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Biochemical and Histopathological Alterations Induced by Tramadol

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Abstract

Background: Drug addiction is a serious public health concern. Tramadol addiction and dependence have been documented in recent years, most commonly in young adults, making tramadol use a significant health concern. The study investigated the long-term effects of tramadol intoxication on the γ-aminobutyric acid (GABA) system and tricarboxylic acid cycle enzymes in the brains of rats, focusing on regions with a high number of GABAergic neurons.

Methods: In this animal study, three treatment groups of adult male rats were considered. Rats were divided into three treatment groups: control no tramadol was given, Gp 25 mg/Kg tramadol was given 25 mg/kg for one month by oral gavage, and Gp 50 mg/Kg tramadol was given 50 mg/kg for one month by oral gavage for one month, and the enzyme activities for GABA transaminase (GABA-T), succinic semialdehyde dehydrogenase (SSA-DH), succinate dehydrogenase (SDH), and isocitrate dehydrogenase (IDH) were measured using ELISA kits, on brain tissue samples from the cerebellum, brain stem, cerebral cortex, thalamus, and hypothalamus. Histopathological analysis of the cerebral cortex was conducted using hematoxylin-eosin and Nauta silver staining. Statistical analysis for GABA shunt enzymes and tricarboxylic acid cycle enzymes was conducted using one-way ANOVA followed by Tukey's multiple means comparisons.

Results: Tramadol significantly ($P < 0.05$) reduced the levels of GABA-T, SSA-DH, and IDH enzymes across various brain regions, with the most pronounced reductions observed in the brain stem and hypothalamus. In contrast, SDH enzyme levels remained largely unchanged in most regions. Additionally, structural changes in the brain were noted, including vascular congestion, neuronal degeneration, and disruption of cortical layers. These alterations were more severe in the high-dose group, suggesting that higher doses of Tramadol may lead to more extensive brain damage.

Conclusion: Tramadol exposure was found to cause biochemical and histopathological alterations in the nervous tissue through impairment of GABA metabolism.

Keywords: Tramadol, Addiction, GABA metabolism, Cerebral cortex

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Introduction

Drug abuse is a major public health issue in low- and middle-income countries. with the expected prevalence to rise by 43% by 2030, according to estimates from the United Nations Office on Drugs and Crime (UNODC) (1).

Drug abuse develops after prolonged and consistent use of drugs, progressing from occasional and recreational use to more regular use, and finally to persistent use characteristic of addiction. The development of a drug use disorder

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↑What is "already known" in this topic:
Drug addiction is a serious public health concern. Although tramadol is a useful tool for managing pain, there is a growing understanding of its significant risk for abuse and dependency, which was initially underestimated because of its different mechanisms of action from other opioids. Additionally, it also induces seizure at high doses, which may result from different mechanisms.

→What this article adds:
Tramadol exposure was found to cause biochemical and histopathological alterations in the nervous tissue through impairment of GABA metabolism.

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has severe consequences for both mental and physical health (2).

A number of well-known molecular mechanisms are known to underlie the actions of drugs of abuse, which include effects on neurotransmitter systems, neuronal plasticity of reward circuits, down- or up-regulation of key receptors or transporters, modifications to membrane permeability or ion conductance, and feedback-mediated inhibition of endogenous opioids and peptides (3).

These changes not only contribute to addiction and dependence but also many of the adverse consequences of chronic drug use, including opioids (4).

A common synthetic prescription medication for moderate to severe pain is tramadol. Tramadol produces analgesia through a number of mechanisms, which are thought to make it safer than "pure" opioid analgesics, at least with respect to abuse and dependence (5).

Tramadol is an agonist of the μ opioid receptor and the GABA-A receptor, but an antagonist of N-methyl-D-aspartate (NMDA) glutamate receptors and GABA-A receptors at high concentrations while also inhibiting the re-uptake of serotonin and norepinephrine, among other mechanisms (6).

Based on actions at the μ opioid receptor and monoamine transporters, tramadol is used to treat post-operative pain in orthopedic patients (7).

Although tramadol is a useful tool for managing pain, there is a growing understanding of its significant risk for abuse and dependency, which was initially underestimated because of its different mechanisms of action from other opioids. Additionally, it also induces seizure at high doses, which may result from GABA inhibition (8, 9).

Several studies documented the main side effects of tramadol resulting from chronic use or acute intoxication, including nephrotoxicity, hepatotoxicity, testicular toxicity, and neurotoxicity, as well as adverse effects on many other systems (10-12).

