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

Asmaa Mahmoud Meghawry*, Ibrahim Sadek Elgendy, Marcell Ramsis Haroun, Abdelmonem Goda Madboly, Eman Ibrahim Elgendy**

*Forensic Medicine and Clinical Toxicology Department, and pathology department**, Faculty of Medicine, Benha University, Egypt*

***Corresponding Author: Asmaa Mahmoud Meghawry**

Email: asma.omar87@gmail.com. 01278679214

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 study aimed to assess the chronic toxic effects of tramadol on the brains of adult albino rats of both sexes (males & 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 & 28 females) weighing between 180-200 gm. Rats were randomly divided into seven groups of 8 rats each: three control groups [negative, solvent & 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 centrally acting synthetic opioid that has proved to be highly effective in pain treatment. Its efficiency as analgesic ranges between weak opioid and morphine action ***(Mohamed and Mohamed, 2021).***

Tramadol is used as a worldwide analgesic. It has fewer side effects and better tolerability than oral nonsteroidal anti-inflammatory drugs (NSAIDs) or traditional opioids ***(Edinoff et al., 2021).***

Tramadol has been approved by the Food and Drug Administration (FDA) since 1995 for the management of pain ***(Manouchehri et al., 2022).***

Because of an increased incidence of tramadol related overdoses and deaths, it has been classified as a controlled substance in several countries ***(Nakhaee et al., 2021).***

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

The main side effects of tramadol treatment are nausea, vomiting, constipation, tachycardia, headache, ataxia, dizziness, drowsiness, somnolence, and loss of consciousness. Central nervous system is one of the most vulnerable systems to tramadol. Long-term tramadol abuse can significantly alter the structure and function of the brain. Tramadol abuse deteriorates nervous tissues including cerebral cortex and that symptoms of tramadol abuse are generated by neurodegeneration of this area ***(Aghajanpour et al., 2020).***

Melatonin (N-acetyl-5-methoxytryptamine; MT) is an endogenous indole amine, mainly secreted at night and plays a key role in regulating sleep. In addition, it is involved in numerous physiological functions, including antioxidant effects, anti-apoptotic activities, strengthening of the immune system, stopping the growth of cancer cells, and preventing aging

(Koohsari et al., 2020).

In the Egyptian community, tramadol abuse is considered an increasingly alarming phenomenon. The popularity and massive use of tramadol especially among Egyptian youth is alleged to be used for treatment of premature ejaculation also for the extension of orgasm & increase sexual pleasure. In Egypt, 20% to 40% of adults and 83% of adolescents with substance use disorders were found use tramadol. So, tramadol moved from schedule 3 to 1 by the Egyptian Ministry of Health as it is highly addictive substance. Tramadol was considered a very popular street drug in Cairo according to the information from the United Nations Office for Drugs and Crime (UNODC) that found 5 billion pills available for use in 2014 ***(Ahmed et al., 2018).***

2.AIM OF THE WORK

The purpose of this study was to assess the chronic toxic effects of tramadol on the brains of adult albino rats of both sexes (males & 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

The Research Ethics Committee at the Faculty of Medicine, Benha University (REC- FOMBU), Egypt, gave its approval to the experimental design study with approval number MS 20-2-2022.

3.1 Animals

A 56 healthy adult healthy albino rats of different sexes (28 males & 28 females) were used with an average body weight between 180 and 200 gm.

Before the experiment began, all the animals at the animal bread house at Benha Faculty of Veterinary underwent a week of passive preliminaries (consuming food and water without any drugs) to ensure their physical well-being and to weed out any ill animals. All the animals received the same food (Wheat, Bread & Milk). Medication administration for all animals was planned to start at noon. The animals were given ether anesthesia before being put to death 24 hours following the last treatment.

3.2 Chemicals

Sigma-Aldrich Chemical Co. sourced via EICI and HIMEDIA lab chemicals & 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 & 4 females"):*** left without intervention to measure the basic parameters, free access to food and distilled water were allowed during the whole period of the study.

2- ***Group Ⅱ (solvent control group): (n=8 rats "4 males & 4 females"):*** received a single oral daily dose of 0.5 ml distilled water by gavage tube for the whole period of study.

3- ***Group III (Melatonin-treated male group): (n=8 rats "4 males & 4 females"):*** treated with melatonin (10mg/kg/day) dissolved in distilled water orally by gastric gavage for 6 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 dissolved in distilled water orally by 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 dissolved in distilled water orally by gavage tube for 6 months according to ***Ghoneim et al. (2014).***

6- ***Group Ⅵ (Tramadol and melatonin treated male group): (n= 8 rats):*** treated with a single daily dose of melatonin (10 mg/kg/day), 30 minutes

after treatment with a single daily dose of tramadol (50 mg/kg/day) 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):*** treated with a single daily dose of melatonin (10 mg/kg/day), 30 minutes after treatment with a single daily dose of tramadol (50 mg/kg/day) 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 till the end of the experiment using 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 according to 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 subjected 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, constituted of the 4 central squares, and a periphery zone, constituted of the 12 surrounding 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) by reverse 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 using enzyme-linked immunosorbent assay (ELISA) kit from Glory Science Co., Ltd. (Del Rio, USA) using ELISA reader (photometer) (Ray Biotech, Canada) according to the manufacturer's instructions ***(Abdel-Salam et al., 2019).***

