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Ameliorative Effects of FMRFamide on 3,4-Methylenedioxyl-Methamphetamine /Tramadol-Induced Neurodegeneration in the Hippocampus

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Abstract

Objective: Tramadol and 3,4-methylenedioxy-methamphetamine (MDMA) use over an extended period is linked to deficits in memory encoding and retrieval. We aimed to determine the neurotoxic effects of co-administration of MDMA and tramadol (TRAM) on hippocampal function and investigate the potential of FMRFamide in attenuating resulting alterations in the Wistar rat model.

Methods: Thirty adult male Wistar rats were grouped into six (n=5): Control, FMRFamide, TRAM, MDMA, TRAM+MDMA, and TRAM + MDMA + FMRFamide groups. The opiates were administered orally at 20mg/kg each, while 2mg/kg of FMRFamide was administered intraperitoneally for 12 days using normal saline as a vehicle. The Barnes and Morris water mazes were used to evaluate spatial learning and memory functions, followed by H&E staining, and immunohistochemical staining for glial fibrillary acid protein (GFAP).

Results: The opiates significantly increased total latencies in the Barnes Maze test, indicating that short- and long-term memory functions were impaired. Also, high levels of escape latency were observed following MDMA administration, suggesting that MDMA reduced the spatial navigation ability of the animals. These discrepancies were noticeably extreme in animals that received co-administration of opiates. However, FMRFamide showed significant potential in attenuating the damage induced by opiates, thus repairing and restoring memory formation and retention functions.

Conclusion: FMRFamide may attenuate the Tramadol- and MDMA-mediated memory dysfunction by enhancing cholinergic and glutaminergic synthesis and transporting and restoring exploratory and navigational abilities in the hippocampus of male Wistar rats.

Keywords: 3,4-methylenedioxy-methamphetamine, Tramadol, FMRFamide, Hippocampus, Memory, Neurodegeneration

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Plain English Summary
Tramadol is a centrally acting synthetic opioid analgesic and serotonin/norepinephrine reuptake inhibitor that is similar structurally to codeine and morphine. Due to its ability to modulate the perception and response to pain and affect other pain modulators in the central nervous system, it is used in the treatment of several kinds of pain such as neuropathic pain, post-operative pain, lower back pain, osteoarthritis, fibromyalgia, and cancer. However, Tramadol can be abused alone or in combination with other substances such as 3,4-methylenedioxyl-methamphetamine, of which prolonged use can lead to memory impairment. “In this study, ameliorative effects of FMRFamide were investigated on adult Wistar rats, including its effect on the co-administration of Tramadol and 3,4-Methylenedioxyl-Methamphetamine by assessing their neurobehavioural changes and biochemical changes. The findings from this study showed that as a result of the coadministration of Tramadol and 3,4-methylenedioxy-methamphetamine, short- and long-term memory functions were impaired, and reduced spatial navigation ability of the animals was noticed, However, FMRFamide showed significant potential in ameliorating the damages induced by the opiates.

