

Histological, Immunohistochemical, and Biochemical Assessment of the Amygdala in Tramadol-Induced Neurotoxicity

T. Ammani¹ and M. S. Saleh²

¹Department of Anatomy, Usmanu Danfodiyo University, Sokoto, Sokoto State, Nigeria.

²Department of Anatomy, Bayero University Kano, Kano State, Nigeria.

Corresponding author email: tijjaniammani76@gmail.com ; Tel: +2348065262734

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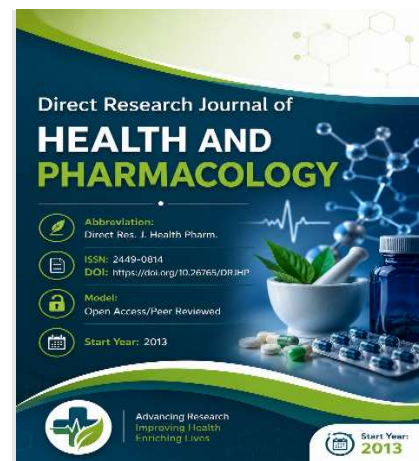
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ABSTRACT

Herbal Tramadol, a synthetic opioid analgesic widely used for pain management, is increasingly associated with neurotoxicity due to oxidative stress, particularly affecting the amygdala, a key brain region for emotional processing, fear response, and memory modulation. This study investigates the dose-dependent effects of tramadol on amygdala integrity in adult albino mice, focusing on histological, immunohistochemical, and biochemical alterations. Eighteen mice were divided into three groups (n=6 per group): Group 1 (Control, distilled water), Group 2 (Tramadol 20 mg/kg body weight), and Group 3 (Tramadol 40 mg/kg body weight), treated daily for three weeks. Hematoxylin and eosin (H&E) staining assessed neuronal morphology, glial fibrillary acidic protein (GFAP) immunohistochemistry evaluated astrocyte reactivity, and biochemical assays measured oxidative stress markers (glutathione, superoxide dismutase, catalase, malondialdehyde [MDA]). Results revealed dose-dependent neuronal damage, with Group 3 showing severe pyknotic nuclei, cytoplasmic vacuolation, and spongiform neuropil. GFAP staining indicated progressive astrocyte hypertrophy and gliosis, most pronounced in Group 3, reflecting robust neuroinflammation. Biochemical analyses showed significant reductions in antioxidants (glutathione, superoxide dismutase, catalase) and elevated MDA levels, with Group 3 exhibiting the most severe oxidative stress. These findings highlight tramadol's neurotoxic potential in the amygdala, with higher doses exacerbating damage through oxidative and inflammatory pathways, underscoring the need for neuroprotective strategies.

Keywords: Tramadol, neurotoxicity, amygdala, oxidative stress, neuronal damage, astrocyte reactivity, gliosis, glutathione, superoxide dismutase, catalase, malondialdehyde, histology, immunohistochemistry, H&E staining, GFAP, opioid abuse



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INTRODUCTION

Tramadol, a synthetic opioid analgesic, is widely used for managing moderate to severe pain due to its dual mechanism as a mu-opioid receptor agonist and an inhibitor of serotonin and norepinephrine reuptake (Dayer *et al.*, 1997; Grond & Sablotzki, 2004). Its clinical efficacy stems from its ability to modulate pain perception through opioid and monoaminergic pathways, making it a versatile analgesic for conditions ranging from postoperative pain to

chronic musculoskeletal disorders (Budd & Langford, 1999). However, its widespread use, particularly in regions with high opioid misuse rates such as Nigeria, has raised significant concerns about its neurotoxic effects, especially with chronic administration or abuse (Nakhaee *et al.*, 2021; Akindele *et al.*, 2020). Tramadol's metabolism, primarily via hepatic cytochrome P450 enzymes (CYP2D6 and CYP3A4), produces active metabolites like O-

desmethyltramadol, which contribute to its pharmacological effects but also generate reactive oxygen species (ROS) (Subrahmanyam *et al.*, 2001; Wu *et al.*, 2002). These ROS trigger oxidative stress through lipid peroxidation, protein oxidation, and mitochondrial dysfunction, processes implicated in neuronal damage (Hussein & Abdel Aal, 2017; El-Sayed *et al.*, 2019).

The amygdala, a limbic structure critical for emotional processing, fear conditioning, and memory modulation, is particularly vulnerable to tramadol's neurotoxic effects (Elliott *et al.*, 2011; LeDoux, 2000). Its high metabolic rate, rich in polyunsaturated fatty acids, and limited antioxidant capacity make it susceptible to oxidative damage (Tanner, 1992; Zaidi & Banu, 2004). Chronic tramadol exposure induces neuronal apoptosis, synaptic dysfunction, and glial activation, contributing to emotional and cognitive impairments observed in both animal models and human users (Nakhaee *et al.*, 2021; Mohamed *et al.*, 2015). Histological studies have documented tramadol-induced neurodegeneration in limbic regions, characterized by pyknotic nuclei, vacuolated cytoplasm, and spongiform neuropil, indicative of cellular stress and injury (Hosseinzadeh *et al.*, 2019; Awadalla & Salah-Eldin, 2016). Immunohistochemical analyses using glial fibrillary acidic protein (GFAP) reveal increased astrocyte reactivity, a hallmark of neuroinflammation, with reactive astrocytes releasing pro-inflammatory cytokines such as IL-1 β and TNF- α , exacerbating neuronal damage (Valentim *et al.*, 2024; Sofroniew & Vinters, 2010). Biochemically, tramadol depletes key antioxidants glutathione, superoxide dismutase (SOD), and catalase while elevating malondialdehyde (MDA), a marker of lipid peroxidation, further compromising neuronal integrity (Khazdair *et al.*, 2021; Bameri *et al.*, 2022; Abdel-Zaher *et al.*, 2011).

The global rise in tramadol abuse, particularly in Africa and the Middle East, has amplified its public health impact (UNODC, 2017). In Nigeria, tramadol misuse is prevalent among young adults and workers in high-risk occupations, driven by its accessibility and perceived safety compared to other opioids (Akindele *et al.*, 2020; WHO, 2018). This has led to neurological complications, including anxiety, depression, and memory deficits, potentially linked to amygdala dysfunction (Elliott *et al.*, 2011; Saletu *et al.*, 2010). Tramadol's neurotoxicity is mediated by multiple mechanisms, including excitotoxicity via glutamate dysregulation, blood-brain barrier disruption, and oxidative stress-induced mitochondrial damage (Mehdizadeh *et al.*, 2017; Ghoneim *et al.*, 2014). Excitotoxicity results from excessive glutamate release, which overactivated NMDA receptors, leading to calcium overload and neuronal death (Olney, 1990; Lipton & Rosenberg, 1994). Blood-brain barrier disruption, evidenced by perivascular oedema, facilitates the entry of toxic metabolites, amplifying damage (Sharma *et al.*, 2007). Additionally, tramadol's inhibition of GABAergic signalling may exacerbate neuronal hyperexcitability, further contributing to neurotoxicity (Hara *et al.*, 2005).