IV. Assessment of neurotransmitters in the brain tissues: The brain hemispheres were dissected out, 100 mg of brain tissue of each rat was homogenized in 1 ml of phosphate buffer saline and was stored overnight at -20°C. Serotonin and noradrenaline levels were assayed by a rat ELISA kits (Life Span BioSciences, LSBio Inc.), USA. Using ELISA reader (photometer) (RayBiotech, Canada) ***(Baldo, 2021).***

V. Assessment of oxidative stress markers: Superoxide dismutase (SOD), reduced glutathione (GSH), and malondialdehyde (MDA) were examined using Spectro-nanodrop in brain tissues using commercially available colorimetric methods (diagnostic kits supplied by Bio diagnostic Company, Egypt) following the manufacturer 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 picric acid, acetic acid and formaldehyde in an aqueous solution) which is used to fix organs that need good morphologic analysis. Tissue specimens were fixed for 6-8 hours and then transferred to 70% alcohol before submitting to histology for automated dehydration, paraffin embedding, sectioning and staining ***(Bancroft and Gamble, 2008).***

VII. Immunohistochemical (IHC) analysis for Caspase-3: IHC reactions were carried out using the streptavidin/peroxidase method ***(Happerfield et al., 1993).*** IHC staining for caspase-3 apoptosis was immunohistochemically localized using Caspase-3 antibodies with a species-unspecific rabbit polyclonal antibody (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 using SPSS [Statistical bundle for social science] version 26. The mean and standard deviation were added for quantitative data. Student's t-test was used to compare between mean of two groups of numerical (parametric) data, for continuous non-parametric data, Mann-Whitney U- test was used. The 0.05 threshold for significance was used in this study.

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 & RBW% were comparable between all male studied control groups with non-significant difference among them. There was a statistically highly significant decrease in the body weight & RBW% in tramadol treated group compared to control groups and insignificant increase in tramadol-melatonin treated group compared to the tramadol treated group. There was insignificant difference in the tramadol-melatonin treated group compared to the control groups.

Behavioral test parameters were comparable between all studied control groups with non-significant difference among them. There was a statistically highly significant decrease in all behavioral test parameters in tramadol treated group compared to control groups. In the tramadol-melatonin treated group, all behavioral test parameters showed a significant increase compared to tramadol treated group and there was insignificant statistically difference compared to control groups, as shown in **table (1)**

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

Groups	Control-1	Control-2	Control-3	Tramadol	Tramadol& 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.

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 showed a

significant increase in the tramadol treated group compared to control groups and showed a significant decrease in tramadol-melatonin treated group compared to 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 compared to control groups and showed an insignificant increase in tramadol-melatonin treated group compared to tramadol treated group, with insignificant difference as compared to control group, as shown in **table (2).**

Table (2) Biochemical parameters in different studied male groups

Groups	Control-1	Control-2	Control-3	Tramadol	Tramadol & 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.

Histopathological studies in different studied male groups

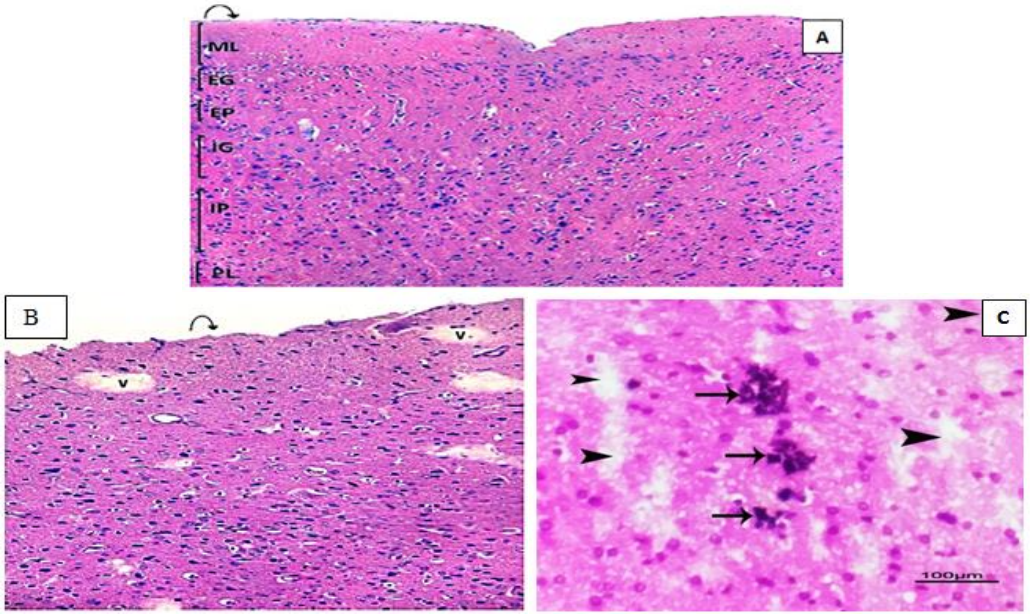

Fig (1) [A]- A photomicrograph of a section in the frontal lobe of cerebral cortex of an adult male control albino rat showing [A]: well-organized regularly arranged six layers from outer to inner surface: Molecular layer (ML), external granular (EG), external pyramidal (EP), internal granular (IG), internal pyramidal (IP) and polymorphic layer (PL). The pia mater covers the molecular layer (curved arrow), **(H & E × 100).**