Introduction
3,4-Methylenedioxyl-methamphetamine (MDMA) is a synthetic psychoactive drug that alters the mood and perception (awareness of surrounding objects) of its user; it causes euphoria, increased energy, and empathy and its effect can last for more than 3 hours (1). MDMA is illegal in most countries and although some theory claims it can be used to combat post-traumatic stress disorder (PTSD), the fact remains that as of 2017, has no medical uses (2). MDMA is known on the street as ‘ecstasy’, ‘molly’ or scientifically as 3,4-methylenedioxyl-methamphetamine. MDMA is readily absorbed from the intestinal tract and reaches its peak concentration in the plasma about two hours after oral administration. The major metabolites of MDMA are 3,4-methylenedioxyamphetamine (MDA), 4-hydroxy-3-methoxyamphetamine (HMA), and 4-hydroxy-3-methoxymethamphetamine (HMMA). The drug is broken down metabolically mainly in the liver where an enzyme designated CYP2D6 is chiefly responsible. CYP2D6 is the main enzyme involved in MDMA metabolism and therefore an important determinant of MDMA induced toxicity 3(3).
Long-term use of MDMA in humans has been shown to produce marked neurodegeneration in occipital, prefrontal, hippocampal and striatal serotonergic axon terminals (4, 5). Studies have shown that the hippocampus is one of the regions of the brain that suffers extensive serotonergic neuron damage following MDMA consumption (6).
Tramadol is a management therapy for moderate to severe pain. This medicine can be ingested orally or injected into the body. Long-term continued use of this opioid can result in dependence and addiction. Tramadol acts by altering the serotonin neurotransmitter system. Both enantiomers of tramadol are agonists of the μ-opioid receptor and its M1 metabolite, O-demethylate, is also a μ-opioid receptor agonist but is 6 times more potent than tramadol itself. By independently enhancing noradrenergic and serotonergic activity, they work together to produce effects of analgesia in the central nervous system (CNS). Tramadol is converted by CYP450 enzymes 3A4 and 2D6 into 3 major metabolites; O-desmethyltramadol (M1), N, N-didesmethyltramadol (M3) and N, O-didesmethyltramadol (M5), 2 of which are active (7). Tramadol administration impairs memory function in rodent models by activation of μ-opioid receptors (8, 9). Chronic administration of tramadol has been associated with histological abnormalities such as increasing apoptosis in rat cerebral cortex and hippocampus (10, 11).
FMRFamide is a neuropeptide that has been described as an anti-opioid peptide. It plays a role in opioid anti-nociception, dependence and tolerance. It was first isolated out of molluscs and has a lot of functions from anti-opiate functions to modulation of muscle contraction in cardiac and non-cardiac muscles such as gut contraction and heart rate. Previous research showed that FMRFamide exhibits therapeutic potential in pain management because it mimics the analgesic benefits of opiates without causing opiate dependence. FMRFamides are also regarded as a type of anti-opiate peptide, because of their capacity to block opioid signalling in research on mammals. Some FMRFamides may lessen opiate tolerance, which makes them useful in drug addiction treatment plans (12, 13, 14).
Over 20 million people worldwide suffer from a substance use disorder, which includes alcohol, methamphetamines, and opioids. When taking prescription painkillers, some people become dependent on them. It has been demonstrated that medication-assisted treatment increases patient compliance, lowers opioid use and overdose rates, and lowers the risks of opioid use disorder. However, the potential of FMRFamide as an alternative treatment to the neurotoxic effects caused by opioids has not been explored despite its powerful anti-opioid properties. This research aimed to study the combinatory effects of MDMA and Tramadol on hippocampal integrity and the potential of FMRFamide to alleviate the damages associated with this condition. Our specific objectives

were to assess the behavioural inconsistencies in the animals and access the neurotransmitter activity levels.

Materials and Methods

Experimental animals

Thirty (30) adult male Wistar rats each weighing between 145g-165g were obtained, housed and maintained in the Institutional Animal Holding Facility at Babcock University, Ilishan-Remo, Ogun State, using standard-sized home cages under suitable environmental conditions (at 23 ± 1^0 C, a 12-h light/dark cycle and *ad libitum* food and water).

Procurement, Preparation, and Administration of Opioids and FMRFamide

Tramadol capsules were obtained from the pharmaceutical department of Babcock University Teaching Hospital (BUTH) with a prescription for research purposes. MDMA tablets were purchased from Sigma-Aldrich, St Louis, Missouri, USA. These opioids were administered to the animals at 20mg/kg in a ml of normal saline (vehicle) with the aid of an oral cannula (11, 15). Synthetic FMRFamide powder was obtained from St Louis, Missouri, USA. FMRFamide was administered to the rats at 2mg/kg in 0.5ml of normal saline (vehicle) intraperitoneally using a needle and syringe.

Research Design

The thirty (30) Wistar rats were grouped into six (6), n=5, to prevent overcrowding and provide easy identification during the research process in correlation with the experimental design. Control group received 1ml of normal saline (vehicle only), Group B received 2mg/kg of FMRFamide in normal saline, Group C received 20mg/kg of Tramadol (TRAM) in normal saline, Group D received 20mg/kg of MDMA in normal saline, Group E received 20mg/kg each of MDMA and Tramadol in normal saline, Group F received 20mg/kg each of MDMA and Tramadol and 2mg/kg of FMRFamide using normal saline as vehicle. MDMA and Tramadol were administered orally while FMRFamide was done intraperitoneally consecutively for 12 days.