This study provides a comprehensive assessment of tramadol's neurotoxicity in the amygdala, integrating histological (H&E), immunohistochemical (GFAP), and biochemical (glutathione, SOD, catalase, MDA) analyses. The findings contribute to understanding opioid-related brain damage, with implications for managing tramadol use and addressing its abuse. This study investigates tramadol's dose-dependent neurotoxicity in the amygdala of adult albino mice, aiming to evaluate histological effects on neuronal morphology using hematoxylin and eosin (H&E) staining, assess astrocyte reactivity via glial fibrillary acidic protein (GFAP) immunohistochemistry, and measure biochemical changes in oxidative stress markers (glutathione, superoxide dismutase, catalase, malondialdehyde), highlighting the need for neuroprotective interventions to mitigate tramadol's adverse effects on emotional and cognitive functions.

MATERIALS AND METHODS

The study utilized tramadol hydrochloride (Sigma-Aldrich, purity $\geq 98\%$, CAS 27203-92-5), sterile saline (0.9% NaCl), and distilled water. Standard rodent chow (Vital Feeds, Nigeria) and water were provided ad libitum. Histological equipment included a rotary microtome (Leica RM2235), light microscope (Olympus BX51 with DP72 digital camera), and staining reagents: hematoxylin (Sigma-Aldrich), eosin (Merck), xylene, ethanol (70–100%), paraffin wax, and DPX mounting medium. Immunohistochemical reagents comprised citrate buffer (pH 6.0, SigmaAldrich), primary GFAP antibodies (rabbit anti-GFAP, 1:200, Abcam ab7260), secondary antibodies (goat anti-rabbit IgG, HRP-conjugated, 1:500, Abcam ab205718), 3,3'-diaminobenzidine (DAB, Sigma-Aldrich), and 3% hydrogen peroxide. Biochemical assays used Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid, Sigma-Aldrich) for glutathione, a superoxide dismutase (SOD) assay kit (Cayman Chemical, 706002), a catalase (CAT) assay kit (SigmaAldrich, CAT100), and a malondialdehyde (MDA) assay kit (Sigma-Aldrich, MAK085) for lipid peroxidation. Phosphate-buffered saline (PBS, pH 7.4) was prepared for tissue processing (Hussein *et al.*, 2020).

Study Design

A randomized controlled trial was conducted using 18 adult albino mice (Swiss strain, 25–30 g, 8–10 weeks old), sourced from the Animal House of Usmanu Danfodiyo University, Sokoto. Mice were acclimatized for one week and randomly assigned to three groups (n=6 per group, Table 1) using a computer-generated randomization sequence (Random.org). Group 1 (Control) received distilled water, Group 2 received tramadol 20 mg/kg body weight, and Group 3 received tramadol 40 mg/kg body weight, administered subcutaneously daily for three weeks (Hirst *et al.*, 2014).

Table 1: Groupings of the Experimental Animals.

Group	Treatment
1 Control (distilled water)	Tramadol 20 mg/kg
2 Tramadol 20 mg/kg	Tramadol 20 mg/kg
3 Tramadol 40 mg/kg	Tramadol 20 mg/kg

Table 2: Biochemical Analysis of Oxidative Stress Markers in the Amygdala.

Group	Glutathione (mg/m protein)	SOD (U/mg protein)	Catalase (U/mg protein)	MDA (U/mg protein)
1 (Control)	0.25 ± 22.90	128.67 ± 12.42	2.00 ± 1.23	0.50 ± 0.10
2 (Tramadol 20 mg/kg)	0.15 ± 15.40	110.50 ± 10.30	1.80 ± 1.10	0.80 ± 0.15
3 (Tramadol 40 mg/kg)	0.10 ± 10.20	95.33 ± 9.50	1.50 ± 0.90	1.20 ± 0.20
p-value	0.979	0.250	0.750	0.320

Administration

Tramadol was dissolved in sterile saline to achieve concentrations of 2 mg/mL (Group 2) and 4 mg/mL (Group 3). Injections were administered subcutaneously in the dorsal neck region using a 1 mL insulin syringe (BD Micro-Fine, 29G) at 9:00 AM daily. Control mice received distilled water (0.1 mL/10 g body weight). Animals were housed in polycarbonate cages (3 mice per cage, 22 ± 2°C, 50–60% humidity, 12-hour light/dark cycle) with wood shavings as bedding. Body weights were recorded weekly using a digital scale (Ohaus Scout Pro, 0.01 g precision) (Hirst *et al.*, 2014).

Histological Analysis

Mice were anesthetized with ketamine (50 mg/kg, intraperitoneal) and euthanized by cervical dislocation after three weeks. Brains were dissected using stereotaxic coordinates (bregma -0.8 to -2.8 mm) to isolate the amygdala. Tissues were fixed in 10% neutral buffered formalin (48 hours, 4°C), dehydrated through graded ethanol, cleared in xylene, and embedded in paraffin wax. Coronal sections (5 µm) through the basolateral amygdala were cut using a rotary microtome and mounted on poly-L-lysine-coated slides. Sections were stained with hematoxylin (5 minutes) and eosin (2 minutes), mounted with DPX, and examined at 250x and 400x magnification. Neuronal morphology, neuropil integrity, and vascular changes were analyzed using ImageJ software (NIH) (Hussein *et al.*, 2020).

Immunohistochemical Analysis

GFAP immunohistochemistry assessed astrocyte reactivity. Sections were deparaffinized, rehydrated, and subjected to antigen retrieval in citrate buffer (pH 6.0, 95°C, 20 minutes). Endogenous peroxidase was blocked with 3% hydrogen peroxide (10 minutes), and non-specific binding with 5% bovine serum albumin (1 hour). Sections were incubated with primary GFAP antibodies (1:200) overnight at 4°C, followed by secondary antibodies (1:500, 1 hour at room temperature). Staining was visualized with

DAB (5 minutes), counterstained with hematoxylin (1 minute), and mounted with DPX. Astrocyte density and morphology were analyzed at 250x and 400x magnification using ImageJ, with counts per 0.1 mm² in the basolateral amygdala (Hussein *et al.*, 2020).

Biochemical Analysis

Amygdala tissues were homogenized in ice-cold PBS (pH 7.4, 1:10 w/v), centrifuged at 10,000 x g (4°C, 15 minutes), and supernatants collected. Glutathione levels were measured using Ellman's reagent (412 nm, Shimadzu UV-1800). Superoxide dismutase activity was assessed using a SOD assay kit (450 nm). Catalase activity was determined by hydrogen peroxide decomposition (240 nm). Malondialdehyde (MDA) levels were quantified using an MDA assay kit, measuring thiobarbituric acid reactive substances (TBARS) at 532 nm. Protein content was quantified using the Bradford assay (595 nm) to normalize results, expressed as mg/m protein (glutathione, MDA) or U/mg protein (SOD, catalase). Assays were performed in triplicate with intra-assay coefficients of variation <5% (Hussein *et al.*, 2020; Bameri *et al.*, 2022).