[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 & E × 100). **(H & 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&E × 100).**

Immunohistochemical reaction for caspase-3 in different studied male groups

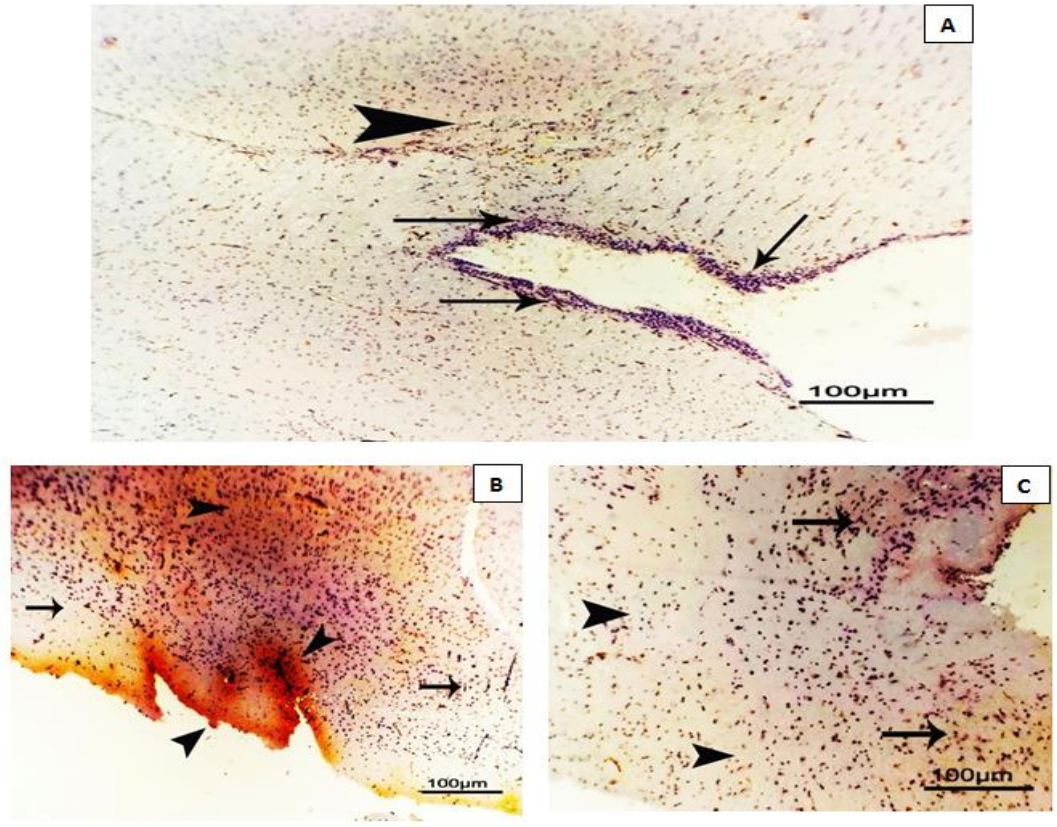

Fig (2) [A]- A Photomicrograph of peroxidase-stained section in the frontal lobe of cerebral cortex of an adult male control albino rat showing diffuse negative reaction (arrows) minimal positive reaction (arrow head) for caspase-3, **(IHC × 100)**.
[B]- A Photomicrograph of peroxidase-stained section 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)**.

Female groups

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

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

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

Groups	Control-1	Control-2	Control-3	Tramadol	Tramadol & 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.

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 compared to tramadol treated female group, with an insignificant difference as compared to control groups. Meanwhile brain tissue reduced GSH and SOD levels showed a significant decrease in the tramadol treated group compared to control groups and showed an insignificant increase in tramadol-melatonin treated group compared to tramadol treated group, with insignificant difference as compared to control group, as shown in **table (4).**

Table (4) Biochemical parameters in female groups

Groups	Control-1	Control-2	Control-3	Tramadol	Tramadol & 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.

