Histopathological and Biochemical Investigations of the Brain upon Sub-acute Pentazocine Administration.

Comment [1]:

ABSTRACT

Objective: Despite its common use among sickle-cell disease patients, pentazocine addiction in Nigeria has become a health concern among youths battling with diverse forms of pain. This study aimed to evaluate the toxicological effects of pentazocine on the cerebral cortex. **Methods**: Twenty-eight (28) adult Wistar rats were acclimatized and grouped into four. Group 1 (control) was administered 1ml of normal saline (0.9% NaCl) while groups 2 – 4 served as the experimental groups and administered graded doses of 30 mg/kg, 60 mg/kg, and 90 mg/kg pentazocine (PZ) intramuscularly for 14 days. Brain tissues were obtained and analyzed for biochemical parameters such as catalase (CAT), superoxide dismutase (SOD), and malondialdehyde (MDA), as well as histopathological analyses of sample tissues. A one-way analysis of variance (ANOVA) was used to analyze the differences between the groups using the Statistical Package for Social Sciences (SPSS) version 23.0. **Results**: In PZ-treated groups, the activities of CAT and SOD in the brain decreased while MDA levels increased ($p < 0.05$). The cerebral cortex of PZ-treated groups showed granular cell aggregations, moderate neuronal cell loss, and pyramidal cell loss. **Conclusion**: Sub-acute pentazocine administration was shown to cause moderate toxicity and could lead to brain inflammatory responses.

Keywords: Pentazocine, Toxicity, Brain, Sub-acute

INTRODUCTION

Analgesics are a diverse collection of medications and treatments recommended by medical professionals to relieve pain. The mechanism of action is the activation of certain receptors in the central nervous system by these compounds, therefore altering the perception of pain and initiating relief (Argoff, 2011; Wolkerstorfer et al., 2016). Analgesics often have a depressant effect on the central nervous system, leading to tiredness and inhibition of respiratory function. Moreover, they possess the capacity to influence cognitive functions such as preserving working memory and modulating attention (Boom et al., 2012; Webster and Karan, 2020). The relationship between opioid dependence and brain alterations is particularly relevant in connection with the management of sickle cell disease (SCD) patients, who often experience chronic pain and may be prescribed opioids for managing the pain.

SCD is usually correlated with recurrent painful episodes and patients frequently rely on opioids for pain relief, which can lead to long-term use and potential dependence (Smith, 2014; Lakkakula et al., 2018; Carroll, 2020). Several studies have shown that chronic opioid use can lead to changes in brain structure and function, particularly in areas related to pain processing, reward, and emotional regulation (Baldacchino et al., 2012; Mohamed and Mahmoud, 2019; Yang and Chang, 2019). Furthermore, it is important to state that while investigating the relationship between opioid dependence and brain changes in SCD patients, several truths into the neurobiological mechanisms of pain and addiction can be elucidated in addressing the

duration-specific effects of opioid use on brain health in SCD patients towards pain management, improvement of mental health, and overall quality of life.

Among the classes of opioid analgesics, pentazocine is specifically known as a mixed agonist-antagonist, and acts on the κ-opioid receptors while acting mildly on μ-opioid receptors (Mori et al., 2015; Cruz and Granados-Soto, 2022). This allows it to provide effective analgesia while potentially minimizing the dysphoric and psychotomimetic effects that can occur with pure μ-opioid agonists (Cruz and Granados-Soto, 2022). Pentazocine has been known to produce fewer side effects compared to stronger opioids like morphine, making it a more tolerable option for patients who may already be dealing with multiple health issues related to SCD (Riley III et al., 2010; King et al., 2013). In Nigeria, pentazocine is a potent opioid analgesic often used to relieve moderate to severe pain related to the medical therapy of sickle cell disease (Adewoyin et al., 2019; Mba et al., 2024). A recent study had indicated that the use of pentazocine during the sub-acute period may result in pathological issues connected to the cardiovascular system (Hart and Adheke, 2024).