Table 1: The Experimental Design

GROUPS (n=5)	TREATMENT SCHEDULE
Control	Normal saline (0.9% NaCl) (16).
FMRFamide	Intraperitoneal administration of FMRFamide in normal saline at 2mg/kg (16).
TRAM	Oral administration of Tramadol in normal saline at 20mg/kg (11).
MDMA	Oral administration of MDMA in normal saline at 20mg/kg (15).
TRAM+MDMA	Oral administration of MDMA at 20 mg/kg+ Oral administration of Tramadol at 20mg/kg in normal saline.
TRAM+MDMA+FMRfamide	Oral Co-administration of MDMA 20mg/kg + Tramadol at 20mg/kg with FMRFamide at 2mg/kg using normal saline as vehicle

The rats were allowed to acclimatize for seven days upon procurement. Administration lasted for twelve days. After the end of administration, Barnes Maze and Morris Water Maze tests were carried out.

Neurobehavioral Testing Procedure

Barnes maze test was done to assess memory formation and retention (short and long term) and the Morris water maze was carried out to test spatial memory and spatial navigation.

For the Barnes maze, the rats were first trained about three times with different time limits to get them accustomed to the position of the escape box. On the probe day, the amount of time that it took the rat to locate and enter the escape hole was recorded (*Total latency*) (17)**.**

For the Morris water maze, the rats first went through a learning probe, with a set time (120 secs). The probe day for memory and spatial navigation is done 24 hours after the training or last acquisition period. The rats were placed at each cardinal point and the time taken for the rat to find the escape platform **(***Escape Latency***)** was then recorded with a time limit of 60secs (18).

Animal Sacrifice

Sacrifice was carried out the next day after the completion of the neurobehavioral test. Experimental rats were anaesthetized with diethyl ether, after which, they were perfused intracardially with 100 mL phosphate buffered saline (PBS; 0.1 M, pH 7.4), followed by 250 mL of neutral buffered formalin.

Biochemical Procedure

The hippocampus was dissected and homogenized. It was then centrifuged (4,000 rpm for 10 min), and its supernatant was preserved at −20°C for further biochemical analysis. The neurotransmitters (serotonin, dopamine, glutamate) were analysed via Enzyme-Link Immunosorbent Assay (ELISA) at 450 nm using a microplate reader. The analysis was done according to the manufacturer's instructions in the ELISA kits.

Statistical Analysis

In this research, all the results were represented as grouped data and analysed using the GraphPad Prism 8.0 software using one-way analysis of variance (ANOVA). The results were expressed as Mean±SEM.

Results

Relative Brain Weight

Fig 1 shows the relative brain weight of the animals across the groups; Control (0.787 ± 0.009), FMRFamide only (0.946 ± 0.033), Tramadol only (0.89 ± 0.063), MDMA only (0.96 ± 0.105), Tramadol + MDMA (0.86 ± 0.023), and Tramadol + MDMA + FMRFamide (0.837 ± 0.018). There was a slight observable increase in the relative brain weights of animals that were treated compared with the control group. However, these changes were not significant at $p<0.05$.

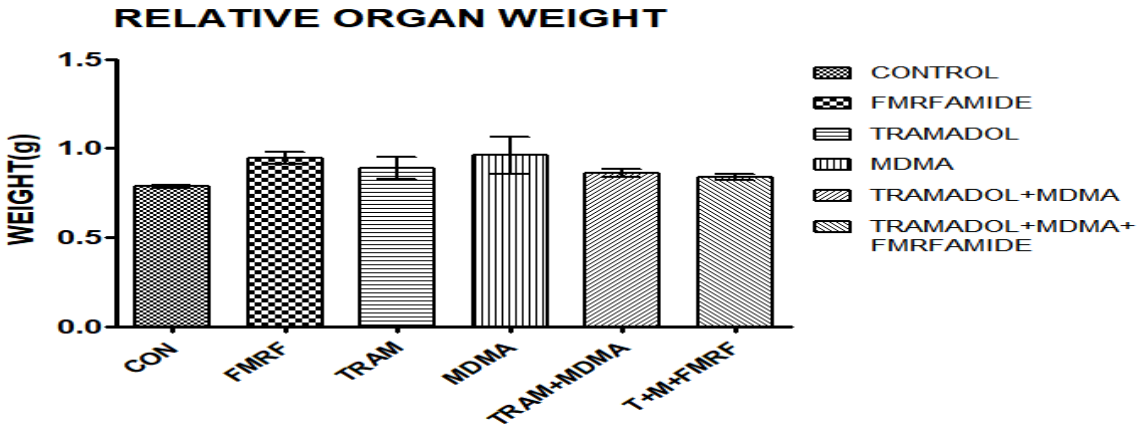

Figure 2: Graph Illustrating the Relative Brain Weights Across the Groups
Values expressed as Mean±SEM. CON: Control; FMRF: FMRFamide only; TRAM: Tramadol only; MDMA: MDMA only; TRAM+MDMA: Tramadol + MDMA; TRAM+MDMA+FMRF: Tramadol + MDMA + FMRFamide