Histopathological studies in different studied female groups

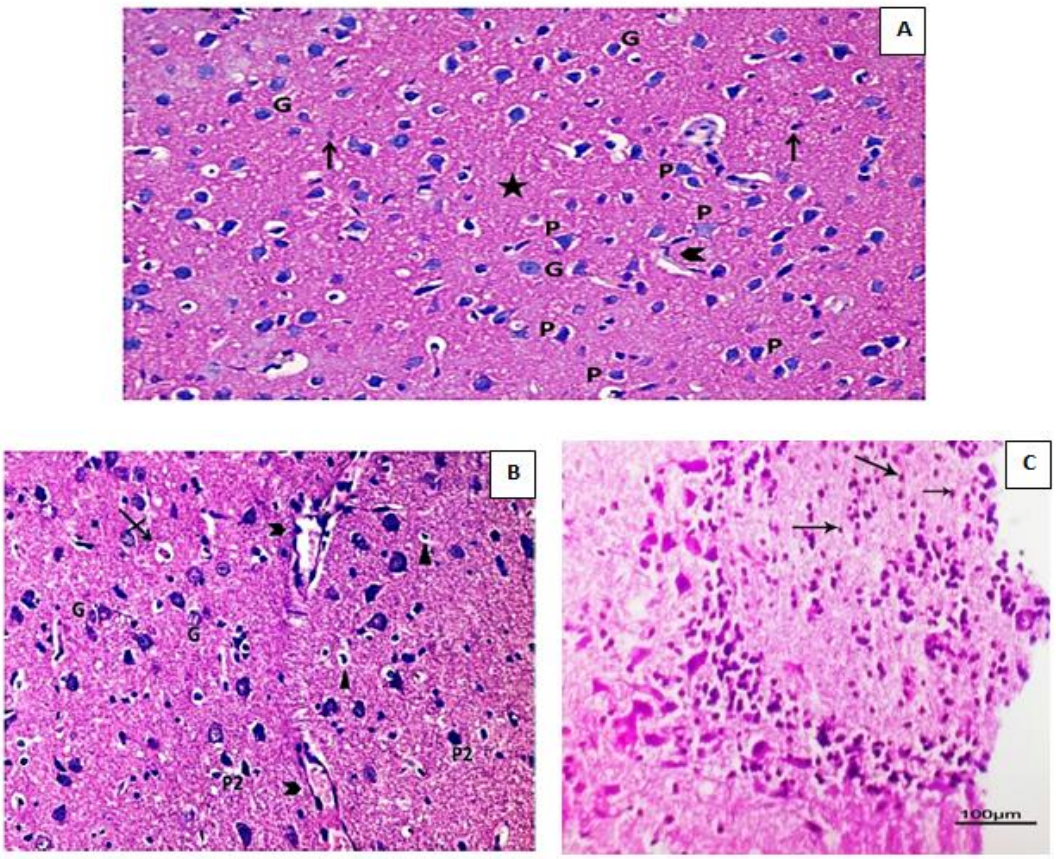

Fig (3) [A]- A photomicrograph of a section in the frontal lobe of cerebral cortex of an adult female control albino rat showing: well organized outer molecular layer, rounded granular cells (G) with open face nuclei in the internal granular layer. The internal pyramidal layer contains large sized pyramidal cells with triangular cell bodies, basophilic cytoplasm, rounded nuclei and long apical dendrites (P). Glial cells can be seen (thin arrows). Normally distributed neurons & neuroglia cells in the neuropil (star). Normal non congested blood vessel can be seen (arrow head) **(H & 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 & 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 & E× 100).**

Immunohistochemical reaction for caspase-3 in different studied female groups

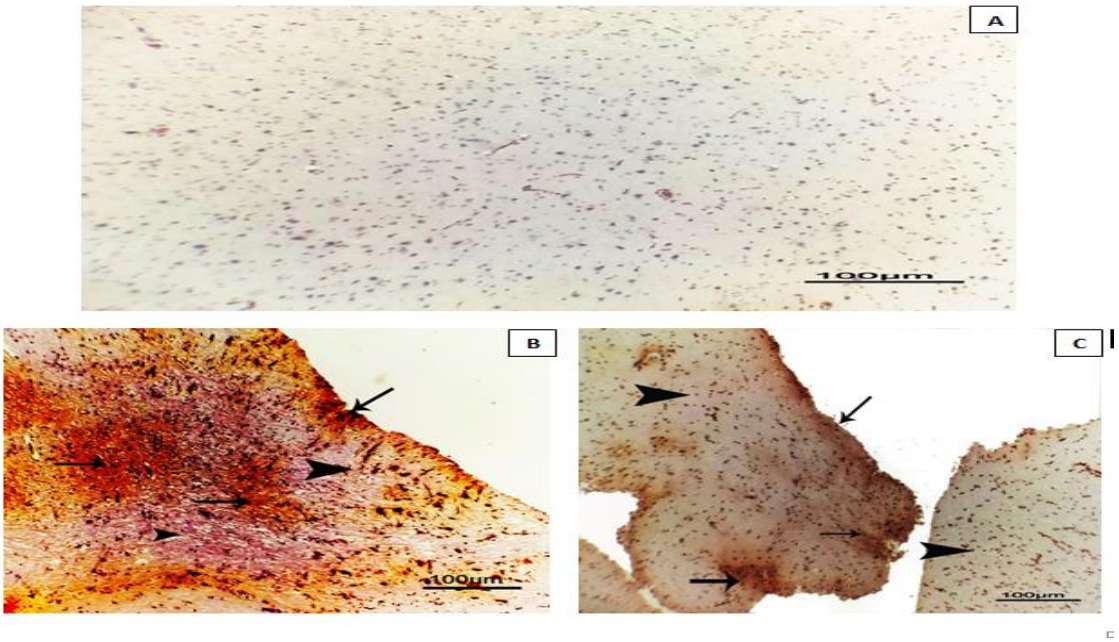

Fig (4): (A)- A Photomicrograph of peroxidase stained section in the frontal lobe of cerebral cortex of an adult female control albino rat showing negative reaction (-) for caspase-3, **(IHC × 100)**.
(B)- A Photomicrograph of peroxidase stained section in the frontal lobe of cerebral cortex of an adult 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)**.

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).**

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.

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 shown in **table (6).**

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.

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 study (either in male or female studied groups) showed that there was a statistically highly significant decrease in the body weight and RBW% in tramadol treated groups compared to control groups, meanwhile in the tramadol-melatonin treated groups, body weight and RBW% showed an insignificant increase compared to the tramadol treated groups.

The results of the present study agreed with ***Ibrahim and Salah-Eldin (2019*)** who showed that tramadol treated group had a lower body weight compared to the control group. ***Mraisel et al. (2021)*** revealed a significant decrease in the relative brain weight in the tramadol-treated group rats with increasing the time of administration compared to the control group. ***Elsukary et al. (2022)*** observed that the final body weight significantly declined in the tramadol misuse groups as compared to the controls.