In association with some literature, chronic exposure to analgesics results in oxidative stress in the brain, exerting a significant impact on neurological health through mitochondrial damage, brain inflammation, and other inflammatory mechanisms including the release of neurotransmitters, including glutamate (Joshi et al., 2014; Vašková et al., 2016; Mohamed and Mahmoud, 2019). Notably, the administration of sub-acute analgesics has been associated with decreases in the activity of antioxidant enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase (GPx), which are involved in oxidative stress disorders (Yousef et al., 2010; Samarghandian et al., 2014; Hart and Adheke, 2024). Despite the well-documented analgesic properties of pentazocine, the potential short-term consequences of its inappropriate use on neurological well-being have not been well investigated. Although this therapy has shown efficacy in reducing pain, its use during the sub-acute period is not without potential negative consequences. The main aim of this study is to examine the toxicological effects of sub-acute pentazocine administration on the brain of Wistar rats.

MATERIALS AND METHODS

Drugs and Chemicals

Injections of pentazocine (PZ) (marketed as Pentabeta-30) were obtained from Dooka Pharmacy, Port-Harcourt. The drug was dissolved in normal saline (0.9% NaCl) and administered to the experimental animals (albino rats) intramuscularly. Normal saline served as a placebo drug.

Animals and Animal Handling

The current study used a randomized controlled trial (RCT) research methodology. The experimental rat sample size was determined by using the resource equation technique, utilizing degrees of freedom (E) as specified in the procedures defined by Festing et al. (2006). The study included four groups, each consisting of seven rats, denoted as “k”. Hence, the mathematical representation for E was derived as kn-k = (28-4) = 24. Given that the value of E is more than 20, it was considered sufficient to use seven rats per group to determine the ideal sample size. After receiving authorization, a cohort of twenty-eight albino Wistar rats, with weights ranging from 100 to 140g, were used as experimental animal models. The animals were then accommodated at the Zoo-research Laboratory of Basic Medical Sciences, situated at the

University of Port-Harcourt. All the animals were separately housed in four wooden cages, each particularly built to fit seven creatures. In line with the regulations for the protection of animals used in scientific procedures in the Directive 2010/63/EU, proper handling of cages was done and the conditions under which animals are kept were favorable throughout the research process. Cages should be designed to provide adequate space, comfort, and enrichment for the animals. To maintain thermal equilibrium, the temperature of the surroundings was consistently regulated at roughly 25°C, while the daily relative humidity fluctuated between 40% and 48%. Trained personnel were responsible for the handling of animals and the maintenance of their cages. The animals had two weeks of physiological adaption before the trial began. Furthermore, apart from their usual meals, they were mandated to be provided with unlimited amounts of water for consumption. It is essential that the management techniques and procedures for experimental animals strictly comply with the criteria established by the Animal Use and Care Committee of the National Veterinary Research Institute in Vom, Nigeria. Authorization has been granted by the University of Port Harcourt's Research Ethics Committee, under reference number UPH/CEREMAD/REC/MM95/023.

Experimental Model and Drug Treatment

In a preliminary study conducted by Hart and Adheke (2024), it was determined that the terminal dose necessary to induce mortality in fifty percent (50%) of the laboratory rats was 175 mg/kg. Therefore, Wistar rats were randomly allocated to four groups. One cohort was assigned as the control group and administered a 1 ml dosage of a normal saline solution containing 0.9% sodium chloride concentrate. Over 14 days, pentazocine was delivered intramuscularly to treatment groups 2, 3, and 4 at dosages of 30 mg/kg (0.1ml PZ), 60 mg/kg (0.25ml PZ), and 90 mg/kg (0.4ml PZ) accordingly.

The low dose was measured for group 2 using the formulae of calculating dose volume from 30 mg/ml;

Dosage (mg) = $\frac{Average\ Body\ weight\ of\ animal\ (g)}{1000g}$ x dose = $\frac{120\ g}{1000g} x\ 30mg$ = 3.6 mg

If 30 mg of stock solution = 1 ml of dosage; therefore, 3.6 mg of stock solution will give a calculated volume of 3.6mg divided by 30 mg of stock solution which is approximately 0.1 ml.

The medium dose was measured for group 3 using the same formulae as shown above to calculate the dose volume from 60 mg/ml to give 7.2 mg. If 30 mg of stock solution gives 1 ml of dosage, 7.2 mg of stock solution will give a calculated volume of approximately 0.25 ml.

