
[B]- A photomicrograph of a section in the frontal lobe of cerebral cortex of an adult female albino rat treated with tramadol hydrochloride for six months showing: pyknotic granular cells with deeply stained nuclei in the external granular layer. granular cells with karyolytic nuclei (G) in the internal granular layer. In the internal pyramidal layer, pyramidal cells show deeply stained pyknotic nuclei (P2). Some neurons are pyknotic and surrounded by haloes (triangles). Dilated and congested blood vessels (arrow heads) and a red neuron (crossed arrow) can be detected **(H** and **E× 400).**
[C]- A Photomicrograph of a section in the frontal lobe of cerebral cortex of an adult female albino rat treated with tramadol hydrochloride and melatonin for six months showing:focal gliosis which is represented in over distribution of glia cells within the external granular layer (arrows), also focal dark stained cellular and non-cellular deposits. **(H** and **E× 100).**

Immunohistochemical reaction for caspase-3 in different studied female groups

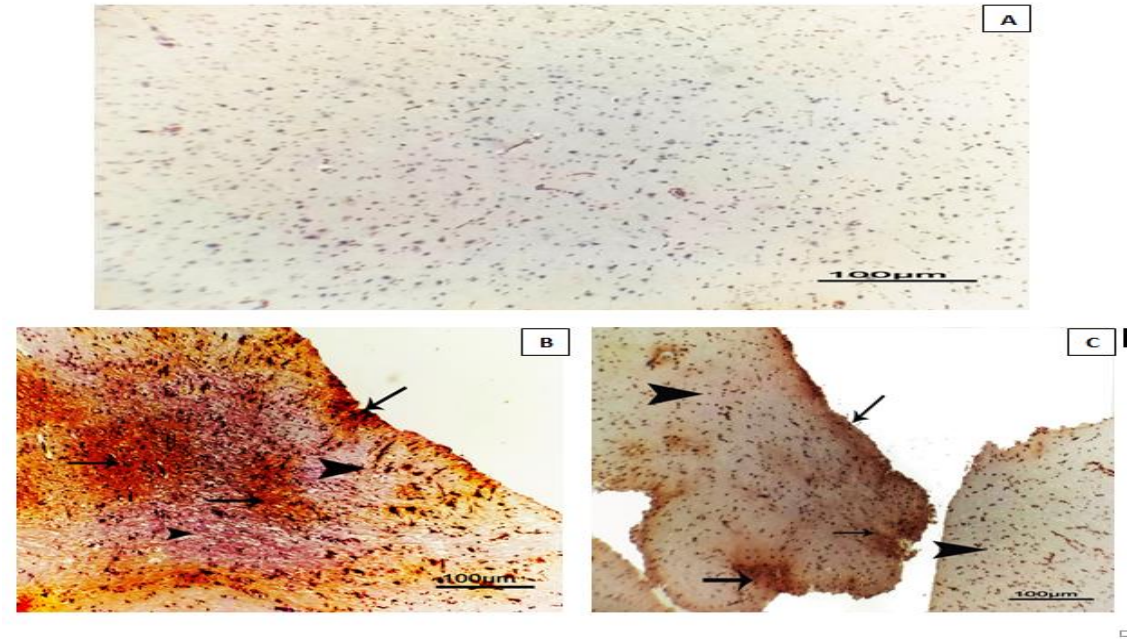

Fig (4): (A)- A Photomicrograph of peroxidase stained section in the frontal lobe of cerebral cortex of an adult female control albino rat showing negative reaction (-) for caspase-3, **(IHC × 100)**.
(B)- A Photomicrograph of peroxidase stained section in the frontal lobe of cerebral cortex of an adult female albino rat treated with tramadol hydrochloride for six months showing focal severe positive reaction (+++) (arrows) to moderate (++) reaction (arrows head) for caspase-3, **(IHC × 100)**.
(C)- A Photomicrograph of peroxidase-stained section in the frontal lobe of cerebral cortex of an adult female albino rat treated with tramadol hydrochloride and melatonin for six months showing minimal focal positive reaction (+) (arrows) to negative (-) reaction (arrows head) for caspase-3, **(IHC × 100)**.