Also, ***Paulis and Abbas (2015)*** *and* ***El-Mottaleb et al. (2019)*** in their studies showed a progressive decrease of body weight of female rats treated with tramadol. In contrast***, Drobnis et al. (2017)*** found that Wistar rats treated with tramadol at doses of 40 mg/kg and 80 mg/kg for 8 weeks experienced a significant increase in body weight as compared to the control group.

Furthermore, ***Mohamed and Mahmoud (2019)*** in their study on adult male albino rates found that tramadol chronic administration has been linked to increased body weight. One of the major effects of opiates abuse on the body is damage to the user's eating habits and appetite leading to irregular eating patterns and poor nutrition resulting in significant health problems which may include fluctuations in the body weight ***(Balogun et al., 2020)***.

Common side effects of opiate abuse include constipation, nausea, and vomiting. These symptoms can lead to a lack of nutrients and an imbalance of electrolytes and often decrease the user's appetite, making it difficult to maintain a healthy diet ***(FitzHenry et al., 2020)***. Another side effect of opiate abuse is fatigue. This can lower the user's

metabolic rate and causes changes in eating habits due to lack of appetite ***(Balogun et al., 2020)***.

As regard behavioral test parameters, either in male or female studied groups, there was a statistically highly significant decrease in behavioral test parameters in tramadol treated groups as compared to control groups with a significant increase of these tests in the tramadol-melatonin treated groups compared to 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 regarding all the tested parameters as compared to the tramadol treated group. It was also evident that tramadol negatively affected all open field-test parameters (the speed, the total distance traveled, and the number of line crossings) as these parameters showed a statistically significant decrease in the tramadol group compared to the control group.

This effect could be attributed to the anxiolytic activity of tramadol mediated by its effect on monoamine and opioid levels ***(Mowaad et al., 2023)***. The changes in neurotransmitters along with the inflammatory status are associated with mental and emotional health abnormalities ***(Mohamed and Mahmoud, 2019)***. In contrary**, *Ibrahim and Salah-Eldin (2019)*** reported that tramadol treated group appeared with hyperactivity and increased excitability when compared to control animals, which exhibited a normal behavior.

The results of the present study either in male or female studied groups showed a statistically highly significant increase in serum UCH-L1 levels in tramadol treated groups compared to the control groups and showed a significant decrease in serum UCH-L1 levels in tramadol-melatonin treated groups compared to tramadol treated groups.

This was in accordance with ***Abdel-Salam et al. (2019)*** who reported that the brain tissue levels of UCH-L1 were significantly increased after tramadol (5, 10, or 20 mg/kg) administration compared to the control group. The increase in brain UCH-L1 after repeated tramadol use suggests that this biomarker could be a sensitive or an early indicator of neurotoxicity.

Studies showed that UCHL-1 has several functions including acting as a ubiquitin ligase and stabilizing mono-ubiquitin, which are crucial to the degradation of damaged or misfolded proteins. UCHL-1 can recover synaptic function and contextual memory formation from Aβ- induced oxidation ***(Gong et al., 2006)***. Hence, the down-regulated expression of UCHL-1 may contribute to altered synaptic function ***(Zhuo et al., 2012)***.

Moreover, UCH-L1 is abundant in neuronal cell body and increased release of this protein in CSF or serum therefore reflects neuronal damage ***(Roberts et al., 2015)***. The results of the present study 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 compared to control groups and showed a significant decrease of brain tissue serotonin and noradrenaline levels in the tramadol-melatonin treated groups compared to tramadol treated groups.

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

A previous study done by ***Arakawa et al. (2019)*** showed that tramadol administration increased the binding and functional assays at the 5-HT and the NA transporters. ***Hussein and Abdel Aal (2017)*** stated that tramadol exerts its analgesic effect through at least two complementary and synergistic mechanisms; the first is by activating the μ-opioid receptor, and the second is by inhibition of the neuronal uptake of

noradrenaline and serotonin.

The results of the present study either in male or female studied groups and as regard malondialdehyde (MDA) levels showed a statistically highly significant increase in tramadol treated groups compared to control groups and showed a significant decrease in tramadol-melatonin treated groups compared to tramadol treated groups.

This agreed with ***Mraisel et al. (2021)*** who reported that MDA was significantly increased in the cerebrum of rats received either 30 or 60 mg/kg tramadol for 8 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 tramadol administered group as compared to the controls.

Also, ***Awadalla and Salah-Eldin (2016)*** reported a significant increase in serum MDA levels, the last metabolite of LPO chain, in the tramadol treated group as compared to control group. ***Assi (2016)*** reported that tramadol induces oxidative stress through free radicals. Brains can be a target to the free radicals, besides the high oxygen consumption and high polyunsaturated fatty acids (PUFA) levels present, that makes nerve tissues suffer even more from oxidative stress. Elevated MDA indicates an increase of free radical generation, and it is considered a useful measure of oxidative stress status ***(Awadalla and Salah-Eldin, 2016)***.

The results of the present study either in male or female studied groups as regard superoxide dismutase (SOD) and reduced glutathione (GSH) levels in the brain tissue showed a statistically highly significant decrease in tramadol treated groups compared to control groups and showed a significant increase in tramadol-melatonin treated groups compared to tramadol group.