Finally, the high dose was measured for group 4 using the above formulae of calculating dose volume from 90 mg/ml to give 10.8 mg. If 30 mg of stock solution gives 1 ml of dosage, 10.8 mg of stock solution will give an approximate volume of 0.4 ml.

All treatments were administered once daily between 11 am and 12 pm, and the experimental rats were monitored for 3 hours after the drug delivery. After the regular daily meal, the animals were furnished with their customary access to drinkable water. Nevertheless, their usual dietary routine was decreased and carefully supervised. Upon completion of the experiment, after the animals had been given each dose, they were only provided with water for drinking and thereafter subjected to rigorous monitoring over the next 24 hours until they were all executed

the next day. Following the sacrificial process, surgical acquisition of neurological and intestinal tissue samples was performed. These samples were then homogenized using an Ultra-Turrax homogenizer with a cold 50 mM phosphate buffer in a 1:4 (m/v) ratio for further biochemical analysis.

Biochemical Assessment of the Brain

The Sinha (1972) method was used to quantify the activity of catalase (CAT). Here, the brain tissues were obtained and homogenized in phosphate buffer (at pH 7.0) to create a tissue homogenate of about 1.0 mg protein/ml. The homogenate was then centrifuged to remove debris to obtain a clear supernatant. Later, the Bradford assay was used to determine the protein concentration in the supernatant. A dichromate/acetic acid reagent was prepared by mixing a 5% solution of potassium dichromate ($K_2Cr_2O_7$) with glacial acetic acid in the ratio of 1:3 (by volume). Then, a 0.2 M hydrogen peroxide (H_2O_2) solution was also prepared. Inside small test tubes, we add a specific volume of the tissue supernatant to a series of tubes containing varying amounts of hydrogen peroxide. The reaction was monitored for about 2 minutes at room temperature. Subsequently, the reaction was stopped by adding the dichromate/acetic acid reagent to the mixture and heated for some time. Finally, the absorbance of the resulting chromic acetate was measured at a wavelength of between 570-610 nm using a spectrophotometer. Then, the catalase activity (K) was determined based on the decrease in hydrogen peroxide concentration.

The Misra and Fridovich (1972) method were used to compute the activity of superoxide dismutase (SOD). The brain tissues were homogenized in phosphate buffer to extract the superoxide dismutase. The homogenate was then centrifuged to remove debris, and the supernatant was collected for analysis. The protein concentration of the tissue extract was determined using the Bradford assay method. Later, the SOD activity was assessed by measuring its ability to inhibit the autoxidation of epinephrine. Here, a specific concentration of epinephrine is prepared in a buffered solution (e.g., Tris or sodium carbonate buffer). The tissue extract containing the SOD is added to the epinephrine solution. The reaction was monitored spectrophotometrically by measuring the increase in absorbance at 480 nm. The rate of increase in absorbance was recorded, and the percentage of inhibition of epinephrine oxidation is calculated. The results were plotted to determine the concentration of SOD.

The methodology of reactive components of thiobarbituric acid (TBA) (Buege and Aust, 1978) was employed to evaluate the lipid peroxidation marker, malondialdehyde (MDA). Here, the brain tissues were first homogenized in phosphate buffer to create a tissue extract to help release lipids and proteins from the tissue cells to produce a homogenate. The homogenate was then subjected to a solvent extraction process using a mixture of chloroform and methanol to separate the lipids from proteins and other cellular components. Later, the solution is allowed to settle, and the lower organic phase (containing the lipids) was collected. The protein concentration in the remaining aqueous phase can be quantified using the Bradford assay. The TBA reagent was prepared and combined with the lipid extract. The mixture is then heated using a boiling water bath for about 2 minutes. After heating, the solution was cooled and centrifuged to remove any precipitate. Later, the absorbance of the supernatant was measured at 535 nm using a spectrophotometer. The concentration of MDA was calculated using a standard curve generated from known concentrations of MDA.