One of the most susceptible systems to tramadol is the central nervous system, as the brain structure and function can be profoundly altered by long-term tramadol use, including degeneration of the cerebral cortex (13-15).

Neurotoxicity has been observed in 8–81% of tramadol overdose cases, suggesting that this may be a consequence of acute tramadol actions at high doses, something that may become more likely in cases of abuse and tolerance. These neurotoxic effects likely result from the robust inhibition of GABA-A receptors at high tramadol doses that are associated with seizure (16, 17).

GABA is the main inhibitory neurotransmitter in the central nervous system and, as such, is a critical regulator of brain excitability (18). GABA is generated in a metabolic mechanism called the GABA shunt via glutamate decarboxylation by the glutamate decarboxylase (GAD) enzyme in the cytosol (19).

GABA is metabolized in mitochondria by GABA transaminase (GABA-T) into succinic semialdehyde (SSA) and then into succinate (Suc) via succinate-semialdehyde dehydrogenase (SSA-DH). Suc enters the tricarboxylic acid (TCA) cycle, a crucial mechanism for producing cellular energy and neurotransmitter regulation (20).

The TCA cycle is regulated by citrate synthase, isocitrate dehydrogenase (IDH), α-ketoglutarate (αKG) dehydrogenase, and succinate dehydrogenase (SDH) (21).

A previous study on rats treated with morphine revealed that the activity of GABA-T, SSA-DH, SDH, and IDH enzymes, and thus GABA catabolism, can be significantly disrupted by morphine intoxication (22).

In our previous study, morphine intoxication evoked a significant increase in GABA-shunt activity in the thalamus and brain stem, followed by activation of TCA cycle enzymes. These effects were brain-region dependent as those enzymes were inhibited in the cerebellum, suggesting that chronic morphine exposure may exacerbate both GABA shunt and TCA cycle turnover in the region (23). Another study observed neuronal degeneration resulting from long-term tramadol exposure in rats, which was linked to the accumulation of oxidative stress intermediates in the brain (24).

These histopathological changes highlight the importance of examining the toxic mechanisms of Tramadol that may involve alterations in GABA metabolism and activity. Therefore, this study was designed to investigate the long-term effects of tramadol intoxication on the GABA shunt and TCA cycle enzymes in rats, focusing on regions with abundant GABAergic neurons (cerebellum, brain stem, cerebral cortex, thalamus, and hypothalamus).

Methods

Animals

This experimental study used twenty-four Sprague Dawley male rats at the age of 21-30 days, weighing 150 g, and was housed in the Department of Laboratory Animal Resources, Applied Science Private University, Amman, Jordan. Pairs of rats were housed in a standard plastic cage and had free access to food and water throughout the experiment. The room temperature was maintained at 21°C and 50% humidity with a 12-hour light-dark cycle.

Experimental design

Three groups of eight rats were used in this study in order to induce chronic intoxication, receiving Tramadol by oral gavage (0, 25, or 50 mg/kg PO, daily for 30 days) (25, 26).

At the end of the experiment, one hour after the final tramadol dose, the animals were euthanized using isoflurane followed by cervical dislocation, and brains were collected; eight brains from each group were snapped frozen and then transferred to -80°C, for further analysis. The remaining two brains from each group were fixed in 9% neutral buffered formol for histochemical analysis.

Isolation of the brain and determination of biochemical indicators

The frozen eight brains from each group samples from the thalamus, hypothalamus, cerebral cortex, brainstem, and cerebellum were identified and isolated according to the Rat Brain Atlas (27).

The other two brains from each group were fixed in 9% neutral buffered formol, dehydrated in ascending grades of alcohol, clearing in xylol, prior to processing for histochemical analysis by paraffin embedding, sectioning

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through microtome (4-6 µm), and staining of alternate sections with either hematoxylin (Hx) and Eosin (E) for general structural changes or Nauta silver stain for detection of degenerative changes in axonal fibers.

Protein quantification

Following the identification and isolation of brain regions, all samples from the same region and group were pooled and stored frozen at -80 C°, creating three different samples for each region. Tissues of the brain regions were used to prepare homogenates for determining the level of GABA-T, SDH, IDH, SSA-DH, and protein content.

Pooled samples were homogenized using a glass homogenizer with a Teflon pestle in chilled 0.1 M phosphate buffer saline (PBS) (Dulbecco's phosphate buffered saline w/o Calcium, w/o Magnesium, ECB4004L, Euroclone, Italy) pH 7.4. Dulbecco′s PBS without these ions is used to wash and rinse suspended cells and maintain the pH in the physiological range. Cells were then centrifuged for 15 min at 4 C°. The resulting supernatant was kept at -80 C° and used to determine enzyme level using ELISA kits. The BCA protein assay kit (ab102536, Abcam company. BioVision) was used to determine the overall concentration of proteins in solutions using a small portion of the homogenate.