Table (1) Physical and Behavioral test parameters in different studied male groups:

Groups	Control-1	Control-2	Control-3	Tramadol	Tramadol and Melatonin	P Value	Post hoc
Physical parameters							
Body Weight (gm)	312.3±12.5	312.5±11	315.5±11.9	216.7±21.2*	276.6±25.7	P<0.001*	P1<.001* P2=0.341 P3=0.277
RBW%	22.7±.46	21.06±2.3	20.6±1.2	16.9±1.3	18.5±1.8	P<0.001*	P1<0.001* P2=0.932 P3=0.081
Behavioral test parameters							
Speed (cm/s)	2.21 ± 0.19	2.51 ± 0.17	2.71 ± 0.2	0.61 ± 0.11	1.50 ± 0.12	< 0.001	P1<0.001* P2=0.051 P3=0.07
Total distance traveled (m)	6.62 ± 0.58	6.72 ± 0.5	6.71 ± 0.53	2.61 ± 0.33	4.80 ± 0.37	< 0.001	P1<0.001* P2=0.032 P3= 0.054
Number of line crossing	54.50 ± 7.06	52.50 ± 6.06	53.80 ± 7.36	20.70 ± 2.75	35.30 ± 5.83	< 0.001	P1<0.001* P2=0.041 P= 0.061
Number of immobile episodes	6 (4–9)	7 (5–9)	6 (3–8)	16 (10 –17)	11 (7–13)	< 0.001	P1<0.001* P2=0.034 P= 0.082
Time in the peripheral zone (s)	240.65 ± 6.40	244.65 ± 6.10	242.65 ± 6.50	296.33 ± 5.07	252.84 ± 9.23	< 0.001	P1<0.001* P2=0.022* P= 0.072

Data are presented as mean± SD or median (range). RBW%: relative brain weight, P1: significance between controls and tramadol treated group, P2: significance between tramadol treated group and tramadol-melatonin treated group, P3: significance between tramadol-melatonin treated group and controls.

Table (2) Biochemical parameters in different studied male groups:

Groups	Control-1	Control-2	Control-3	Tramadol	Tramadol and Melatonin	P Value	Post hoc
Serum tramadol (mg/l)	0.0	0.0	0.0	0.62±0.07	0.58±0.06	P=0.072	----------
UCH-L1 (G-MLN)	2.6±0.4	3±0.73	2.9±0.55	3.7±0.45	2.9±0.52	0.005	P1=0.415 P2=0.991 P3=0.783
ST (ng-mg)	2.2±.61	2.7±.63	3.7±1.08	10.4±.92	4.6±.83	P<0.001*	P1<0.001* P2<0.001* P3=0.156
NOR (pg-mg)	73.5±19.6	58.4±4.6	80.3±9.3	236.7±28.2	100.7±5	P<0.001*	P1=.496 P2<0.001* P3=0.114
MDA (nmol-mg)	0.32±0.07	0.48±0.08	0.67±0.12	3±1.1	0.87±0.32	P<0.001*	P1<0.001* P2<0.001* P3=0.302
Reduced GSH (ng-mg)	243.7±27.8	268.5±41.1	278.3±15.3	117.4±16.7	226.7±22.6	P<0.001*	P1<0.001* P2<0.001* P3= 0.7
SOD (u-mg)	246.8±8.1	218.9±85.2	275.4±7.4	128.7±30.8	228.4±20.9	P<0.001*	P1<0.001* P2<0.001* P3=0.904

Data are presented as mean± SD or median (range). UCH-L1: Ubiquitin C-terminal hydrolase L1, ST: serotonin, NOR: noradrenaline, MDA: malondialdehyde, GSH: glutathione, SOD: superoxide dismutase. P1: significance between controls and tramadol treated group, P2: significance between tramadol treated group and tramadol-melatonin treated group, P3: significance between tramadol-melatonin treated group and controls.