Histopathological Analysis of the Cerebral Cortex

To conduct standard histopathology procedures, brain samples were obtained from sacrificed rats. After being preserved in a 10% formaldehyde solution, the samples were hydrated using ethanol at different percentages, namely 75%, 90%, 95%, and 100%. After the dehydration process, the materials underwent further purification by two cycles of xylene extraction processing. The samples were then submerged and made translucent by being covered with molten paraffin wax while being subjected to pressure. Divisible paraffin slices, each measuring 5 µm in thickness, were generated using a sled microtome. Moreover, the slices were positioned on glass slides and subjected to a sequential staining procedure using hematoxylin and eosin-colored dyes. Following an exhaustive examination of the stained slides, their images were then compared. Utilizing an Accu-Scope 3000 digital microscope, photomicrographs were produced.

Methods of Data Analysis

This study was conducted using Version 23.0 of the Statistical Package for Social Sciences (SPSS). An exhaustive examination of the obtained data was carried out using both descriptive and inferential statistical methods. Next, the comparisons between the groups were evaluated using the least significant difference (Post hoc) test after doing a one-way analysis of variance (ANOVA). Statistical significance is shown by a p-value below 0.05, and a 95% confidence range will be used.

RESULTS

Changes in Oxidative Stress Parameters of Brain

Table 1 demonstrates that all experimental groups exhibited a reduction in the antioxidant enzyme activity of CAT and SOD when compared to the control groups. While the control group had an average serum CAT level of 1.07 nmol/mg, the experimental groups in this study, who were administered pentazocine (PZ) at low, medium, and high dosages, had average serum CAT levels of 0.87, 0.85, and 0.88 nmol/mg, respectively.

Furthermore, the control group had an average SOD content of 0.36 nmol/mg. In contrast, the experimental groups in this study, which were administered very low, moderate, and high doses of pentazocine (PZ), had average superoxide dismutase (SOD) levels of 0.31, 0.29, and 0.28 nmol/mg, respectively.

Notably, all experimental groups exhibited considerable improvements in the rate of MDA lipid peroxidation relative to the control group. The average MDA levels of the experimental groups exposed to dosages of PZ ranging from low to high were 0.48, 0.48, and 0.49 nmol/mg, respectively, compared to the average MDA values of 0.40 nmol/mg seen in the placebo group.

Histopathology of the Cerebral Cortex

In comparison with the control group (A), the histopathological architecture of cerebral regions of B, C, and D showing moderate neuronal cell loss and vacuolations of the outer molecular layer.

Table 1. Effects of pentazocine treatment on antioxidant enzyme activity and lipid peroxidation status in the brain of rats

Treatment Groups	CAT (nmol/mg)	SOD (nmol/mg)	MDA (nmol/mg)
	Mean ± S.E.M	Mean ± S.E.M	Mean ± S.E.M
1mL saline (control)	1.07 ± 0.14	0.36 ± 0.05	0.40 ± 0.06
0.1mL PZ (low dosage)	0.87 ± 0.24**a**	0.31 ± 0.01**a**	0.48 ± 0.02**a**
0.25mL PZ (medium dosage)	0.85 ± 0.16**a**	0.29 ± 0.01**a**	0.48 ± 0.02**a**
0.4mL PZ (high dosage)	0.88 ± 0.15**a**	0.28 ± 0.04**a,b**	0.49 ± 0.04**a**

a = indicates the least significant difference in comparison with the control group at $p < 0.05$
b = indicates the least significant difference in comparison with 0.1mL PZ group at $p < 0.05$

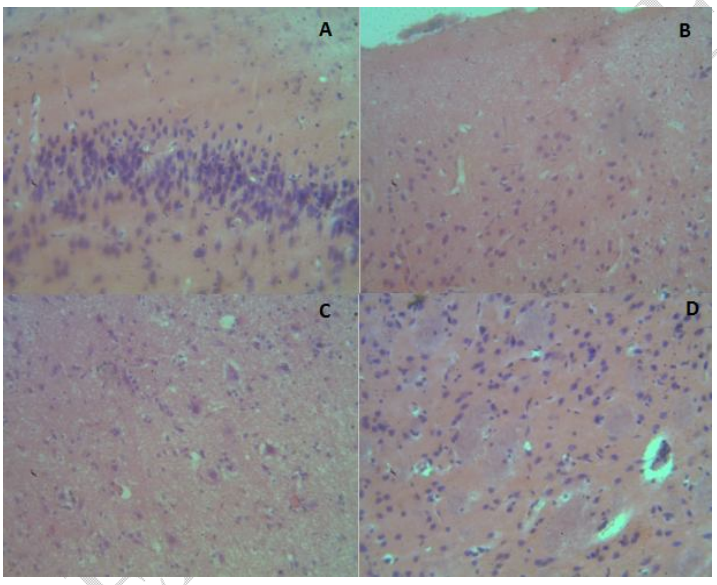

Figure 1. Photomicrograph of Cerebral cortex showing the control (A) and experimental groups (B, C, and D).