Neurobehavioral Analysis

Table 2 summarizes the data obtained from the behavioural studies of the animals across the groups. Under our experimental conditions, Tramadol and MDMA significantly increased the short- and long-term latencies of the animals. However, FMRFamide showed strong attenuating potential from our findings. From the Barnes maze, there was a significant increase in total latencies following opioid administration when compared with the control group and the animals that received FMRFamide. More so, the Morris Water Maze showed a significant increase in the escape latencies of animals that received MDMA when compared with other groups. These findings indicate deficits in short-term and long-term memory as well as reduced spatial navigation ability of the animals following opioid administration at 20mg/kg.

However, under our experimental conditions, FMRFamide at 2mg/kg restored memory and navigation function as suggested by the obvious significant increase in escape latencies of animals that received FMRFamide after opioid administration.

Table 2: The Latencies of the animals across the groups

Groups	Short-Term Latency	Long-Term Latency	Escape Latency
Control	24.40 ± 4.250	50.00 ± 4.858 $^{\$}$	11.53 ± 3.566
FMRFamide only,	104.4 ± 20.61	56.80 ± 7.768 $^{\$}$	9.133 ± 3.540
Tramadol only	291.6 ± 6.787 $^{*\#\$}$	300.0 ± 0.0 $^{*\#\$}$	27.00 ± 6.089
MDMA only	228.6 ± 43.87 $^{*\#\$}$	225.8 ± 45.47 $^{*\#\$}$	36.67 ± 7.391$^{*\#\$}$
Tramadol + MDMA	274.4 ± 25.60 $^{*\#\$}$	283.4 ± 11.73 $^{*\#\$}$	27.20 ± 4.212
Tramadol + MDMA + FMRFamide	33.80 ± 11.52	151.6 ± 33.69 $^{*\#}$	10.40 ± 2.004

Values expressed as Mean±SEM. #: Statistical significance when compared to Control. #: Statistical significance when compared to FMRF. $: Statistical significance when compared to T+M+ FMRF

Neurotransmitter Analysis

There was an obvious increase in glutamate levels (µgg-1) of the animals that received Tramadol (0.134 ± 0.005), MDMA (0.132 ± 0.003), and opioid co-administration (0.140 ± 0.008) at 20mg/kg when compared with the control (0.128 ± 0.001) and FMRFamide (0.129 ± 0.002) groups but these differences were not significant as shown in Figure 3.

Similarly, in Figure 4, there was a slight increase in acetylcholine levels (µgg-1) of the animals that received Tramadol (0.129 ± 0.007), MDMA (0.128 ± 0.007), and opioid co-administration (0.129 ± 0.008) at 20mg/kg when compared with the control (0.12 ± 0.003) and FMRFamide (0.123 ± 0.006) groups but these differences were not significant.

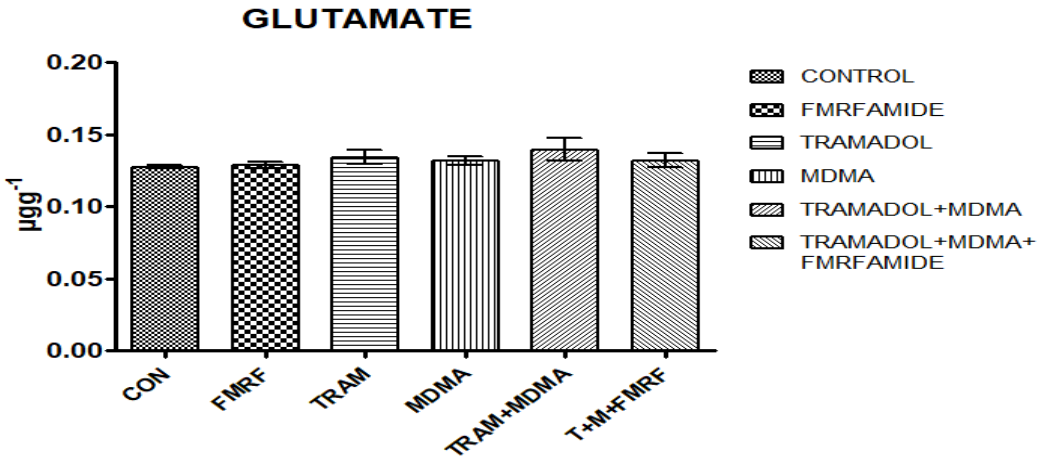

Figure 3: Glutamate Levels Across the Groups.
Values expressed as Mean±SEM. CON: Control; FMRF: FMRFamide only; TRAM: Tramadol; MDMA; TRAM+MDMA: Tramadol + MDMA; TRAM+MDMA+FMRF: Tramadol + MDMA + FMRFamide