Levels of GABA shunt enzymes (GABA-T and SSA-DH)

GABA-T Levels

The levels of GABA-T were determined using a Rat GABA-T ELISA Kit (MyBioSource, Inc., MBS3809445, Species Reactivity Rat). According to the manufacturer protocol, a 50 µL sample was added to 100 µL of HRP-conjugate reagent and incubated for 60 minutes at 37°C in 96- Micro Elisa strip plate. Each sample was measured in duplication. After washing, 50 µl of chromogen solutions were added to each well, gently mixed, and incubated for 15 min; finally, 50 µL of Stop Solution was added to each well, and the optical density (OD) read at 450 nm using an ELISA reader (Epoch Microplate Spectrophotometer, Biotek/Agilent, USA).

SSA-DH levels

The SSA-DH ELISA Kit (MyBioSource Inc., MBS2516109) was used to assay SSA-DH enzyme levels. A 100 µL sample was added to each well and incubated for 90 min at 37 ℃. Each sample was measured in duplication. 100 µL of biotinylated detection antibody was added to the wells after removing the liquid, and the solution was incubated for 1 hr. After three repeated washing steps, 100 µL of HRP Conjugate was added, and the solution was incubated for 30 min at 37℃. A 90 µL substrate reagent was added, and the solution was incubated for 15 min at 37 ℃, followed by five repeated washing steps. Finally, 50 µL of stop solution was added to each well, and the OD was read at 450 nm using an ELISA reader (Epoch Microplate Spectrophotometer, Biotek/Agilent, USA).

Determination of TCA cycle enzyme levels (SDH and ISD)

SDH levels

SDH levels were determined using a rat SDH ELISA Kit (Genochem World SL, GW1645Ra). According to the manufacturer protocol, 40 µL of sample dilution buffer was added to each 10 µL of sample solution and incubated for 30 min at 37 ℃. Each sample was measured in duplication, and after five repeated washing steps, 50 µL of HRP-conjugate reagent was added to each well, and the solution was incubated for 30 min at 37 ℃. After another five repeated washing steps, 50 µl of chromogen solution A and B were added to each well, gently mixed, and incubated for 15 min. For the final step, 50 µL of stop solution was added to each well, and the OD was read at 450 nm using an ELISA reader (Epoch Microplate Spectrophotometer, Biotek/Agilent, USA).

IDH levels

IDH levels were measured using a human ISD 1, Soluble (IDH1) ELISA Kit (MyBioSource Inc., MBS450967). A 100 µL sample was added to each well and incubated for 90 min at 37 ℃. Each sample was measured in duplication, and the liquid was removed without washing, and 100 µL of detection reagent A working solution was added to each well and incubated for 1 hr at 37 ℃. Following, the wells were washed three times, and 100 µL of detection reagent B working solution was added to each well prior to incubation for 1 hr at 37 ℃. The wells were washed five times again, and 90 µL of substrate solution was added to each well and incubated for 30 min. Finally, 50 µL of stop solution was added to each well, and OD was read at 450 nm using an ELISA reader (Epoch Microplate Spectrophotometer, Biotek/Agilent, USA).

Statistical analysis

GABA shunt enzymes and tricarboxylic acid cycle enzymes were analyzed using one-way ANOVA followed by Tukey's multiple means comparisons. Data were presented as mean + SD, and all data were analyzed using GraphPad Prism 9.0; significance was set at $p < 0.05$.

Results

The effect of chronic administration of tramadol on the level of GABA shunt enzymes and tricarboxylic acid cycle enzymes

GABA-T levels

Tramadol treatment decreased the GABA-T enzyme level in the thalamus. This effect showed a significant decrease in GABA-T enzyme levels after tramadol treatment with 50 mg/kg po compared to 25 mg/kg PO, but GABA-T levels did not differ significantly from control values. Similar results were shown in the hypothalamus. However, in this case, both tramadol doses decreased GABA-T enzyme levels compared to control values. This effect significantly decreases GABA-T enzyme levels after both tramadol treatments compared to the control group. In the cerebellum, tramadol treatment again decreased GABA-T enzyme levels. This effect revealed a significant main effect of

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Treatment, showing a significant decrease in GABA-T enzyme levels after 25 and 50 mg/kg po tramadol treatment compared to the control group. In the cerebral cortex, tramadol again decreased GABA-T enzyme levels significantly, showing that 50 mg/kg po tramadol reduced GABA-T levels compared to the control group. Tramadol treatment also decreased GABA-T enzyme levels in the brain stem, showing a significant decrease in GABA-T enzyme levels after treatment with 25 and 50 mg/kg po tramadol compared to the control group (Table 1).