Comment [2]: Mention the lesions in the experimental group.
Mention the stains used and the magnification.

DISCUSSIONS

The results of this study demonstrate a significant decrease in the activity of the antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD) in the brain across all experimental groups, in comparison to the control group. Catalase (CAT) and superoxide dismutase (SOD) are crucial enzymes that have a fundamental function in safeguarding against oxidative stress by eliminating reactive oxygen species (ROS) (Puppel et al., 2015). Significant reductions in mean catalase (CAT) and superoxide dismutase (SOD) levels were seen when comparing the control group to the experimental groups prescribed low, medium, and high doses of pentazocine. The polymer pentazocine functions as an inhibitor of the antioxidant defense mechanisms mediated by catalase (CAT) and superoxide dismutase (SOD). Oxidative stress-induced lipid peroxidation, protein oxidation, and DNA damage responses may directly harm neurons in the brain. The

compromise of neuronal activity, decrease in synapse regeneration capacity, and obstruction of neurotransmitter transmission caused by oxidative damage may affect cognitive function and mood regulation (Chen et al., 2012; Singh et al., 2019). Furthermore, oxidative stress may trigger neuroinflammation in the brain, marked by the activation of microglia and the release of pro-inflammatory cytokines. Neuropathic inflammation may disrupt the electrical impulse communication between neurons, expedite the terminal degeneration of neurons, and contribute to the pathogenesis of neurodegenerative diseases (Teleanu et al., 2022).

Conversely, the laboratory findings showed significant increases in lipid peroxidation activity, as evaluated by malondialdehyde (MDA) levels, across all experimental groups as compared to the control group. Lipid peroxidation is the process of lipids being oxidatively broken down by a series of biological processes, which include the production of reactive oxygen species (ROS). A subsequent product of lipid peroxidation, malondialdehyde (MDA) is often used as a biomarker to assess the degree of oxidative stress (Niki, 2020). The present study found that the experimental groups receiving pentazocine therapy had higher average MDA levels compared to the control group. The experimental findings indicate that the administration of pentazocine resulted in heightened lipid peroxidation activities. Prior investigations have shown that the regular administration of different dosages of tramadol via diverse delivery methods results in decreased brain superoxide dismutase (SOD) activity and increased brain malondialdehyde (MDA) levels in mice and rat models (Ghoneim et al., 2014; Nafea et al., 2016; Hussein et al., 2017; Ali et al., 2020). Furthermore, further scientific investigations have shown that oxidative stress may compromise the structural integrity of the blood-brain barrier, therefore enabling the penetration of inflammatory chemicals and toxins into the brain. Furthermore, this might expedite the progression of neuroinflammation and neuronal degeneration, therefore endangering the whole of the brain's health and functionality (Teleanu et al., 2022).

Dosage-dependent pentazocine administration to different groups of rats and subsequent examination of the histology of the cerebral cortex showed pathological characteristics such as aggregation of granular cells with a moderate decrease in neuronal cells on the cerebral matrix, vacuolations in the outer molecular layer, and loss of pyramidal cells. Previous experimental studies have shown that the administration of both tramadol and tapentadol by injection results in histological changes in the cerebral cortex, such as neuronal degeneration and gliosis (Awadalla and Salah-Eldin, 2016; Faria et al., 2017). Combined exposure to tramadol and tapentadol triggers glial activation marked by the rapid proliferation of microglia and is associated with the formation of dysfunctional and asymmetrical neurons. Significant histological changes were shown to be directly proportional to the amount of tapentadol administered. Moreover, in a similar study, the treatment of tramadol led to neuronal expansion (Barbosa et al., 2021).