In agreement with the present work, ***Ibrahim and Salah-Eldin (2019)*** showed that the SOD and reduced glutathione levels decreased significantly in the tramadol treated group compared to the control group. Also, ***Adikwu and Bokolo (2017)*** reported that the administration of tramadol resulted in a decrease in SOD and reduced GSH levels. Furthermore, melatonin administration resulted in an increase in SOD and reduced GSH levels compared to the tramadol treated group with no significant difference compared to the control group.

Reduced glutathione is the most important free radical scavenger in the cortex. Thus, its reduced level after the administration of tramadol could be due to its consumption in scavenging the produced free radicals. The reduced level of GSH has been recorded in many neurodegenerative diseases. Therefore, the present deficiency in GSH content indicates the induction of neurodegeneration by tramadol ***(Mowaad et al., 2023)***.

Antioxidant enzymes were found to contain a transition metal as a cofactor. The interaction between tramadol and metals of these enzymes may interpret why these enzymes inhibition observed ***(Ismail et al., 2010).***

The results of the present study either in male or female studied groups as regard brain histopathological findings in the tramadol treated groups showed loss of organization of all layers of cerebral cortex. The molecular layer showed pyknotic neurons surrounded by haloes with red neurons. The external granular layer showed pyknotic granular cells with deeply stained nuclei. In the external pyramidal layer, pyramidal cells appeared pyknotic with deeply stained nuclei and surrounded by haloes. Neuropil appears vacuolated. The internal granular layer showed granular cells with karyolytic nuclei. In the internal pyramidal layer, pyramidal cells were shrunken & 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 results were in agreement with ***Elsukary et al. (2022)*** who showed that in the tramadol treated group, the pyramidal cells are shrunken with darkly stained nuclei. The granular cells had reduced in size and were surrounded by vacant areas. Apoptotic cells were also seen and vascular congestion.

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

The possible mechanism of tramadol induced brain damage is the decrement in the rat brain activities of Na+/K+-, Mg2+ and Ca2+- dependent ATPases with subsequent decrease in ATP turnover and energy metabolism as well as loss of mitochondrial membrane transport functions ***(Chetan et al., 2007)***. In addition, tramadol and/or its active metabolite may produce excessive release of ROS leading to single- or double-strand DNA breaks and cell damage as a result of oxidative stress ***(Klaunig and Kamendulis, 2004)***.

In the present study after treatment 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, there was a large vesicular nucleus, prominent nucleoli, and basophilic Nissel's granules in a comparable way to the control group.

Also, ***Bekheet et al. (2023)*** reported that administration of melatonin 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 either 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 & 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 after tramadol administration in tissues compared to the control group. ***Liu et al. (2013)*** stated that opioids may induce the mRNA expression of pro-apoptotic receptors in the lymphocytes, spleen, lung, heart, and brain of rats through the activating opioid receptors. They observed many apoptotic neurons in the hippocampus of these rats, and alteration 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 chronic treatment of rats with opioids is associated with neuronal degeneration and apoptosis in the rat brain with a remarkable upregulation of the pro-apoptotic Fas receptor, as well as intracellular pro-apoptotic elements such as caspase-3, combined with an opposing moderate downregulation of the anti-apoptotic oncoprotein Bcl-2.

6. CONCLUSION

The present study provided evidences that chronic tramadol 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

Depending on the results of this study, the following guidelines are recommended*:*

- 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 side and adverse 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 side effects that might occur.
- Physicians must be aware of tramadol adverse effects, substantial abuse potential, and drug interactions to weigh its risk-benefits for pain management.
- The utility of serum UCH-L1 as valuable markers or indicators that could reflect early neurotoxicity of tramadol in humans could be recommended for further research.
- 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 studies are required to clarify the mechanisms of the protection rules of melatonin against tramadol toxicity.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENT

Our deep appreciation to the staff members of Forensic Medicine and Clinical Toxicology, Faculty of medicine, Benha University. https://bu.edu.eg/

REFERENCE

1. ***Abdel-Salam, O.; Youness, E. R.; Mohammed, N. A.; and et al., (2019):*** Citicoline protects against tramadol-induced oxidative stress and organ damage. Reactive Oxygen Species J., *7* (20):106-120.
2. ***Abdel-Wahhab, M. A.; Abdel-Galil, M. M. & El-Lithey, M. (2005):*** Melatonin counteracts oxidative stress in rats fed an ochratoxin A contaminated diet. Pineal Res J., 38, 130-5.
3. ***Adikwu, E. and Bokolo, B. (2017):*** Prospects of N-acetylcysteine and melatonin as treatments for tramadol-induced renal toxicity in albino rats. Pharmaceutical SciencesJ., 23 (3): 172-181.
4. ***Aghajanpour, F.; Boroujeni, M.E.; Jahanian, A.; and et al. (2020):*** Tramadol: a potential neurotoxic agent affecting prefrontal cortices in adult male rats and PC-12 cell line. Neurotoxicity research J., 38, 385-397.
5. ***Ahmed, A. I.; El-Dawy, K.; Fawzy, M. M.; Aand et al. (2018):*** Retrospective review of tramadol abuse. Slov Vet Res J., 55 (20): 471-483.
6. ***Arakawa, R.; Takano, A. and Halldin, C. (2019):*** Serotonin and norepinephrine transporter occupancy of tramadol in nonhuman primate using positron emission tomography. International Journal of Neuropsychopharmacology., 22 (1): 53-56.
7. **Assi, M. (2016):** The Impact of Physical Activity and Antioxidants on Tumor-Skeletal Muscle Crosstalk during Cancer: Deciphering Signaling Pathways Involved in Tumor Growth and Muscle Wasting Université Rennes 2.
8. ***Awadalla, E. A. and Salah-Eldin, A.E. (2015):*** Histopathological and molecular studies on tramadol mediated hepato-renal toxicity in rats. Pharm Biol Sci J., 10 (6): 90-102.
9. ***Baldo, B.A. (2021):*** Toxicities of opioid analgesics: respiratory depression, histamedine release, hemodynamic changes, hypersensitivity, serotonin toxicity. Archives of toxicology J., 95 (8): 2627-2642.
10. ***Balogun, S. K.; Osuh, J. I.; and Onibokun, O. O. (2020):*** Effects of Separate and Combined Chronic Ingestion of Codeine and Tramadol on Feeding Behaviour of Female Albino