Succinate-semialdehyde dehydrogenase SSA-DH levels

Tramadol treatment did not affect SSA-DH enzyme levels in the thalamus. There was no significant effect of Treatment. Differences were seen in other brain regions, but the responses were brain region-dependent. In the hypothalamus, tramadol treatment increased SSA-DH enzyme levels at the 25 mg/kg po dose, but levels returned to baseline at the 50 mg/kg tramadol dose. Significant increases in SSA-DH enzyme levels in the 25 mg/kg tramadol treatment group compared to the control group and the 50 mg/kg treatment group. In contrast to this change, tramadol decreased SSA-DH levels in other brain regions. In the cerebellum, 25 and 50 mg/kg po tramadol treatment for one month decreased SSA-DH enzyme levels, and significant means comparisons for both doses versus the control group. In the cerebral cortex, 25 mg/kg and 50 mg/kg tramadol treatment decreased SSA-DH enzyme levels significantly compared to the control group. A significant decrease in SSA-DH enzyme levels at the 25 mg/kg and 50 mg/kg doses of tramadol compared to the control group.

Tramadol treatment for one month also decreased SSA-DH enzyme levels in the brain stem significantly (Table 2).

Succinate dehydrogenase determination levels

Tramadol treatment, 25 mg/kg and 50 mg/kg for one month did not decrease SDH enzyme levels in the thalamus. There was also no effect of tramadol on SDH in the hypothalamus. In the cerebellum, tramadol treatment decreased SDH enzyme levels. There was a significant main effect of Treatment showing that there was a significant decrease in SDH enzyme levels for the 50 mg/kg tramadol treatment group compared to the control group. Tramadol did not affect SDH enzyme levels significantly in the cerebral cortex (Table 3).

Table 1. GABA-T enzyme levels (ng/mg) after 25 and 50 mg/kg po tramadol treatment for one month

GABA-T enzyme Variable		Control	Gp 25 mg/Kg	Gp 50 mg/Kg	*P*-value
Thalamus	Mean ± SD	0.07784 ± 0.007	0.08310 ± 0.001	0.06385 ± 0.0008*	0.045
Hypothalamus	Mean ± SD	0.1195 ± 0.006	0.09050 ± 0.002#	0.09700 ± 0.0014*	0.011
Cerebellum	Mean ± SD	0.1115 ± 0.002	0.08000 ± 0.001#	0.06300 ± 0.0084#	0.006
Cerebral Cortex	Mean ± SD	0.07550 ± 0.003	0.06300 ± 0.009#	0.04500 ± 0.0014#	0.035
Brain stem	Mean ± SD	0.1080 ± 0.005	0.07800 ± 0.002#	0.06950 ± 0.0021#	0.004

(data expressed as mean ± SEM). A) Thalamus; B) Hypothalamus; C) Cerebellum; D) Cerebral Cortex; E) Brain Stem (Significant P < 0.05).
* Significant compared to Gp 25 mg/Kg.
# Significant compared to control.

Table 2. SSA-DH enzyme levels (ng/mg) after 25 and 50 mg/kg po tramadol treatment for one month

SSA-DH enzyme Variable		Control	Gp 25 mg/Kg	Gp 50 mg/Kg	*P*-value
Thalamus	Mean ± SD	3.370 ± 0.94	3.244 ± 0.56	3.520 ± 1.55	0.969
Hypothalamus	Mean ± SD	4.283 ± 0.38	9.631 ± 0.94#	3.013 ± 0.35*	0.003
Cerebellum	Mean ± SD	4.376 ± 0.46	2.744 ± 0.20#	2.343 ± 0.11#	0.013
Cerebral Cortex	Mean ± SD	4.565 ± 1.22	1.700 ± 0.03#	1.655 ± 0.20#	0.043
Brain stem	Mean ± SD	8.417 ± 1.37	2.955 ± 0.05#	2.890 ± 0.13#	0.010

(data expressed as mean ± SEM). A) Thalamus; B) Hypothalamus; C) Cerebellum; D) Cerebral Cortex; E) Brain Stem (Significant P < 0.05).
* Significant compared to Gp 25 mg/Kg.
# Significant compared to control.