Statistical Analysis

Data were analyzed using SPSS version 25. Normality was confirmed with the Shapiro-Wilk test, and homogeneity with Levene's test. One-way ANOVA with post-hoc Tukey tests compared outcomes across groups. Results were expressed as mean ± SEM, with p < 0.05 considered significant.

RESULTS

Biochemical assays showed dose-dependent changes in oxidative stress markers (Tables 2-3). Group 1 (Control) had glutathione at 0.25 ± 22.90 mg/m protein, superoxide dismutase at 128.67 ± 12.42 U/mg protein, catalase at 2.00 ± 1.23 U/mg protein, and MDA at 0.50 ± 0.10 nmol/mg protein. Group 2 (Tramadol 20 mg/kg) showed reduced antioxidants and increased MDA: glutathione (0.15 ± 15.40 mg/m protein, 40% reduction), superoxide dismutase

Table 3: Experimental groups and the plates that illustrate them. Group 3 dose is taken from the Plate VI legend; the Plate III legend states none.

Group	Treatment	H&E plate (×400)	GFAP plate (×250)
Group 1	Control (no tramadol)	Plate I	Plate IV
Group 2	Tramadol 20 mg/kg body weight	Plate II	Plate V
Group 3	Tramadol 40 mg/kg body weight	Plate III	Plate VI

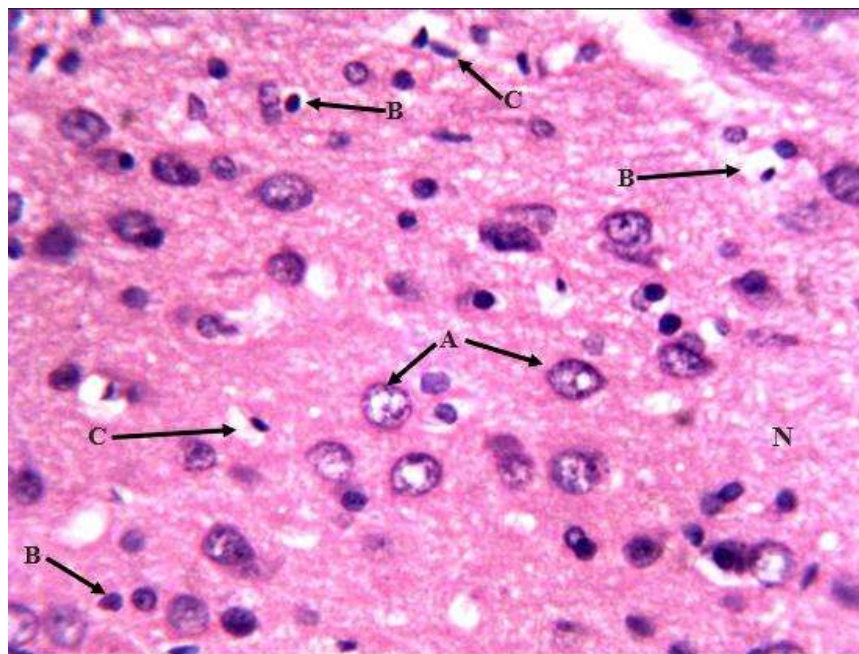


Plate I: Photomicrograph of the amygdala of adult albino mice (Group 1, control), H&E ×400. Arrows (A): neuronal cell bodies with central basophilic nucleoli; arrows (B): oligodendrocytes with darkly stained nuclei and central halo; arrow (C): glial cell with eccentric, elongated nucleus; (N): eosinophilic neuropil containing axons, synapses and glial cells.

(110.50 ± 10.30 U/mg protein, 14% reduction), catalase (1.80 ± 1.10 U/mg protein, 10% reduction), and MDA (0.80 ± 0.15 nmol/mg protein, 60% increase). Group 3 (Tramadol 40 mg/kg) had the most severe changes: glutathione (0.10 ± 10.20 mg/m protein, 60% reduction), superoxide dismutase (95.33 ± 9.50 U/mg protein, 26% reduction), catalase (1.50 ± 0.90 U/mg protein, 25% reduction), and MDA (1.20 ± 0.20 nmol/mg protein, 140% increase). Differences were non-significant ($p = 0.250-0.979$), likely due to small sample size and variability.

Histological Findings (H&E Staining)

Qualitative light-microscopic examination of the basolateral amygdala of adult albino mice showed a dose-related progression of neuronal injury and reactive astrogliosis after tramadol administration. In the control group (Group 1), neurons were well organised with large, round nuclei and central basophilic nucleoli (Plate I), and GFAP immunoreactivity was low, confined to small astrocytes with fine, elongated processes (Plate II). Tramadol at 20 mg/kg (Group 2) produced mixed pathology: shrunken, pyknotic, vacuolated and

karyorrhectic neurons, dilated capillaries with perivascular astrocytes and inflammatory cells, and probable microglia (Plate II), accompanied by moderate GFAP staining and hypertrophied reactive astrocytes with thickened processes (Plate V). At 40 mg/kg (Group 3), neuronal degeneration was severe, with pyknotic hyperchromatic neurons, actively necrotic somata and a glial-populated neuropil (Plate III); GFAP staining was intense, with marked cellular enlargement, stunted or lost processes and widespread reactive gliosis (Plate VI). These patterns agree with published reports of tramadol-induced neurodegeneration and astrogliosis in the hippocampus, cerebral cortex, prefrontal cortex and cerebellum. In the literature searched for this report, no amygdala-specific histological study of tramadol was identified, so the present data extend the regional evidence.