Table (3) Physical parameters and behavioral test parameters in different studied female groups:

Groups	Control-1	Control-2	Control-3	Tramadol	Tramadol and Melatonin	P Value	Post hoc
Physical parameters							
Body Weight (gm)	302.8±8.3	333.7±5.9	321.7±5.4	181.3±7.3*	238.7±13.9	P<0.001*	P1<.001* P2=0.149 P3=0.056
RBW%	24.4±.71	24.2±1.21	24.8±.45	14.6±.52	20.5±1.3	P<0.001*	P1<0.001* P2=0.261 P3=.084
Behavioral test parameters							
Speed (cm/s)	3.21 ± 0.19	3.51 ± 0.17	3.71 ± 0.2	0.91 ± 0.11	1.80 ± 0.12	< 0.001	P1<0.001* P2=0.041 P3=0.09
Total distance traveled (m)	7.62 ± 0.58	7.52 ± 0.5	7.41 ± 0.53	3.61 ± 0.33	5.23 ± 0.37	< 0.001	P1<0.001* P2=0.032 P3= 0.054
Number of line crossing	56.50 ± 7.36	54.50 ± 6.46	55.80 ± 7.56	25.70 ± 2.55	38.30 ± 5.73	< 0.001	P1<0.001* P2=0.041 P= 0.061
Number of immobile episodes	7(4–9)	8 (6–9)	7 (4–8)	17 (11 –18)	10 (7–12)	< 0.001	P1<0.001* P2=0.024 P= 0.062
Time in the peripheral zone (s)	246.65 ± 6.50	243.65 ± 6.40	245.65 ± 6.20	285.33 ± 4.07	256.84 ± 7.23	< 0.001	P1<0.001* P2=0.018* P= 0.082

Data are presented as mean± SD or median (range). RBW%: relative brain weight, P1: significance between controls and tramadol treated group, P2: significance between tramadol treated group and tramadol-melatonin treated group, P3: significance between tramadol-melatonin treated group and controls.

Female groups

Body weight and RBW% were comparedrable between all female studied control groups with non-significant difference among them. There was was statistically highly significant decrease in the body weight and RBW% in tramadol treated group compared with control groups and insignificant increase in tramadol-melatonin treated group comparing with the tramadol treated group, with an insignificant difference in the TR-melatonin treated group comparing with the control groups.

Behavioral test parameters were comparable between all studied control groups with a non-significant difference among them. There was a statistically greatly significant reduction in all behavioral test parameters in tramadol treated group compared to control groups. In the TR-melatonin treated group, all behavioral test parameters showed a significant increase comparing with TR treated group and there was an insignificant statistically difference comparing with control groups, as shown in **table (3).**

Serum tramadol, serum UCH-L1 levels, brain tissue serotonin, NOR, MDA, reduced GSH and SOD levels were comparable between all studied female control groups with a non-significant difference among them. Serum tramadol, serum UCH-L1 levels, and brain tissue serotonin, NOR, MDA levels showed a significant increase in the tramadol treated group compared to control groups and showed an insignificant decrease in tramadol-melatonin treated group comparing with TR treated female group, with an insignificant difference as compared to control groups. Meanwhile brain tissue reduced GSH and SOD levels detected a significant decline in the TR treated group comparing with control groups and showed an insignificant increase in tramadol-melatonin treated group comparing with tramadol treated group, with insignificant difference comparing with control group, as shown in **table (4).**

Comparison between male and female studied groups

There was an insignificant difference in all studied parameters between male and female control groups.