Table 3. SDH enzyme levels (ng/mg) after 25 and 50 mg/kg po tramadol treatment for one month

SDH enzyme Variable		Control	Gp 25 mg/Kg	Gp 50 mg/Kg	*P*-value
Thalamus	Mean ± SD	0.2180 ± 0.008	0.2355 ± 0.033	0.2225 ± 0.002	0.691
Hypothalamus	Mean ± SD	0.4335 ± 0.130	0.2900 ± 0.008	0.3025 ± 0.031	0.271
Cerebellum	Mean ± SD	0.2525 ± 0.017	0.2245 ± 0.003	0.1955 ± 0.007#	0.034
Cerebral Cortex	Mean ± SD	0.2205 ± 0.038	0.1685 ± 0.031	0.1395 ± 0.004	0.144
Brain stem	Mean ± SD	0.3025 ± 0.036	0.2805 ± 0.012	0.2610 ± 0.053	0.605

(data expressed as mean ± SEM). A) Thalamus; B) Hypothalamus; C) Cerebellum; D) Cerebral Cortex; E) Brain Stem (Significant P < 0.05).
* Significant compared to Gp 25 mg/Kg.
# Significant compared to control.

Table 4. IDH enzyme levels (ng/mg) after 25 and 50 mg/kg po tramadol treatment for one month

IDH enzyme	Variable	Control	Gp 25 mg/Kg	Gp 50 mg/Kg	*P*-value
Thalamus	Mean ± SD	0.4355 ± 0.18	0.8605 ± 0.09	4.454 ± 1.05*,#	0.013
Hypothalamus	Mean ± SD	0.4830 ± 0.16	2.187 ± 0.74	1.295 ± 0.58	0.119
Cerebellum	Mean ± SD	0.5680 ± 0.04	0.8525 ± 0.05	0.6765 ± 0.25	0.312
Cerebral Cortex	Mean ± SD	2.620 ± 0.96	0.5270 ± 0.10	0.4490 ± 0.10	0.051
Brain stem	Mean ± SD	3.253 ± 0.93	0.7535 ± 0.09#	1.192 ± 0.16	0.038

(data expressed as mean ± SEM). A) Thalamus; B) Hypothalamus; C) Cerebellum; D) Cerebral Cortex; E) Brain Stem (Significant P < 0.05).
* Significant compared to Gp 25 mg/Kg.
# Significant compared to control.

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Isocitrate dehydrogenase levels

Tramadol treatment increased IDH enzyme levels in the thalamus significantly for the 50 mg/kg tramadol treatment group compared to the control group. Tramadol did not significantly affect IDH in the hypothalamus, cerebellum, or cerebral cortex results. Tramadol treatment significantly decreased IDH enzyme levels in the brain stem, showing that there was a significant decrease in the 25 mg/kg tramadol treatment group compared to the control group (Table 4).

Structural changes in the brain

In the cerebral cortex of the control subjects, groups of nerve cells were regularly arranged in six clear layers, with few signs of neuronal or other pathology. The molecular layer, covered with pia mater, had few neurons or neuroglia cells but more extensive white matter than other cortical layers. The inner and outer granular layers were formed of stellate-shaped nerve cells with large round nuclei. at the same time, the inner and outer pyramidal layers were formed of medium-sized pyramidal cells. The pleomorphic layer is the innermost layer and is formed of a variety of types (shapes) of nerve cells. Low-dose tramadol group produced vascular congestion, mildly degenerated neurons with apoptotic cells, and mild disorganization of cortical layers. These changes were more pronounced in the high-dose tramadol group, which showed a marked loss of organization of cerebral layers and dilated blood vessels with wide perivascular space. The molecular layer contained deformed neurons surrounded by haloes and other deformed neurons with acidophilic cytoplasm.

The external granular layer showed granule cells with deeply stained pyknotic nuclei. In the external pyramidal layer, pyramidal cells appeared with pyknotic deeply stained nuclei and surrounded by haloes. The neuropil was vacuolated. The internal granular layer showed granule cells with dark nuclei and others with karyolitic nuclei, deformed neurons, dilated and congested blood vessels, and other neurons with acidophilic cytoplasm (red) were seen. In the internal pyramidal layer, deformed neurons appeared with deeply stained nuclei and were surrounded by haloes. Dilated congested blood vessels and red neurons could be seen (Figure 1).