Rats. Journal of Medical Research and Health Sciences., 3 (7): 1009−1020. doi: 10.15520/jmrhs.v6i7.220.

11. ***Bancroft, J.D. and Gamble, M. (eds.) (2008):*** Fixatives. In: Theory and Practice of Histology Techniques. Churchill Livingstone, Elsevier, China, 8th ed., chapter (10), 72-75.
12. ***Bekheet, E.A. (2023):*** Role of Melatonin in Ameliorating the Harmful Effects of Tramadol on the Frontal Cortex of Adult Male Albino Rat (Histological and Immunohistochemical study). Egyptian Journal of Histology,1-42.
13. ***Bloms-Funke, P.; Dremencov, E.; Cremers, T. I.; and et al. (2011):*** Tramadol increases extracellular levels of serotonin and noradrenaline as measured by in vivo microdialysis in the ventral hippocampus of freely-moving rats. Neurosci Lett J., 490 (3):191-195. doi: org/10.1016/j.neulet.2010.12.049.
14. ***Chetan, P.; Ramakrishna, B.; Reddanna, P.; and et al. (2007):*** Tramadol effects on the activity levels of ATPases in mitochondrial fractions of rat brain areas during non-induction of pain. International Pharmacology J., 3, 341-346.
15. ***Drobnis, E. Z.; Nangia, A. K.; Drobnis, E. Z.; and et al. (2017):*** Pain medications and male reproduction. *Impacts of Medications on Male Fertility*, 39-57.
16. ***Edinoff, A.N.; Kaplan, L.A.; Khan, S.; and et al. (2021):*** Full opioid agonists and tramadol: pharmacological and clinical considerations. Anesthesiology and Pain MedicineJ., 11(4). doi: 10.5812/aapm.119156.
17. ***El-Mottaleb, A.; Ahmed, H. A.; Mahmoud, S. F.; and et al. (2019):*** Effects of Chronic Use of Tramadol on Uterus and Ovary of Albino Rats. The Egyptian Hospital Medicine J., 76 (1): 3184-3190.
18. ***Elsukary, A. E.; Helaly, A. M.; El Bakary, A. A.; and et al. (2022)****:* Comparative study of the neurotoxic effects of pregabalin versus tramadol in rats. neurotoxicity researc J., 40 (5): 1427-1439.
19. ***Elsukary, A. E.; Helaly, A. M.; El Bakary, A. A.; and et al. (2022):*** Comparative study of the neurotoxic effects of pregabalin versus tramadol in rats. neurotoxicity researc J., 40 (5): 1427-1439.
20. ***FitzHenry, F.; Eden, S. K.; Matheny, M. E; and et al. (2020):*** Prevalence and risk factors for opioid-induced constipation in an older national veteran cohort. Pain Research and Management J., 1-11.
21. ***Ghoneim, F.M; Khalaf, H.A; Elsamanoudy, A.Z; and et al. (2014):*** Effect of choronic usage of tramadol on motor cerebral cortex and testicular tissues of adult male albino rats and the effect of its withdrawal: histological, immunohistochemical and biochemical studt. Int Clin Exp Pathol J., 7 (11):7323-41.
22. ***Gong, B.; Cao, Z.; Zheng, P.; and et al. (2006):*** Ubiquitin hydrolase UCH-L1 rescues β-amyloid-induced decreases in synaptic function and contextual memory. Cell J., 126 (4): 775-788.
23. ***Happerfield, L.c.; Boborow, L.g.; Bains, R.M.; and et al. (1993):*** Peroxidase labeling immunocytochemistry: a comparison of eleven commercially available avidine-biotin systems. British journal of biomedical science., 50 (1): 21-26.
24. ***Hashem, H. (2018):*** The possible protective role of melatonin on the changes in the cerebral cortex and meninges of streptozotocin-induced diabetes in adult male albino rats (histological and immunohistochemical study). Egyptian Journal of Histology., 41(4): 533-545.
25. ***Hussein, O. A.; Abdel Mola, A. F.; and Rateb, A. (2020):*** Tramadol administration induced hippocampal cells apoptosis, astrogliosis, and microgliosis in juvenile and adult male mice, histological and immunohistochemical study. Ultrastructural Pathology J., 44 (1): 81-102.
26. ***Hussein, S. A. and Abdel Aal, S. A. L. (2017):*** Neurodegeneration and oxidative stress induced by tramadol

administration in male rats: the effect of its withdrawal. Benha Veterinary Medical Journal., 33 (2): 149-159.