There was an insignificant difference in the body weight, RBW%, all behavioral test parameters and all studied biochemical parameters between male and female tramadol treated groups ($P > 0.05$), as shown in **table (5).**

Also, there was an insignificant difference in the body weight, RBW%, all behavioral test parameters and all studied biochemical parameters between male and female tramadol- melatonin treated groups, as detected in **table (6).**

Table (4) Biochemical parameters in female groups

Groups	Control-1	Control-2	Control-3	Tramadol	Tramadol and Melatonin	P Value	Post hoc
Tramadol (mg/l)	0.0	0.0	0.0	0.6±.06	0.56±.07	P= 0.059	----------
UCH-L1 (G-MLN)	2.4±.35	**2±.39**	2.5±.35	3.9±.5	2.6±.67	P<0.001*	P1<0.001* P2=0.010 P3=0.911
ST (ng-mg)	3.7±1.05	**2±.31**	1.65±.18	9.7±.96	4.3±1.2	P<0.001*	P1<0.001* P2<0.001* p3=0.501
NOR (pg-mg)	84.1±10.1	**67.59±19.4**	50±15.8	266.2±46.6	118.2±10.1	P<0.001*	P1<0.001* P2<0.001* p3=0.061
MDA (nmol-mg)	0.62±0.07	**0.6±0.1**	0.42±0.09	3.1±0.54	0.94±0.09	P<0.001*	P1<0.001* P2<0.001* P3=0.116
Reduced GSH (ng-mh)	264.8±16.3	**221.9±49.9**	212.6±44.6	107.4±5.5	209.1±12.4	P<0.001*	P1<0.001* P2<0.001* P3=0.999
SOD (u-mg)	241.7±47.3	**264.2±6.8**	254.6±9.2	107.7±7.3	214.8±9.1	P<0.001*	P1<0.001* P2<0.001 P5=0.889

Data are presented as mean± SD or median (range). UCH-L1: Ubiquitin C-terminal hydrolase L1, ST: serotonin, NOR: noradrenaline, MDA: malondialdehyde, GSH: glutathione, SOD: superoxide dismutase. P1: significance between controls and tramadol treated group, P2: significance between tramadol treated group and tramadol-melatonin treated group, P3: significance between tramadol-melatonin treated group and controls.

Table (5): Comparison of physical, behavioral test and biochemical parameters between male and female tramadol treated groups.

Groups	Males treated with Tramadol	Females treated with Tramadol	P value
Physical parameters			
Body Weight (gm)	216.7±21.2	181.3±7.3	P= 0.059
RBW%	16.9±1.3	14.6±.52	P= 0.157
Behavioral test parameters			
Speed (cm/s)	0.61 ± 0.11	0.91 ± 0.11	P=0.061
Total distance traveled (m)	2.61 ± 0.33	3.61 ± 0.33	P=0.134
Number of line crossing	20.70 ± 2.75	25.70 ± 2.55	P=0.221
Number of immobile episodes	16 (10 –17)	17 (11 –18)	P=0.342
Time in the peripheral zone (s)	296.33 ± 5.07	285.33 ± 4.07	P=0.763
Biochemical parameters			
Tramadol (mg/l)	0.62±.07	0.61±.06	P=0.373
UCH-L1 (g-mln)	3.7±.45	3.99±.5	P=0.296
ST (ng-mg)	10.4±.92	9.7±.96	P=0.157
NOR (Pg-mg)	236.7±28.2	266.2±46.4	P=0.148
MDA (nmol-mg)	3±1.1	3.16±.54	P=0.727
GSH (ng-mh)	117.4±16.7	107.3±7.3	P=0.130
SOD (U-mg)	128.7±30.8	107.7±7.3	P=0.082

Data are presented as mean± SD or median (range). RBW%: relative brain weight, UCH-L1: Ubiquitin C-terminal hydrolase L1, ST: serotonin, NOR: noradrenaline, MDA: malondialdehyde, GSH: glutathione, SOD: superoxide dismutase. p: significance between males and females treated with tramadol, p<0.05 is statistically significant.

Table (6): Comparison of physical, behavioral test and biochemical parameters between male and female tramadol–melatonin treated groups.