CONCLUSION

Overall, the groups of rats subjected to sub-acute pentazocine (PZ) showed a moderate degree of toxicity. The treatment of various doses of PZ in numerous groups of rats was associated with distinct changes in anti-oxidative activity, as well as pathological abnormalities in the histology of the cerebral cortex. A limitation of the present study was that the assessment of systemic inflammation markers in the blood such as cytokines was not done to ascertain for brain tissue inflammation. Therefore, it is recommended to conduct more comprehensive investigations on systemic inflammation upon PZ administration should be considered. Furthermore, local healthcare and pharmaceutical institutions need to provide cautionary comments about the use of pentazocine for managing and treating clinical conditions related to pain.

REFERENCES

Comment [3]: FOLLOW THE REFERENCE FORMAT GIVEN BY THE EDITORS

1. Argoff, C. (2011). Mechanisms of pain transmission and pharmacologic management. *Current medical research and opinion*, *27*(10), 2019-2031.
2. Wolkerstorfer, A., Handler, N., & Buschmann, H. (2016). New approaches to treating pain. *Bioorganic & Medicinal Chemistry Letters*, *26*(4), 1103-1119.
3. Boom, M., Niesters, M., Sarton, E., Aarts, L., W. Smith, T., & Dahan, A. (2012). Non-analgesic effects of opioids: opioid-induced respiratory depression. *Current pharmaceutical design*, *18*(37), 5994-6004.
4. Webster, L. R., & Karan, S. (2020). The physiology and maintenance of respiration: a narrative review. *Pain and Therapy*, *9*, 467-486.
5. Adewoyin, A., Adeyemi, O., Davies, N., & Ojo, M. (2019). Clinical and socio-demographic determinants of pentazocine misuse among patients with sickle cell disease, Benin City, Nigeria: A case-control study. *Pan African Medical Journal*, *34*(1).
6. Mba, U. C., Onyebueke, G., Okwesili, O. R., Enyanwuma, I. E., Okorie, G. M., Ohayi, S. R., & Okenwa, W. O. (2024). Tissue necrosis: A burden of pentazocine abuse in South East Nigeria. *Journal of West African College of Surgeons*, *14*(2), 159-165.
7. Hart, J. S., & Adheke, O. M. (2024). Evaluating cardiotoxicity upon chronic administration of graded doses of pentazocine in wistar rats. *The Egyptian Journal of Forensic Sciences and Applied Toxicology*, *24*(2), 73-78.
8. Carroll, C. P. (2020). Opioid treatment for acute and chronic pain in patients with sickle cell disease. *Neuroscience Letters*, *714*, 134534.
9. Smith, W. R. (2014). Treating pain in sickle cell disease with opioids: clinical advances, ethical pitfalls. *Journal of Law, Medicine & Ethics*, *42*(2), 139-146.
10. Lakkakula, B. V., Sahoo, R., Verma, H., & Lakkakula, S. (2018). Pain management issues as part of the comprehensive care of patients with sickle cell disease. *Pain Management Nursing*, *19*(6), 558-572.
11. Yang, S., & Chang, M. C. (2019). Chronic pain: structural and functional changes in brain structures and associated negative affective states. *International journal of molecular sciences*, *20*(13), 3130.
12. Baldacchino, A., Balfour, D. J. K., Passetti, F., Humphris, G., & Matthews, K. (2012). Neuropsychological consequences of chronic opioid use: a quantitative review and meta-analysis. *Neuroscience & Biobehavioral Reviews*, *36*(9), 2056-2068.
13. Mori, T., Itoh, T., Yoshizawa, K., Ise, Y., Mizuo, K., Saeki, T., & Suzuki, T. (2015). Involvement of μ□and δ□opioid receptor function in the rewarding effect of (±)□pentazocine. *Addiction Biology*, *20*(4), 724-732.
14. Cruz, S. L., & Granados-Soto, V. (2022). Opioids and opiates: pharmacology, abuse, and addiction. In *Neuroscience in the 21st Century: From Basic to Clinical* (pp. 4045-4082). Cham: Springer International Publishing.
15. Riley III, J. L., Hastie, B. A., Glover, T. L., Fillingim, R. B., Staud, R., & Campbell, C. M. (2010). Cognitive–affective and somatic side effects of morphine and pentazocine: side-effect profiles in healthy adults. *Pain Medicine*, *11*(2), 195-206.
16. King, C. D., Goodin, B., Glover, T. L., Riley, J. L., Hou, W., Staud, R., & Fillingim, R. B. (2013). Is the pain-reducing effect of opioid medication reliable? A psychophysical study of morphine and pentazocine analgesia. *PAIN®*, *154*(3), 476-483.
17. Joshi, J. C., Ray, A., & Gulati, K. (2014). Differential modulatory effects of morphine on acute and chronic stress induced neurobehavioral and cellular markers in rats. *European Journal of Pharmacology*, *729*, 17-21.