The evidence of these changes in hematoxylin-eosin-stained sections was confirmed by silver staining, showing the pyramidal cells of the low-dose tramadol treatment group showed evidence of degenerated dendrites, with

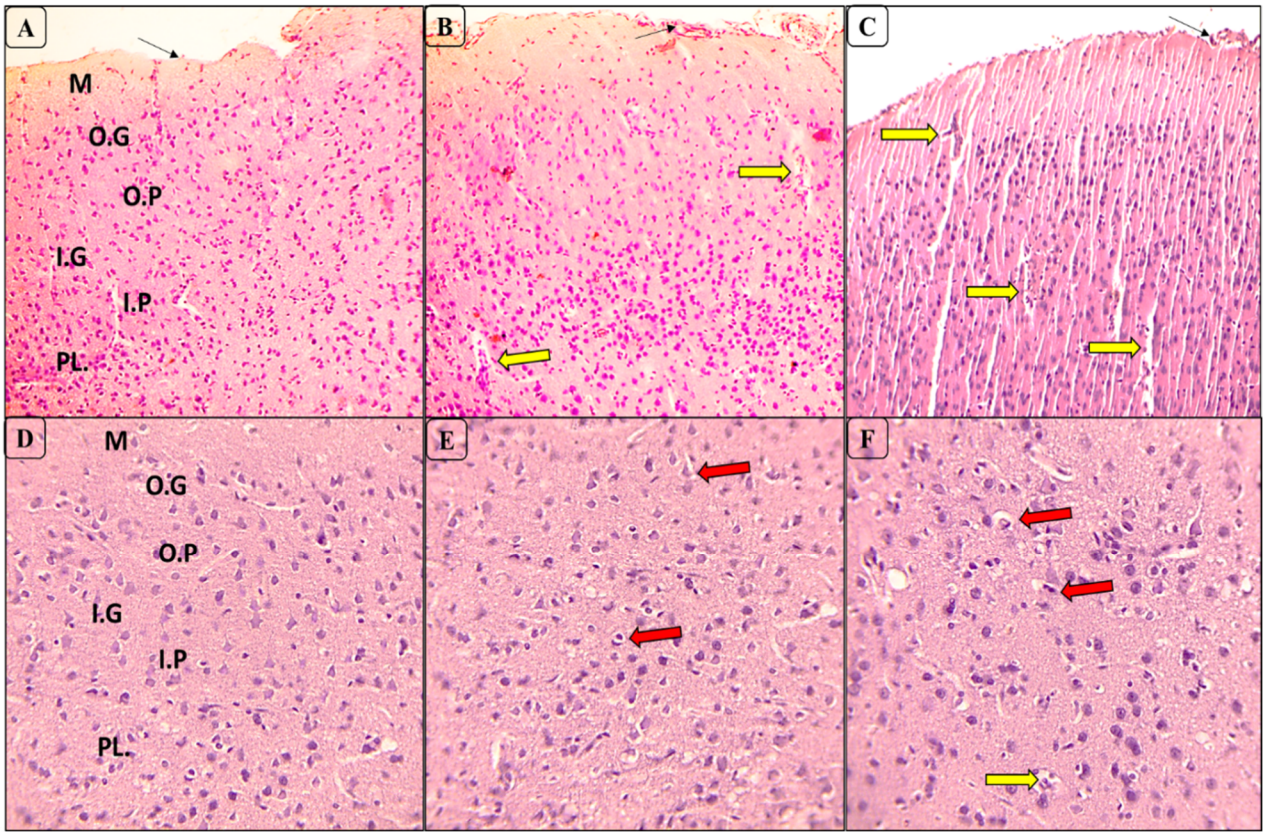

Figure 1. Photomicrographs of rat cerebral cortex after Hematoxylin-Eosin staining. (A, D): No pathological changes were seen in the control cerebral cortex, which was regularly arranged in six layers: the molecular layer (M), had few neurons or neuroglia cells, more extensive white matter, and was covered with pia mater (thin arrow). The inner and outer granular layers (O.G & I.G) contained stellate-shaped nerve cells with large round nuclei, while the inner and outer pyramidal layers (O.P & I.P) contained medium-sized pyramidal cells. The pleomorphic layer (PL) is the innermost layer formed of mixed types of nerve cells. (B, E). The cerebral cortex after low-dose tramadol treatment showed mild thickening of pia matter (thin arrow) and mildly congested blood vessels in the cerebral layers (yellow arrow), mild pericytoplasmic vacuolation around the neuronal cells (red arrow) (C, F). The cerebral cortex after high dose tramadol treatment showed marked thickening and congestion of pia matter (thin arrow), marked congested blood vessels in the vacuolated neuropil of cerebral layers (yellow arrow), loss of organization of cerebral layers and marked pericytoplasmic vacuolation around deeply stained pyknotic nuclei of neuronal cells (red arrow) (Hx.& E stain x 100-200).