Groups	Tramadol-melatonin treated male group	Tramadol-melatonin treated female group	P value
Physical parameters			
Body Weight (gm)	276.7±24.4	238.7±13.9	P=0.078
RBW%	18.5±1.8	20.5±1.3	P=0.641
Behavioral test parameters			
Speed (cm/s)	1.50 ± 0.12	1.80 ± 0.12	P=0.865
Total distance traveled (m)	4.80 ± 0.37	5.23 ± 0.37	P=0.167
Number of line crossing	35.30 ± 5.83	38.30 ± 5.73	P=0.334
Number of immobile episodes	11 (7–13)	10 (7–12)	P=0.776
Time in the peripheral zone (s)	252.84 ± 9.23	256.84 ± 7.23	P=0.897
Biochemical parameters			
Tramadol (mg-l)	0.58±0.08	0.56±0.07	P=0.822
UCHL-1 (g-mln)	2.9±.52	3.2±.56	P=0.310
ST (ng-mg)	7±.81	6.1±1	P=0.065
NOR (Pg-mg)	160.7±19.8	176.6±15.8	P=0.101
MDA (nmol-mg)	0.87±.32	0.94±.09	P=0.573
GSH (ng-mh)	226.7±22.6	209.1±12.4	P=0.074
SOD (U-mg)	228.4±20.9	214.8±9.1	P=0.115

Data are presented as mean± SD or median (range). RBW: relative brain weight, UCH-L1: Ubiquitin C-terminal hydrolase L1, ST: serotonin, NOR: noradrenaline, MDA: malondialdehyde, GSH: glutathione, SOD: superoxide dismutase. p: significance between males and females treated with Tramadol-melatonin, $p<0.05$ is statistically significant.

5. Discussion

The chronic use of tramadol as an analgesic may be abused leading to psychological and physical manifestations such as CNS disturbances, headache, seizures, constipation, nausea, and vomiting ***(Subedi et al., 2019; Mraisel et al., 2021).***

This investigation demonstrated that the body weight and RBW% of tramadol-treated groups were statistically significantly lower than those of control groups, regardless of whether they were male or female, meanwhile in the tramadol-melatonin treated groups, body weight and RBW% showed an insignificant increase compared to the TR treated groups.

The outcomes of the current study agreed with ***Ibrahim and Salah-Eldin (2019*)** that showed that tramadol treated group had a lesser body weight compared to the control group. ***Mraisel et al. (2021)*** revealed a significant decline in the relative brain weight in the TR-treated group rats with rising the administration time comparing with the control group. ***Elsukary et al. (2022)*** observed that the last body weight significantly decreased in the tramadol abuse groups as compared to the controls.

Also, ***Paulis and Abbas (2015)*** *and* ***El-Mottaleb et al. (2019)*** in their studies showed a gradual reduction in the body weight of female rats treated with tramadol. In contrast**, *Drobnis et al. (2017)*** determined that Wistar rats treated with tramadol at doses of 40 mg/kg and 80 mg/kg for 8 weeks exhibited a significant rise in body weight in comparison to the control group.

Furthermore, ***Mohamed*** and ***Mahmoud (2019)*** in their research on adult male albino rates demonstrated that TR chronic

administration has been linked to increased body weight. One of the most significant consequences of misuse of opiates on the body is the impairment of the user's appetite and dietary habits, that may outcome in inconsistent dietary habits and poor nutrition, that may outcome in significant health issues, such as fluctuations in body weight ***(Balogun et al., 2020)***.

Constipation, nausea, and vomiting are among the most prevalent adverse reactions of misuse of opiates. These symptoms can result in an imbalance of electrolytes and a deficiency of nutrients, as well as a reduction in the user's appetite, which may make it challenging to maintain a healthy diet ***(FitzHenry et al., 2020)***. Fatigue is an additional consequence of misuse of opiates. This can reduce the user's metabolic rate and result in changes in dietary habits as a result of a loss of appetite ***(Balogun et al., 2020)***.