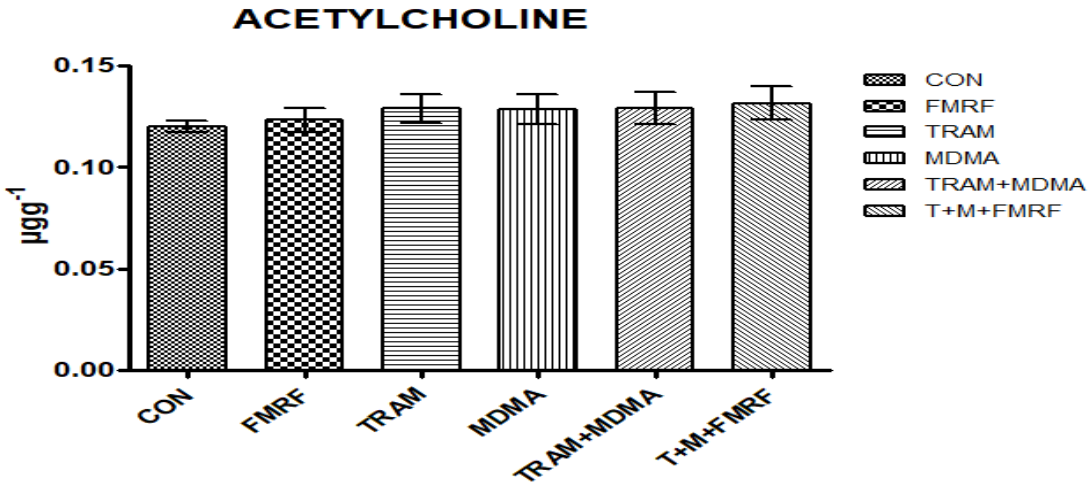

Figure 4: Acetylcholine Levels Across the Groups
Values expressed as Mean±SEM. CON: Control; FMRF: FMRFamide only; TRAM: Tramadol; MDMA; TRAM+MDMA: Tramadol + MDMA; TRAM+MDMA+FMRF: Tramadol + MDMA + FMRFamide

At p-value (<0.05), there was no statistical significance across all the groups when compared to control and each other.

Discussion

Abuse of Tramadol and 3, 4-methylenedioxymethamphetamine (MDMA) singly or together have been associated with deteriorations in memory formation and retention. Previous research has revealed that memory recall is impaired in patients with pain using opioid medication and stimulants. The well-studied positive (pleasure) and negative (stress, depression, and anxiety related to withdrawal) effects and emotion, as well as the incentive salience that distinguishes opiate use, are also connected to the opioid-dependent effects. The hippocampus plays a very important role in memory formation, consolidation, and recall. The opioids' effects on memory and learning are likely mediated by the hippocampus (19, 20).

This research sought to investigate the behavioural changes that the drugs have on the hippocampus and the potential of FMRFamide as an ameliorative agent. The results of this study demonstrated that although, MDMA abuse had more neurotoxic effect than tramadol, co-abuse of MDMA and tramadol elicited the most neurotoxic effect.

The hippocampus is essential for cognition, learning, and spatial navigation. For awareness of conscious knowledge, hippocampal neurons send/receive input to/from higher cortical areas, thus maintaining the balance between memory encoding and retrieval. Damage to these neurons will hamper the memory for the sequence of several recently visited spatial locations (21,22). Under our experimental conditions, the groups Tramadol only, MDMA only, and Tramadol + MDMA had significantly higher total latencies in the Barnes maze suggesting impaired short- and long-term memory functions. These findings are similar to reports on opiate use disorder from both Razavi *et al*. 2014 (23) and Liu *et al.* 2016 (24). Animals that received FMRFamide after co-administration of MDMA and tramadol had significantly lower latencies indicating that FMRFamide was able to repair the discrepancies. Although the total latency was relatively lowered in this group, the damage the opiates caused was still evident. These findings are congruent with findings from Matthys *et al.* 2011 (25). The results from the Morris Water maze test showed a higher escape latency for the groups TRAM only, MDMA only and TRAM+MDMA in comparison to the other groups. However, the difference was only significant for the animals in the MDMA group. This suggests that MDMA has the most negative effect on the spatial navigation and memory of animals which correlates with findings from Arias-cavieres *et al.* 2010 (26) and Baghishani *et al.* 2018 (11).