Experimental Groups and Plates

Three groups of adult albino mice were examined. Sections of the basolateral amygdala were stained with haematoxylin and eosin (H&E ×400) to assess general tissue architecture and with an anti-GFAP

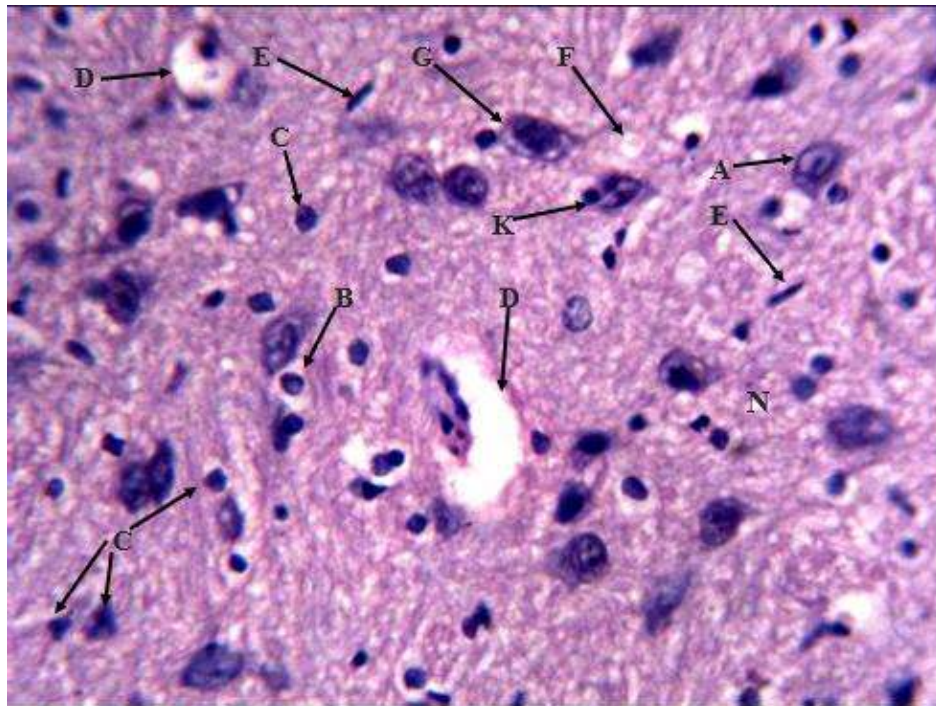


Plate II: Photomicrograph of the amygdala of adult albino mice (Group 2, tramadol 20 mg/kg), H&E ×400. (A, B, G): neurons with prominent basophilic nucleoli surrounded by glial cells; (C, F): shrunken, pyknotic neurons with vacuolated cytoplasm and karyorrhectic nuclei; (D, E): dilated capillaries with probable astrocytes and inflammatory cells, and probable microglia with elongated nuclei; (N): eosinophilic neuropil.

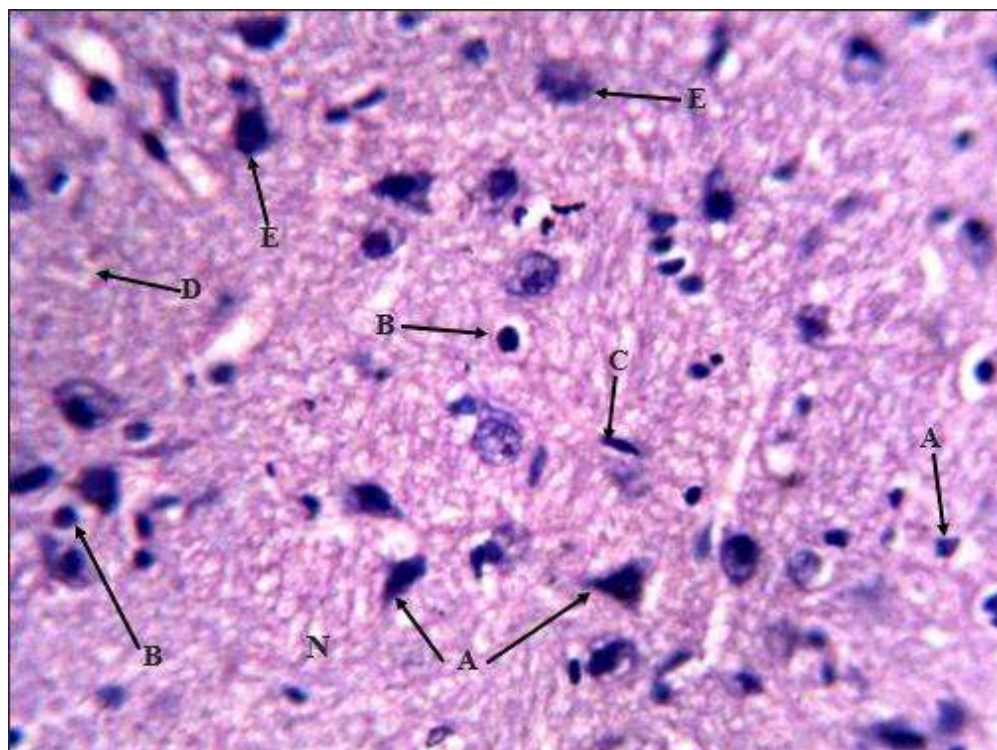


Plate III: Photomicrograph of the amygdala of adult albino mice (Group 3, tramadol 40 mg/kg), H&E ×400. (A): shrunken, pyknotic neurons with hyperchromatic nuclei; (D): actively necrotic soma; (B): cells with dark nuclei and clear halos; (C): elongated microglial nuclei; (E): deeply stained astrocytes with short processes; (N): glial-populated neuropil.

Table 4: Comparative summary of H&E features by group

Feature	Group 1 – control (Plate I)	Group 2 – 20 mg/kg (Plate II)	Group 3 – 40 mg/kg (Plate III)
Neuronal architecture	Well organised; large, round nuclei	Mixed: preserved neurons beside shrunken neurons	Severe degeneration; shrunken neurons
Nucleus / nucleolus	Central basophilic nucleoli	Prominent nucleoli in preserved cells; pyknosis and karyorrhexis in injured cells	Pyknotic, hyperchromatic nuclei
Cytoplasm	Not described	Vacuolated in injured neurons	Not described
Necrosis	None described	Not described	Actively necrotic soma (D)
Glial response	Oligodendrocytes with halo; glia with eccentric elongated nuclei	Glia around neurons; probable astrocytes and microglia	Dark-nucleus cells with halos; microglia; deeply stained astrocytes with short processes
Vascular inflammatory /	None described	Dilated capillaries; inflammatory cells	Not described
Neuropil	Eosinophilic (axons, synapses, glia)	Eosinophilic (axons, synapses, glia)	Glial-populated matrix

immunohistochemical method (×250) to assess astrocytes. Table 3 maps each group to its plates.

Haematoxylin and Eosin Findings

H&E staining of the basolateral amygdala revealed dose-dependent changes across the three groups.

Group 1 (control)

Control sections showed well-organised neurons with large, round nuclei (Plate I). Neuronal cell bodies had central basophilic nucleoli (arrows A). Oligodendrocytes were recognised by darkly stained nuclei with a central halo (arrows B), and glial cells by eccentric, elongated nuclei (arrow C). The surrounding eosinophilic neuropil (N) contained axons, synapses and glial cells.

Group 2 (tramadol 20 mg/kg)

In Group 2, some neurons retained prominent basophilic nucleoli and were surrounded by glial cells (Plate II; A, B, G). Degenerative changes were also present: shrunken, pyknotic neurons with vacuolated cytoplasm and karyorrhectic nuclei (C, F). Dilated capillaries surrounded by probable astrocytes and inflammatory cells, together with probable microglia with elongated nuclei, were observed (D, E). The neuropil (N) remained eosinophilic and contained axons, synapses and glial cells.