As regard behavioral test parameters, regardless male or female studied groups, a statistically greatly significant decline in behavioral test parameters in TR treated groups as compared to control groups with a significant increase of these tests in the tramadol-melatonin treated groups comparing with tramadol treated groups.

This agreed with ***Mowaad et al. (2023)*** who studied the tramadol effect on neurological function using the open field test and concluded that tramadol reduced ambulation significantly. Moreover, ***Elsukary et al. (2022*)** revealed that the open field testing in the control group showed superiority according all the tested parameters comparing with the TR treated group. It was also evident that tramadol adversely impacted all open field-test parameters (the total distance traveled, the speed, and the number of line crossings) as these parameters demonstrated a statistically significant decline in the TR group comparing with the control group.

This impact may be related to the anxiolytic activity of TR, which is controlled by its impact on monoamine and opioid levels **(Mowaad et al., 2023)**. Mental and emotional health abnormalities are linked to variations in neurotransmitters and the inflammatory condition ***(Mohamed and Mahmoud, 2019)***. In contrary**, *Ibrahim and Salah-Eldin (2019)*** reported that In contrast to the control animals, the tramadol-treated group revealed hyperactivity and elevated excitability, whereas the control animals behaved normally.

The results of the present study regardless in male or female studied groups showed a statistically greatly significant rise in serum UCH-L1 levels in TR treated groups comparing with the control groups and showed a significant decrease in serum UCH-L1 levels in tramadol-melatonin treated groups compared to tramadol treated groups.

This agreed with ***Abdel-Salam et al. (2019)*** who documented that the brain tissue UCH-L1 levels were significantly elevated following tramadol (five, ten, or twenty mg/kg) administration compared to the control group. The rise in brain UCH-L1 following repeated tramadol use recommends that this indicator might be a sensitive or an early neurotoxicity indicator.

Studies showed that UCHL-1 has several functions involving functioning as a ubiquitin ligase and maintaining mono-ubiquitin, that's essential for the degradation of harmed or misfolded proteins. UCHL-1 has the ability to restore synaptic function and contextual memory formation as a result of oxidation caused

by Aβ **(Gong et al., 2006)**. Therefore, the altered synaptic function may be a result of the down-regulated expression of UCHL-1 ***(Zhuo et al., 2012)***.

Moreover, UCH-L1 is abundant in the neuronal cell body, and an elevated release of this protein in the cerebrospinal fluid (CSF) or serum is indicative of neuronal injury ***(Roberts et al., 2015)***. The outcomes of the current research either in male or female studied groups showed a statistically highly significant increase in brain tissue serotonin (5-HT) and noradrenaline (NA) levels in the tramadol treated groups comparing with control groups and showed a significant decrease of brain tissue serotonin and noradrenaline levels in the TR-melatonin treated groups comparing with tramadol treated groups.

This agreed with ***Bloms-Funke et al. (2011)*** who reported tramadol time- and dosage-dependently raised extracellular 5-HT and NA levels in the ventral hippocampus in tramadol treated animals as compared to controls. Also, Bloms-Funke et al. (2011) detected that TR administration in animal models of pain, increased extracellular levels of, 5-HT and NA.

Earlier research done by ***Arakawa et al. (2019)*** demonstrated that tramadol administration increased the binding and functional evaluates at the 5-HT and the NA transporters. ***Hussein and Abdel Aal (2017)*** stated that through the activation of the μ-opioid receptor and the inhibition of the neuronal uptake of noradrenaline and serotonin, TR induces its analgesic effects through a minimum two complementary and synergistic mechanisms.

The current study's findings, regarding malondialdehyde (MDA) levels in male and female study groups, demonstrated a statistically significant rise in TR-treated groups in comparison to control groups and a significant decrease in tramadol-melatonin-treated groups comparing with TR-treated groups.