With continued use, opiates trigger dependencies in humans and animals by altering functional neurotransmitter pathways leading to tolerance and later, addiction. Dopamine, Serotonin, gamma-aminobutyric acid (GABA), glutamate and norepinephrine are established pathways affected by opioid use disorder (27, 28). In this study, we checked levels of glutamate and acetylcholine which play important roles in hippocampal mediation of cognition, learning, mood, and spatial navigation (29, 30, 31). There was a slight increase in the levels of glutamate and acetylcholine post-opiate administration; contrary to findings from Olatunji et al., 2020 (32) and Mowaad et al., 2022 (33), respectively. The differences were not significant; consistent with findings from Abdel-Salam *et al.* 2016 (34) who reported that the acetylcholine system was not affected by tramadol. Also, in combination with tramadol, MDMA did not alter brain glutamate and acetylcholine activity. This suggests that tramadol has a modulatory action on the effects of MDMA on glutamate and acetylcholine activity in the hippocampus. The animals who received FMRFamide only had similar results to those of the control. It was observed that FMRFamide treatment after opiate administration slightly lowered the levels of glutamate activity; hinting at the modulatory effects of this neuropeptide reported by Raffa and Bianchi in 1986 (35).

Conclusion

In conclusion, this study examined the effects of Tramadol, MDMA and FMRFamide on the hippocampus. It was observed that Tramadol and MDMA given separately and in co-administration produced alterations in hippocampal structure, which were observed to be alleviated by the introduction of FMRFamide. Also, it examined the effects of Tramadol and MDMA when administered individually and together, as well as the effects of FMRFamide on short, long term and spatial memory. The damaging effects of Tramadol and MDMA on the short-term, long-term and spatial memories of the animals were corrected and restored to a certain extent by FMRFamide. However, in this study, we observed that these drugs caused minimal to no alterations in levels of acetylcholine and glutamate. All these conclusively show that FMRFamide attenuated the spatial and memory dysfunction induced by the co-administration of Tramadol and MDMA in the hippocampus of male Wistar rats.

We recommend that further investigations be carried out on the effectiveness of FMRFamide in the treatment of neurodegeneration caused by these psychoactive drugs. We also recommend that actions targeted at Methamphetamine and opioid use disorder awareness should be done to sway public perceptions on the damaging effects of these psychoactive drugs to influence behaviours and reduce abuse.

List of Abbreviations

ANOVA:	Analysis of Variance
BUTH:	Babcock University Teaching Hospital
CA1:	Cornus Ammonis 1 region;
ELISA:	Enzyme-Link Immunosorbent Assay
FMRFa:	FMRFamide
HMA:	4-hydroxy-3-methoxyamphetamine
HMMA:	4-hydroxy-3-methoxymethamphetamine
MDMA:	3,4-methylenedioxy-methamphetamine;
PBS:	Phosphate buffered saline;
SEM:	Standard Error of Mean
TRAM:	Tramadol

Declarations

Ethics considerations

All experimental activities were carried out in compliance with guidelines set by the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NRC, 2010). The research protocol was approved by the Babcock University

Health Research Ethics Committee (approval number BUHREC 753/19).

Consent for publication
All the authors gave consent for the publication of the work under the Creative Commons Attribution-Non-Commercial 4.0 license. We otherwise convey all copyright ownership, including all rights incidental thereto, exclusively to the journal when published.

Availability of data and materials
Data generated in this study are available from the authors upon reasonable request.

Competing interests
The authors have no conflicts of interest to declare.

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Author Contributions
The study's inception and design involved input from all authors. OJA, OOE, and OJO gave the conceptual framework for this research work. AD handled the material preparation, and data collecting, SO and AT carried out the data analysis. OOE wrote the manuscript's initial draft and AKO, ATP, and DM proofread it with feedback from OSY, EJ, AL, AS, OP, AM, OGA, and SO. The last draft was reviewed by all authors and got their approval.

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