Group 3 (tramadol 40 mg/kg)

Group 3 showed severe neuronal degeneration marked by shrunken, pyknotic neurons with hyperchromatic nuclei (Plate III; A) and an actively necrotic soma (D). Glial pathology was evident as cells with dark nuclei surrounded

by clear halos (B) and elongated microglial nuclei (C). Deeply stained astrocytes with short dendritic processes (E) were interspersed throughout a glial-populated neuropil matrix (N).

GFAP Immunohistochemistry

GFAP immunostaining showed a stepwise increase in astrocyte reactivity with tramadol dose (Plates IV–V).

Group 1 (control)

Staining intensity was low overall, with small, sparse cells and a moderate, evenly distributed cell population (Plate IV). Astrocytes had small cell bodies with fine, elongated processes (A, C), interacted with neighbouring cells to form a mesh-like network (B), and encircled a prominent blood vessel (D).

Group 2 (tramadol 20 mg/kg)

Staining intensity was moderate, with an increased density of reactive cells showing hypertrophied somata and thickened processes (Plate IV). Enlarged cell bodies (A) included some without visible dendrites (D) or with small, short processes (B). Reactive astrocytes with elongated, hypertrophied bodies carried either a few short, thick processes (C) or a moderately thick, extended dendrite (E).

Group 3 (tramadol 40 mg/kg)

Staining intensity was intense, with marked cellular enlargement, stunted or lost processes and widespread reactive gliosis (Plate V). Hypertrophied cell bodies (A) included some completely lacking visible dendrites (D) and

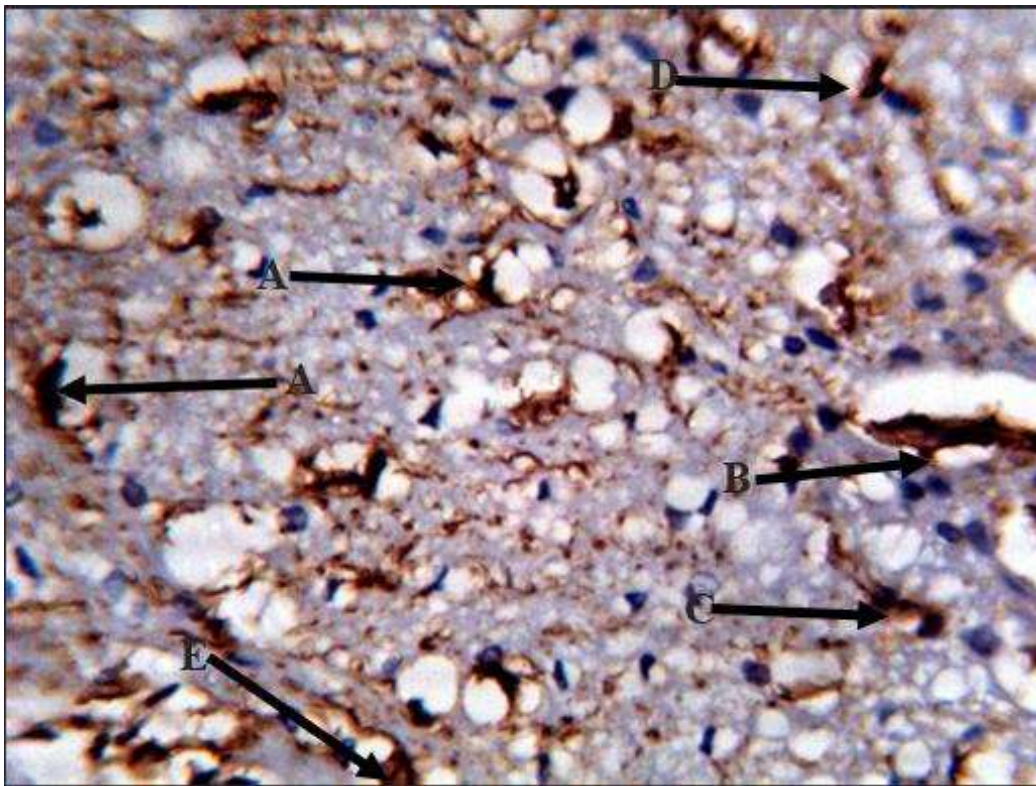


Plate IV: Photomicrograph of the amygdala of adult albino mice (Group 2, tramadol 20 mg/kg), GFAP immunohistochemistry $\times 250$. (A): enlarged cell bodies; (B, D): cells with short or no visible processes; (C, E): reactive astrocytes with hypertrophied bodies and short thick or moderately thick processes.

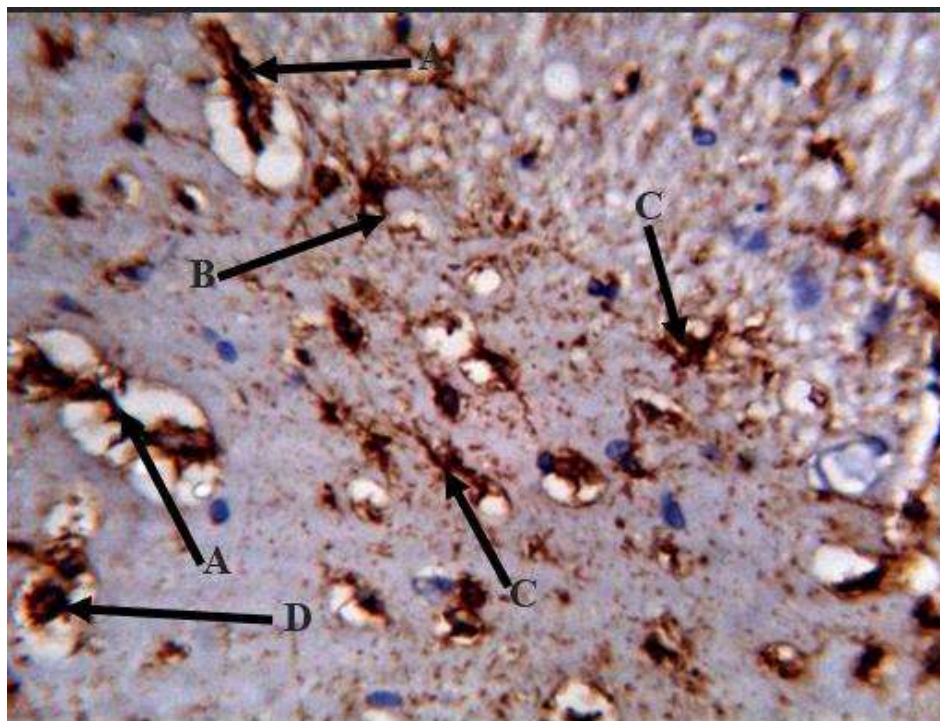


Plate V: Photomicrograph of the amygdala of adult albino mice (Group 3, tramadol 40 mg/kg), GFAP immunohistochemistry $\times 250$. (A): hypertrophied cell bodies; (C, D): cells with abbreviated or absent processes; (B): pairs of enlarged cell bodies joined by short, thickened processes.