This agreed with ***Mraisel et al. (2021)*** who reported that MDA was significantly improved in the cerebrum of rats received either thirty or sixty mg/kg TR for eight weeks when compared with the control rats. ***Xia et al. (2020)*** also illustrated that the oxidative stress is a key factor in tramadol toxicity evidenced by the significant increase in the content of MDA in the brain tissue of the TR administered group as compared to the controls.

Also, ***Awadalla and Salah-Eldin (2016)*** documented a significant rise in serum MDA levels, the last metabolite of LPO chain, in the TR treated group comparing with control group. ***Assi (2016)*** reported that tramadol induces oxidative stress through free radicals. In addition to the high oxygen consumption with elevated polyunsaturated fatty acids (PUFA) levels, brains may be a target for free radicals, which exacerbates the oxidative stress experienced by nerve tissues. Elevated MDA is regarded as a valuable indicator of oxidative stress status, as it suggests a rise in the generation of free radicals ***(Awadalla and Salah-Eldin, 2016)***.

The findings of the current research, regardless of whether the study was conducted on male or female participants, demonstrated a statistically significant reduction in SOD and decreased GSH levels in the brain tissue in the TR-treated groups comparing with the control groups, and a significant rise in the TR-melatonin-treated groups comparing with the tramadol group.

In agreement with the present work, ***Ibrahim and Salah-Eldin (2019)*** showed that the SOD and reduced glutathione

levels declined significantly in the TR treated group comparing with the control group. Also, ***Adikwu and Bokolo (2017)*** documented that the tramadol administration caused a decrease in SOD and reduced GSH levels. Furthermore, melatonin administration resulted in a rise in SOD and declined GSH levels comparing with the TR treated group with no significant variance comparing with the control group.

The cortex's most significant free radical scavenger decreases glutathione. Consequently, its diminished concentration following tramadol administration may be attributed to its consumption in the process of scavenging the free radicals that are generated. Numerous neurodegenerative diseases have been documented to exhibit diminished levels of GSH. Consequently, the current deficiency in GSH content suggests that tramadol has induced neurodegeneration ***(Mowaad et al., 2023)***.

A transition metal was identified as a cofactor in antioxidant enzymes. The noticed inhibition of these enzymes could be explained by the interaction among TR and metals ***(Ismail et al., 2010).***

The results of the current research either in male or female studied groups as regard brain histopathological findings in the tramadol treated groups showed loss of organization in all layers of the cerebral cortex. The molecular layer exhibited pyknotic neurons that were encircled by haloes containing red neurons. Pyknotic granular cells with deeply stained nuclei were observed in the external granular layer. Pyramidal cells were pyknotic in the external pyramidal layer, with deeply stained nuclei and haloes surrounding them. Neuropil appears to be vacuolated. Granular cells with karyolytic nuclei were observed in the internal granular layer. In the internal pyramidal layer, pyramidal cells were shrunken and showed deeply stained pyknotic nuclei. Some neurons are pyknotic and surrounded by haloes. Vascular congestion and dilation, apoptotic cells, and focal acidophilic neuropil were also found.

These outcomes agreed with ***Elsukary et al. (2022)*** that showed that the pyramidal cells are shrunken with darkly stained nuclei in the tramadol-treated group. The granular cells were encircled by vacant areas and had diminished in size. Vascular congestion and apoptotic cells were additionally observed.

Khodeary et al. (2010) studied the impact of administration long-term tramadol on brain morphology and reported focal eosinophilic areas that were intensely stained, degenerated neurocytes, and dilated blood vessels, as well as disorganization of cortical layers. Additionally, neuronal cells in various brain regions exhibited red neurons (neurons with hypoxic changes) and shrunken neurons with pyknotic nuclei and scanty eosinophilic cytoplasm (apoptotic cells).

The potential mechanism of tramadol-induced harm to the brain is the reduction in the activities of Na+/K+-, Mg2+, and Ca2+-dependent ATPases in the rat brain. This decrease in activity leads to a reduction in ATP turnover and energy metabolism, as well as the loss of mitochondrial membrane transport functions (Chetan et al., 2007). Further, TR and/or its active metabolite may induce a high level of ROS, which can result in single- or double-strand DNA breaks and cell damage as a result of oxidative stress ***(Klaunig and Kamendulis, 2004)***.