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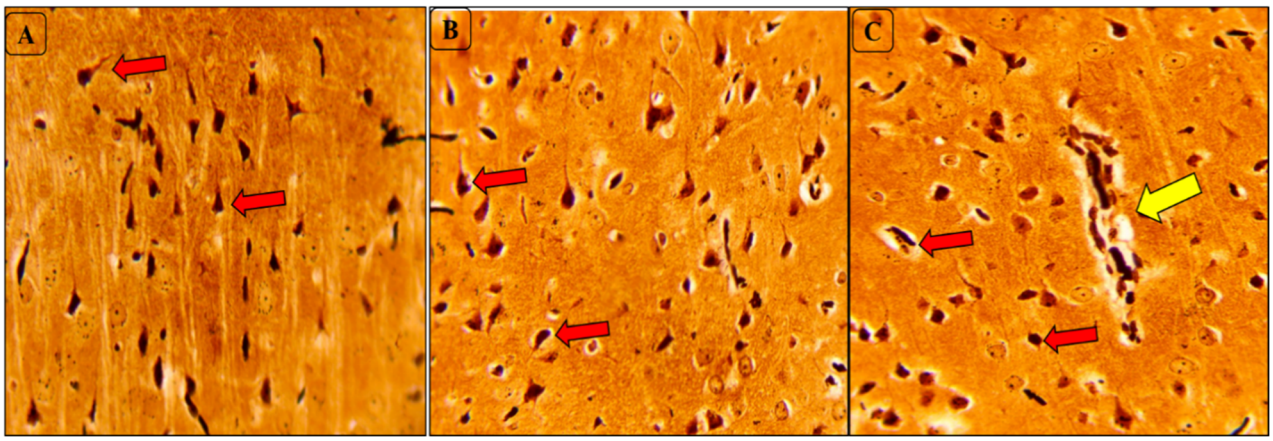

Figure 2. Photomicrographs of rat cerebral cortex after silver Staining (A): Control treatment silver staining in the external pyramidal layer of the cerebral cortex showing clear pyramidal cells with the base of the long apical dendrite (red arrow) surrounded by normal neuropil, (B): the pyramidal cells of the low dose tramadol treatment group showed evidence of degenerated dendrites, with deeply stained pyknotic nuclei surrounded by mild pericytoplasmic vacuolations of the neuropil. (C): pyramidal cells of the high-dose tramadol treatment group showed evidence of even more marked degeneration of pyramidal dendrites, with deeply stained pyknotic nuclei surrounded by marked pericytoplasmic vacuolations of the neuropil (red arrow) and marked congested blood vessels (yellow arrow). (Nauta silver stain x400).

deeply stained pyknotic nuclei surrounded by mild pericytoplasmic vacuolations of the neuropil. Pyramidal cells of the high-dose tramadol treatment group showed evidence of even more marked degeneration of pyramidal dendrites, with deeply stained pyknotic nuclei surrounded by marked pericytoplasmic vacuolations of the neuropil and marked congested blood vessels (Figure 2).

Discussion

Tramadol is a centrally acting analgesic that can be abused despite a perception of greater safety compared to some other opioids, such as morphine (28). Tramadol comprises two enantiomers, each contributing to analgesic efficacy through distinct mechanisms (28). Many of these mechanisms affect the TCA cycle, which has been recognized as a crucial mechanism for producing cellular energy, building blocks for biosynthetic pathways, and neurotransmitter regulation (29).

Different enzymes are involved in the TCA cycle, including GABA-T, IDH, SSA-DH, and SAD (30). GABA is the major inhibitory neurotransmitter in the central nervous system. GABA is metabolized in the mitochondria into SSA by the enzyme GABA-T and then succinate via SSA-DH. This metabolic mechanism is called the GABA shunt (31, 32).

The present study examined the activities of the GABA shunt enzymes (GABA-T and SSA-DH) and TCA cycle enzymes (IDH and SAD) in different brain regions of rats undergoing chronic tramadol intoxication. Using approaches similar to previous studies, tramadol intoxication was produced by multiple oral administrations of tramadol at doses of 0, 25, and 50 mg/kg PO for one month (33).