Table 5. Comparative summary of GFAP immunoreactivity by group (qualitative).

Feature	Group 1 – control (Plate IV)	Group 2 – 20 mg/kg (Plate V)	Group 3 – 40 mg/kg (Plate VI)
Staining intensity	Low	Moderate	Intense
Cell density	Sparse, small cells; moderate, even distribution	Increased density of reactive cells	Widespread reactive gliosis
Cell body	Small	Hypertrophied, enlarged	Marked enlargement
Processes	Fine, elongated; mesh-like network	Thickened; short and thick, or one moderately thick dendrite; some absent	Stunted or lost; short thickened bridges between paired cells
Perivascular astrocytes	Encircling a prominent vessel (D)	Not described	Not described

others of moderate size with abbreviated processes (C). Pairs of enlarged cell bodies were connected by short, thickened dendritic processes (B).

Integrated Interpretation

H&E and GFAP findings progress in parallel. Neuronal injury advances from intact morphology (Group 1), through coexisting preserved and pyknotic or vacuolated neurons (Group 2), to widespread pyknosis and somal necrosis (Group 3). Over the same dose range, astrocytes shift from small cells with fine, branching processes (low GFAP), to hypertrophied cells with thickened processes (moderate GFAP), to markedly enlarged cells with stunted or lost processes (intense GFAP) (Tables 4 and 5).

Comparison with Published Literature

Table 6 compares the present findings with nine peer-reviewed studies of tramadol effects on the brain. Five were published in 2024–2025, and four earlier studies are included because they use mice or doses close to those in this work. All DOIs were taken from the publisher or PubMed record of each article.

Points of Agreement

Neuronal degeneration: Pyknotic, shrunken, vacuolated and degenerating neurons, as seen in Plates II and III, are consistently reported after tramadol in the cerebral cortex, hippocampus, prefrontal cortex and cerebellum (Abdelmoez *et al.*, 2024; Gholami *et al.*, 2024; Mowaad *et al.*, 2023, Aghajani *et al.*, 2020; Hussein *et al.*, 2020). Where molecular markers were measured, degeneration was accompanied by raised pro-apoptotic and inflammatory signals (BAX, caspases, TNF- α , IL-1 β , P53) and reduced Bcl-2 (Abdelmoez *et al.*, 2024; Gholami *et al.*, 2024; Mowaad *et al.*, 2023, Aghajani *et al.*, 2020), which offers a testable mechanism for the necrotic and karyorrhectic cells seen here.

Perivascular and capillary changes: The dilated capillaries with perivascular astrocytes and inflammatory cells in Plate II resemble the swollen capillaries ringed by astrocytes, and the enlarged perivascular astrocyte and microglial processes, described in the rat cerebellum (Abdelmoez *et al.*, 2024). This suggests that the neurovascular unit is affected in more than one brain region.

Reactive astrogliosis: Increased GFAP immunoreactivity with hypertrophied astrocytes (Plates V and VI) agrees with studies in the hippocampus of mice and rats, in the cerebellum and in the prefrontal cortex (Ray *et al.*, 2025; Abdelmoez, 2024, Aghajani *et al.*, 2020; Hussein *et al.*, 2020), and with the review evidence that astrocyte reactivity contributes to tramadol neurotoxicity (Ray *et al.*, 2025). The mouse study at 40 mg/kg (Hussein *et al.*, 2020) is the closest match in species and dose to Group 3.

Glial involvement: The elongated, probable microglial nuclei in Plates II and III are consistent with the microgliosis reported by (Soares-Cardoso *et al.*, 2024; Abdelmoez, 2024; Aghajani *et al.*, 2020; Hussein *et al.*, 2020). Because identification here rests on H&E morphology alone, confirmation with a microglial marker such as Iba-1 would strengthen this point.

Points of Divergence

Three differences deserve comment. First, the damage in (Ates *et al.*, 2025) was described as mild for tramadol alone, whereas Group 3 showed severe degeneration; dose, duration, species and region probably account for this. Second, the cortical GFAP protein response in (Barbosa *et al.*, 2021) was not proportional to dose, whereas GFAP staining here rose from low to moderate to intense. The two studies differ in region and method (Western blot of tissue extracts versus immunohistochemistry of sections), so the findings are not

Table 6: Comparison of the present histological and GFAP findings with recent Web of Science-type journal publications on tramadol neurotoxicity.