In the current research after treating with melatonin either in male or female studied groups, the brain histopathological findings showed attenuated apoptosis, few acidophilic degenerated neurons, focal dark stained cellular and non-cellular deposits with foal area of neuropil vacuolations and focal gliosis.

Hashem (2018) reported that melatonin is a strong free radical scavenger that can relieve the toxicity of brain tissues, as they found that after melatonin treatment in studied animals, in ways that were similar to the control group, the large vesicular nucleus, prominent nucleoli, and basophilic Nissel's granules were present.

Also, ***Bekheet et al. (2023)*** documented that melatonin administration with tramadol attenuated cellular apoptosis, mitochondrial injury that occur with tramadol and preserved the synaptophysin content. Melatonin cellular protective effects were attributed to its ability to increase the anti -apoptotic gene expressions and to diminish pro-apoptotic gene expressions.

The results of the present study regardless in male or female studied groups as regard caspase-3 immuno-histochemical reaction, the control groups showed diffuse negative (-) reaction with minimal positive reaction for caspase-3, while tramadol treated groups showed a severe positive reaction (+++) to moderate (++) reaction and in tramadol and melatonin treated groups there was a focal mild positive reaction (+) to negative (-) reaction for caspase- 3.

This was in accordance with ***Ibrahim and Salah-Eldin (2019)*** who showed that casepase3 level increased markedly following TR administration in tissues comparing with the control group. ***Liu et al. (2013)*** stated that the activating opioid receptors can trigger the mRNA expression of pro-apoptotic receptors in the lymphocytes, spleen, lung, heart, and brain of rats. They noticed numerous apoptotic neurons in the hippocampus of these rats, as well as changes in the expressions of the apoptosis-related proteins, Fas, Bcl-2, and caspase-3.

Sharifipour et al. (2014) and ***Awadalla and Salah-Eldin (2015)*** confirmed that the chronic therapy of rats with opioids correlates with neuronal degeneration and apoptosis in the rat brain. This is accompanied by a significant upregulation of the pro-apoptotic Fas receptor, and also intracellular pro-apoptotic elements like caspase-3, and an opposite moderate downregulation of the anti-apoptotic oncoprotein Bcl-2.

6. Conclusion

The current research provided evidences that chronic TR administration in adult albino rats of both sexes resulted in decrease of the physical parameters (body weight and RBW%), alteration of behavioral test parameters, histological structures and function of the brain with increased UCH-L1, serotonin and noradrenaline levels and increasing the oxidative stress indices, evidenced by the histopathological changes and the increased casepase3 expression, and that all these results could be ameliorated by melatonin administration as antioxidant that improved brain morphology and function.

7. Recommendations

These guidelines are suggested contingent upon the findings of this investigation*:*

- Tramadol hydrochloride should not be used for a long time and should be used only under doctor's prescription with careful supervision since the prolonged daily therapy may result in

many toxic effects especially on the central nervous system.

- Patients with chronic pain and under tramadol therapy; should take the drug with the least effective dose and periodic examinations should be done for early detection of any toxic effects that might occur.
- In order to evaluate the risks and benefits of TR for managing pain, physicians have to be cognizant of its substantial abuse potential, toxic effects, and drug interactions.
- The potential of serum UCH-L1 to serve as important biomarkers or indicators that could indicate the early neurotoxicity of TR in humans might be suggested for additional study.
- It seems reasonable to recommend that melatonin might be a valuable adjuvant in tramadol therapy protocols as evidenced by its valuable role on the chronic tramadol brain toxicity.
- Additional molecular and pathophysiological researches are required to explain the mechanisms of the protection rules of melatonin against tramadol toxicity.

Conflicts Of Interest

The authors declare no conflict of interest.

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