This study showed that chronic tramadol treatment strongly reduces GABA shunt enzyme levels (GABA-T and SSA-DH) broadly across the brain. In contrast, TCA cycle enzymes are impacted less than the GABA shunt enzymes, with more limited effects, in the cerebellum for SDH and in the thalamus and brain stem for IDH. The changes showed in the GABA shunt activity might be caused by the adaptation of the brain regions differing in the opioid receptors contents to protracted tramadol administration (22). This is consistent with a previous study on rats treated with morphine, which revealed that the activity of GABA-T, SSA-DH, SDH, and IDH enzymes, and thus GABA catabolism, can be significantly disrupted by opioid intoxication. Another study in mice similarly showed a dose-dependent disturbance in GABA after repeated treatment with 20 or 50 mg/kg doses of tramadol (34). In our previous study, we found that morphine intoxication caused a change in the activity of GABA-shunt in the thalamus and brain stem, followed by a change in the activity of TCA cycle enzymes (23). These effects were brain-region dependent as those enzymes were inhibited in the cerebellum, suggesting that chronic opioid exposure may exacerbate both GABA shunt and TCA cycle turnover in the affected region (23). Additionally, the neurodegenerative effect of long-term tramadol exposure in rats might lead to the accumulation of oxidative stress intermediates in the brain (24).

The differences in enzyme levels (IDH, SDH, SSA-DH, and GABA-T) in various parts of the brain after tramadol treatment can be due to the tramadol effects on neurotransmitter systems, including serotonin and norepinephrine, which influence enzyme activity in various brain regions. Another reason is that the enzyme expression levels and metabolic activities vary in different brain regions, which can be further modulated by tramadol treatment (34).

These changes in brain enzymes associated with the GABA shunt and the TCA cycle were also associated with neuropathological changes in the cerebral cortex. A histopathological examination found that low-dose tramadol induced mild histopathological changes in rat cerebral cortex in the form of vascular congestion, mildly degenerated neurons, and some disorganization of cortical layering. This is consistent with a previous study showing histological, pharmacotoxicological, neurochemical, and biochemical changes caused by prolonged tramadol administration in male rats that were associated with degeneration of brain

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cells and congestion of blood vessels (25).

Although effects were clearly visible at this low tramadol dose, they were much more pronounced after chronic treatment with a higher tramadol dose, which showed much more marked cellular disorganization of cerebral layering and dilated blood vessels with wide perivascular spaces. Neurons in different cortical layers, including the pyramidal cells, showed marked degeneration of the dendrites, with deeply stained pyknotic nuclei surrounded by marked pericytoplasmic vacuolations of the neuropil.

Several studies have previously shown that chronic tramadol produces structural disorganization of cortical layers in the motor cortex, hypercellularity, and increases in apoptotic cells (35, 36). There was widespread neuropil vacuolization, with degenerated pyramidal cells showing either weakly stained cytoplasm and nuclei or darkly stained cytoplasm with pyknotic nuclei. A noticeable cytoplasmic vacuolization was seen in some undersized pyramidal cells. Along with similarities in terms of these micro- and macrostructural changes, chronic tramadol caused degenerative changes in neurons of the cerebellar cortex with fewer Purkinje cells and loss of their distinctive shape (37). The cause of vacuolation around neurons could be due to shrinkage and damage from exposure to free radicals (38).

This study highlighted changes in some enzyme levels in GABA shunt and TCA in different parts of the brain after tramadol intoxication, which can be crucial for understanding tramadol mechanism of action and potential toxicity, which may not have been extensively studied in the existing body of knowledge.

Conclusion

In conclusion, this work has important implications for negative outcomes associated with chronic tramadol use and adverse effects. This is important since tramadol is considered a safer alternative to other opioids that may have a greater risk for dependence but lack many of the effects of tramadol that may lead to its adverse long-term effects. This work clearly shows, along with other literature, that tramadol has a potential for neurodegeneration that should be considered in the context of more extensive or longer-term use of tramadol for conditions such as chronic pain. The mechanisms involved appear to involve GABA and energy metabolism, which should be more thoroughly investigated, particularly in the context of chronic tramadol use.

Authors' Contributions

Husam Abazid: Concepts, design, experimental studies, supervision, reviewing and editing.

All the authors contributed to the manuscript equally according to their specialty and approved the final version.

Ethical Considerations

The experiments and housing procedures were approved by the Institutional Animal Care and Use Committee of the Applied Science Private University IRB number 2021-PHA-11. All procedures involving animals were conducted in accordance with the ethical guidelines outlined by the Global Helsinki Declaration on Animal Research. Efforts were made to minimize pain and distress by employing appropriate anesthetic and analgesic protocols where necessary. The number of animals used was carefully calculated to achieve statistical significance while adhering to the 3Rs principle (Replacement, Reduction, Refinement) to minimize animal use. Upon completion of the experiments, animals were either humanely euthanized or handled according to approved protocols to ensure minimal suffering.

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Conflict of Interests

The authors declare that they have no competing interests.

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