Ref.	Journal, year and active DOI link	Model, region and dose	Key findings	Relation to present study
1	Ates <i>et al.</i> , (2025) Biomedicines, 2025; 13(7):1690 https://doi.org/10.3390/biomedicines13071690	Rats; whole brain tissue; tramadol alone and combined with citalopram and/or tianeptine	Tramadol alone caused mild brain damage with raised MDA (oxidative stress); astrocyte swelling and oligodendrocyte pericellular oedema were described; tianeptine reduced tramadol-related damage.	Partial agreement (glial swelling and oxidative injury). Injury was milder than the severe changes at 40 mg/kg here, probably reflecting differences in dose, duration and species.
2	Ray <i>et al.</i> , (2025) Cells, 2025; 14(18):1454 (review) https://doi.org/10.3390/cells14181454	Review of astrocytes and astrocyte-derived vesicles across opioids, including tramadol	Summarises tramadol-linked reactive astrocytosis (raised GFAP in hippocampus) and reduced GFAP and neuronal apoptosis with melatonin co-treatment, supporting astrocyte reactivity as a driver of tramadol neurotoxicity.	Supports interpreting Plates V–VI as reactive astrogliosis with a likely contribution to neuronal injury.
3	Soares-Cardoso <i>et al.</i> , (2024) Pharmaceuticals, 2024; 17(6):796 https://doi.org/10.3390/ph17060796	Wistar rats; hippocampal formation; tramadol or tapentadol 50 mg/kg i.p., 8 alternate days	Immunohistochemistry showed increased GFAP and decreased CD11b (astrogliosis and microgliosis); hippocampal GFAP gene expression decreased.	Agrees on increased GFAP immunoreactivity (Plates IV–VI). The GFAP mRNA decrease shows protein staining and gene expression can diverge.
4	Abdelmoez, W. A. (2024) J. Mol Histol, 2024; 55(3):279–301 https://doi.org/10.1007/s10735-024-10189-2	Rat offspring; cerebellar cortex; prenatal tramadol 50 mg/kg/day, gestation days 10–21	Red-neuron degeneration; swollen capillaries surrounded by astrocytes; enlarged perivascular astrocyte processes and microglia; hypertrophic astrogliosis on GFAP; raised GFAP and P53.	Closely matches Plate II (dilated capillaries with astrocytes and inflammatory cells) and the hypertrophic GFAP-positive astrocytes of Plates V–VI.
5	Gholami <i>et al.</i> , (2024) Int J Prev Med, 2024; 15 https://doi.org/10.4103/ijpvm.ijpvm_10_24	Rats; hippocampus (CA1, dentate gyrus); tramadol-induced neurodegeneration	Tramadol altered hippocampal histomorphology and raised TNF- α , IL-1 β , BAX and caspase-3; minocycline reversed these changes.	Consistent with neuronal degeneration in Plates II–III; points to neuroinflammatory and apoptotic mechanisms to test in the amygdala.
6	Mowaad <i>et al.</i> , (2023) Neurochem Res, 2023 https://doi.org/10.1007/s11064-022-03827-2	Wistar rats; cerebral cortex; boldenone and/or tramadol; H&E $\times 400$	Focal neuronal degeneration and fewer intact neurons with tramadol; reduced Bcl-2, oxidative stress and neuroinflammation; memory and motor impairment.	Agrees on neuronal loss and degeneration at the same $\times 400$ H&E magnification (Plates II–III).
7	Barbosa <i>et al.</i> , (2021) Pharmaceuticals, 2021; 14(2):97 https://doi.org/10.3390/ph14020097	Wistar rats; cerebral cortex; tramadol 10, 25 and 50 mg/kg i.p., 14 days	GFAP and glutamine synthetase protein rose at 10 and 25 mg/kg; the response was not simply increasing with dose.	Partial divergence: GFAP rose steadily with dose here (Plates IV–VI), whereas the cortex response was non-monotonic. Region, species and method (Western blot vs immunohistochemistry) may explain this.
8	Aghajanianpour <i>et al.</i> , (2020) Neurotox Res, 2020 https://doi.org/10.1007/s12640-020-00214-z	Rats; prefrontal cortex; tramadol 50 mg/kg daily for 3 weeks	Atrophy and apoptosis (caspase-3 and -8), pro-inflammatory markers, microgliosis, astrogliosis and neuronal death.	Agrees on combined neuronal death, astrogliosis and microglial activation in a limbic-associated region.
9	Hussein <i>et al.</i> , (2020) Ultrastruct Pathol, 2020; 44(1) https://doi.org/10.1080/01913123.2019.1711480	Juvenile and adult male mice; hippocampus; tramadol (reported as 40 mg/kg/day for 1 month in [7])	Degenerative changes in dentate gyrus, CA1 and CA3 neurons; apoptosis, astrogliosis (raised GFAP, astrocytic swelling) and microgliosis; more severe in juveniles.	Closest species match (mice) and dose to Group 3; agrees with Plate VI gliosis and neuronal degeneration.

directly comparable. Third, hippocampal GFAP mRNA fell while GFAP immunoreactivity rose in (Soares-Cardoso *et al.*, 2024), which shows that disagree and that the present conclusions apply to protein-level and gene-level readouts can to protein staining only.

DISCUSSION

This study reveals that tramadol induces dose-dependent neurotoxicity in the amygdala of adult albino mice, with 40 mg/kg causing more severe neuronal damage, astrocyte reactivity, and oxidative stress than 20 mg/kg. Histological findings from H&E staining showed mild pyknosis (20–30% of neurons) and spongiform neuropil in Group 2, escalating to extensive neurodegeneration (60% of neurons), karyolysis, and perivascular oedema in Group 3, aligning with ROS-mediated apoptosis and necrosis (Hussein & Abdel Aal, 2017; Awadalla & Salah-Eldin, 2016). These changes suggest synaptic and blood-brain barrier disruption, consistent with tramadol's vascular effects (Nakhaee *et al.*, 2021; Mehdizadeh *et al.*, 2017; Sharma *et al.*, 2007). The spongiform neuropil and dilated capillaries in Group 3 indicate severe structural compromise, potentially disrupting amygdala-mediated emotional processing (Elliott *et al.*, 2011; LeDoux, 2000). GFAP immunohistochemistry demonstrated progressive astrocyte reactivity, with Group 2 showing moderate hypertrophy (60% increased density) and Group 3 exhibiting intense GFAP staining and gliosis, indicative of robust neuroinflammation (Valentim *et al.*, 2024; Sofroniew & Vinters, 2010). Reactive astrocytes likely release pro-inflammatory cytokines (IL-1 β , TNF- α), amplifying neuronal injury and contributing to excitotoxicity via impaired glutamate uptake (Song & Zhao, 2001; Fattore *et al.*, 2004; Swanson *et al.*, 2004). This glial response may transition from adaptive in Group 2 to maladaptive in Group 3, exacerbating damage at higher doses (Pekny & Nilsson, 2005). These findings parallel those observed with other opioids, such as morphine, where chronic exposure induces gliosis and synaptic dysfunction in limbic regions (Hutchinson *et al.*, 2011; Maze & Nestler, 2011). Biochemical assays confirmed significant antioxidant depletion and elevated MDA levels, with Group 3 showing the most pronounced changes (60% glutathione reduction, 26% SOD reduction, 25% catalase reduction, 140% MDA increase). These results indicate a severe redox imbalance, with glutathione depletion and MDA elevation reflecting oxidative damage to lipid membranes (Khazdair *et al.*, 2021; Bameri *et al.*, 2022; Abdel-Zaher *et al.*, 2011). The correlation between increased MDA and histological neurodegeneration supports the role of lipid peroxidation in tramadol's neurotoxicity (Halliwell & Gutteridge, 2015). Non-significant p-values (0.250–0.979) likely stem from the small sample size (n=6), variability in tramadol metabolism via CYP2D6, and the short three-week treatment duration (Ghosh *et al.*, 2017; Hirst *et al.*, 2014). Chronic exposure, as seen in human abuse, may amplify these effects (Nakhaee *et al.*, 2021; UNODC, 2017).

Conclusion

This study demonstrates that tramadol induces dose-dependent neurotoxicity in the amygdala of adult albino mice, with 40 mg/kg causing more severe neuronal

damage, astrocyte reactivity, and oxidative stress than 20 mg/kg. Histological analyses revealed progressive pyknosis, vacuolation, and spongiform neuropil, while GFAP immunohistochemistry confirmed heightened astrocyte hypertrophy and gliosis, particularly at higher doses. Biochemical assays showed significant antioxidant depletion (glutathione, SOD, catalase) and elevated MDA levels, indicating robust oxidative stress. These findings underscore tramadol's neurotoxic potential, mediated by oxidative damage and neuroinflammation, which may contribute to emotional and cognitive impairments in chronic users. The study highlights the need for cautious tramadol use, especially in regions with high misuse rates, and calls for further research into neuroprotective interventions to mitigate its adverse effects on the amygdala and other brain regions.