27. ***Ibrahim, M. A. and Salah-Eldin, A. E. (2019):*** Chronic addiction to tramadol and withdrawal effect on the spermatogenesis and testicular tissues in adult male albino rats. *Pharmacology* J., 103 (4): 202-211. doi:org/10.1159/000496424.
28. ***Ismail, M.; Al-Naqeep, G. and Chan, K. W. (2010):*** Nigella sativa thymoquinone-rich fraction greatly improves plasma antioxidant capacity and expression of antioxidant genes in hypercholesterolemic rats. Free radical biology and medicine J., 48 (5): 664-672.
29. ***Khalil, A.M.; Mowas, A.S.; Hassan, M.H.; and et al. (2022):*** Neurodegeneration and Oxidative Stress in Brain Tissues Induced by Tramadol with the Protective Effects of Royal Jelly in Albino Rats. Med Pharm. Sci, Saudi J., 8.
30. ***Khodeary, M. F.; Sharaf El-Din, A. A. and El Kholy, S. (2010):*** A histopathological and immunohistochemical study of adult rats' brain after long-term exposure to amadol (tramadol hydrochloride). Mansoura Journal of Forensic Medicine and Clinical Toxicology J., 18 (1): 1-24.
31. ***Klaunig, J. E. and Kamendulis, L. M. (2004):*** The role of oxidative stress in carcinogenesis. Annu. Rev. Pharmacol. Toxicol J., 44, 239-267.
32. ***Koohsari, M.; Ahangar, N.; Mohammadi, E.; and et al., (2020):*** Ameliorative Effect of Melatonin Against Reproductive Toxicity of Tramadol in Rats via the Regulation of Oxidative Stress, Mitochondrial Dysfunction, and Apoptosis-related Gene Expression Signaling Pathway. Addict Health J., 12 (2): 118-129. doi: org/10.22122/ahj.v12i2.265.
33. ***Liu, L.W.; Lu, J.; Wang, X.H.; and et al. (2013):*** Neuronal apoptosis in morphine addiction and its molecular mechanism. International journal of clinical and experimental medicine., 6 (7): 540.
34. ***Manouchehri, A.; Nekoukar, Z.; Malakian, A.; and et al. (2022):*** Tramadol poisoning and its management and complications: a scoping review. Annals of Medicine and Surgery J., 10-1097.
35. ***Mohamed, H.M. and Mahmoud, A.M. (2019):*** Chronic exposure to the opioid tramadol induces oxidative damage, inflammation and apoptosis, and alters cerebral monoamine neurotransmitters in rats. Biomedicine & Pharmacotherapy J., 110, 239-247.
36. ***Mowaad, N. A.; El-Shamarka, M. E.; and Khadrawy, Y. A. (2023):*** The behavioral and neurochemical changes induced by boldenone and/or tramadol in adult male rats. Neurochemical Research J., 48 (5): 1320-1333.
37. ***Mraisel, A. C. Ibrahim, A.H. and Dawood, M. (2021):*** Histopathological changes in brain tissues associated with oral administration of tramadol in male rats. Indian Journal of Forensic Medicine & Toxicology J., 15 (4): 1032-1039.
38. ***Nakhaee, S.; Farrokhfall, K.; Miri-Moghaddam, E.; and et al. (2021):*** The effects of naloxone, diazepam, and quercetin on seizure and sedation in acute on chronic tramadol administration: an experimental study. Behavioral and Brain Functions J., 17 (1): 5.
39. ***Owusu-Afriyie, O.; Quayson, S. E.; Acheampong, E.; and et al. (2018):*** Expression of immunohistochemical markers in non-oropharyngeal head and neck squamous cell carcinoma in Ghana. PloS one J., 13 (8): e0202790.
40. ***Paulis, M. G. and Abbas, M. F. (2015):*** Tramadol subchronic toxicity on pituitary-gonadal axis and ovarian functions in adult female rats. The Egyptian Journal of Forensic Sciences and Applied Toxicology, 15 (1): 67-77.
41. ***Rahimi, H. R.; Soltaninejad, K. and Shadnia, S. (2014):*** Acute tramadol poisoning and its clinical and laboratory findings. Res Med Sci J., 19 (9): 855-859.
42. ***Roberts, R. A.; Guilarte, T. R.; Hanig, J. P.; and et al. (2015):***

Translational biomarkers of neurotoxicity: a health and environmental sciences institute perspective on the way forward. Toxicological Sciences J., 148 (2): 332-340.

43. ***Sharifipour, M., Izadpanah, E.; Nikkhoo, B.;and et al. (2014):*** A new pharmacological role for donepezil: attenuation of morphine-induced tolerance and apoptosis in rat central nervous system. Journal of biomedical science., 21 (1): 1-9.

11): 3351-3364.

44. ***Xia, W.; Liu, G.; Shao, Z.; and et al., (2020):*** Toxicology of tramadol following chronic exposure based on metabolomics of the cerebrum in mice. Scientific reports J., 10 (1): 11130.

45. Zhuo, H.Q.; Huang, L.; Huang, H.Qand et al. (2012): Effects of chronic tramadol exposure on the zebrafish brain: a proteomic study. Journal of proteomics., 75