Limitations of the Study

This study has several limitations that should be considered when interpreting its findings. First, the small sample size of six mice per experimental group may have limited the statistical power to detect significant differences in the biochemical parameters. Although the observed results may suggest dose-related changes, the non-significant p-values (0.250–0.979) indicate that these differences were not statistically established under the conditions of the present study. Biological variability, including differences in tramadol metabolism and individual responses to treatment, may also have contributed to the observed variations (Ghosh *et al.*, 2017). Consequently, the findings should be interpreted cautiously, and the observed trends should not be regarded as definitive evidence of dose-dependent biochemical effects.

Second, the three-week experimental period may not have been sufficient to establish the long-term consequences of tramadol exposure or determine whether the observed changes are reversible following withdrawal. Third, the investigation focused primarily on the amygdala, leaving the effects of tramadol on other brain regions involved in cognition, memory, motor coordination and emotional regulation outside the scope of the study (Eisch *et al.*, 2008). Furthermore, the use of male mice alone limits the generalisability of the findings, given that sex-related differences may influence opioid pharmacodynamics, neurotoxicity and behavioural responses (Hosseinzadeh *et al.*, 2019).

An additional limitation concerns the qualitative assessment of the histological plates. The observations of neuronal pyknosis, tissue alterations and apparent astrocytic reactivity were based on representative photomicrographs rather than a comprehensive, blinded quantitative assessment. Without systematic stereological analysis or validated image-based measurements, the magnitude of neuronal injury and astrogliosis cannot be

reliably quantified. Similarly, the absence of clearly documented section and field counts, scale bars on all photomicrographs, and quantitative immunohistochemical measurements limits the reproducibility and comparative interpretation of the histological findings. Where GFAP immunostaining is used, quantitative assessment of GFAP-positive area fraction or optical density would strengthen the evidence for astrocytic reactivity. Additional markers, including NeuN for neuronal identification, Iba-1 for microglial assessment and cleaved caspase-3 for apoptotic activity, could provide complementary evidence regarding the cellular mechanisms of injury.

Future investigations should therefore employ adequately powered experimental designs, longer exposure and recovery periods, both male and female animals, and quantitative histological and immunohistochemical methods. Statistical comparisons should be conducted using methods appropriate to the experimental design and data distribution, with effect sizes, confidence intervals and exact p-values reported where possible. These improvements would strengthen the reliability, reproducibility and biological interpretation of the findings.

Contribution to Knowledge

This study contributes preliminary experimental evidence concerning the potential neurotoxic effects of tramadol exposure on the mouse amygdala. The histological observations presented in Plates I–VI suggest differences in the appearance of amygdalar tissue across the experimental groups, including neuronal pyknosis and features interpreted as astrocytic reactivity. The higher-dose group (40 mg/kg) appeared to exhibit more pronounced histological alterations than the lower-dose group (20 mg/kg). However, because these observations were not supported by quantitative histological measurements, the apparent dose-related differences should be interpreted as qualitative findings rather than statistically confirmed dose-dependent effects. An important aspect of the study is its focus on the amygdala, a brain region involved in fear processing, anxiety, emotional learning and memory. Previous studies cited in this report have investigated tramadol-associated changes in other brain regions, including the hippocampus, cerebral and prefrontal cortex, and cerebellum. Within the literature examined for this study, which was not a systematic or exhaustive review, limited directly comparable evidence concerning tramadol-associated histological alterations and GFAP immunoreactivity in the amygdala was identified. The present findings therefore provide a basis for further investigation of the amygdala as a potential target of tramadol-associated neurotoxicity. The study also provides a preliminary framework for investigating possible relationships between tramadol exposure, oxidative stress and neuroinflammatory responses in the brain. Nevertheless, the contribution of oxidative stress and

neuroinflammation to the observed histological changes requires further confirmation through quantitative biochemical analyses and specific cellular markers. In addition, although amygdalar injury may plausibly be associated with alterations in emotional processing, the present study did not report behavioural assessments. Any relationship between the observed tissue changes and anxiety-related or other emotional behaviours must therefore be regarded as a hypothesis requiring direct experimental verification.

Recommendations

Based on the findings and limitations of the present study, the following recommendations are proposed:

1. Increase the sample size: Future studies should use an appropriate sample-size calculation based on the primary outcome, expected effect size, statistical power and anticipated variability. This would improve the ability to detect biologically meaningful differences between experimental groups.
2. Extend the experimental duration: Longer exposure periods and post-treatment recovery assessments should be considered to investigate the persistence, progression and potential reversibility of tramadol-associated alterations.
3. Include both sexes: Male and female animals should be studied to determine whether sex influences the histological, biochemical and behavioural responses to tramadol.
4. Introduce quantitative histological analysis: Future investigations should document the number of animals, tissue sections and microscopic fields examined per animal. All photomicrographs should include calibrated scale bars. Blinded assessment should be performed using predefined criteria, with ImageJ or equivalent validated software employed to quantify neuronal density, the proportion of pyknotic neurons and GFAP-positive area fraction or optical density, as appropriate. Quantitative measurements should be based on biological replicates rather than treating multiple fields from the same animal as independent experimental units.
5. Strengthen cellular and molecular validation: Immunohistochemical analyses using NeuN, Iba-1 and cleaved caspase-3 should be considered to investigate neuronal integrity, microglial responses and apoptotic activity, respectively. GFAP assessment should be accompanied by appropriate controls and clearly defined quantification procedures.
6. Improve statistical reporting: Future studies should use statistical methods appropriate to the experimental

design and distribution of the data. Where applicable, one-way analysis of variance followed by a suitable post hoc test may be used to compare three independent groups, provided the relevant assumptions are adequately assessed. Exact p-values, effect sizes and confidence intervals should be reported. If assumptions are not met, suitable alternative methods should be considered.

7. Investigate behavioural correlates: Behavioural tests assessing anxiety-like behaviour, fear responses and emotional memory should be incorporated to determine whether amygdalar histological changes are associated with measurable functional alterations.

8. Evaluate potential neuroprotective interventions: Further studies may investigate candidate antioxidant or neuroprotective agents, such as N-acetylcysteine and *Nigella sativa*, to determine whether they can attenuate tramadol-associated oxidative or histological changes. Such investigations should include appropriate controls and direct measurements of the proposed protective mechanisms before therapeutic benefits are inferred (Abdel-Zaher *et al.*, 2011; Khazdair *et al.*, 2021).

9. Promote responsible tramadol use: Public health education and appropriate clinical monitoring should continue to emphasise the risks associated with tramadol misuse and unsupervised exposure. However, recommendations regarding prescribing practice should be based on the totality of available clinical and preclinical evidence rather than on the present study alone.

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