18. Vašková, J., Kočan, L., & Vaško, L. (2016). Oxidative stress and opioids. *Global Journal of Anesthesiology*, *3*(1), 020-029.
19. Mohamed, H. M., & Mahmoud, A. M. (2019). Chronic exposure to the opioid tramadol induces oxidative damage, inflammation and apoptosis, and alters cerebral monoamine neurotransmitters in rats. *Biomedicine & pharmacotherapy*, *110*, 239-247.
20. Yousef, M. I., Omar, S. A., El-Guendi, M. I., & Abdelmegid, L. A. (2010). Potential protective effects of quercetin and curcumin on paracetamol-induced histological changes, oxidative stress, impaired liver and kidney functions and haematotoxicity in rat. *Food and Chemical Toxicology*, *48*(11), 3246-3261.
21. Samarghandian, S., Afshari, R., & Farkhondeh, T. (2014). Effect of long-term treatment of morphine on enzymes, oxidative stress indices and antioxidant status in male rat liver. *International journal of clinical and experimental medicine*, *7*(5), 1449.
22. Festing, M. F. (2006). Design and statistical methods in studies using animal models of development. *Ilar Journal*. *47*(1), 5-14.
23. Sinha, A. K. (1972). Colorimetric assay of catalase. *Analytical biochemistry*. *47*(2), 389-94.
24. Misra, H. P., Fridovich, I. (1972). The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *Journal of Biological chemistry*. *247*(10), 3170-5.
25. Buege, J. A., Aust, S. D. (1978). Microsomal lipid peroxidation. *In Methods in enzymology*.*52*, 302-310.
26. Puppel, K., Kapusta, A., & Kuczyńska, B. (2015). The etiology of oxidative stress in the various species of animals, a review. *Journal of the Science of Food and Agriculture*, *95*(11), 2179-2184.
27. Chen, X., Guo, C., & Kong, J. (2012). Oxidative stress in neurodegenerative diseases☆. *Neural regeneration research*, *7*(5), 376-385.
28. Singh, A., Kukreti, R., Saso, L., & Kukreti, S. (2019). Oxidative stress: a key modulator in neurodegenerative diseases. *Molecules*, *24*(8), 1583.
29. Teleanu, D. M., Niculescu, A. G., Lungu, I. I., Radu, C. I., Vladâcenco, O., Roza, E., & Teleanu, R. I. (2022). An overview of oxidative stress, neuroinflammation, and neurodegenerative diseases. *International journal of molecular sciences*, *23*(11), 5938.
30. Niki, E. (2020). Dual stressor effects of lipid oxidation and antioxidants. In *Oxidative stress* (pp. 249-262). Academic Press.
31. Ghoneim, F. M., Khalaf, H. A., Elsamanoudy, A. Z., Helaly, A. N. (2014). Effect of sub-acute usage of tramadol on motor cerebral cortex and testicular tissues of adult male albino rats and the effect of its withdrawal: Histological, immunohistochemical and biochemical study. *International Journal of Clinical and Experimental Pathology*. *7*, 7323–7341.
32. Nafea, O. E., ElKhishin, I. A., Awad, O. A., & Mohamed, D. A. (2016). A study of the neurotoxic effects of tramadol and cannabis in adolescent male albino rats. *Int J Sci Rep*, *2*(7), 143-154.
33. Hussein, S. A., Ismail, H. K., & Abdel Aal, S. A. (2017). Effect of tramadol drug on some biochemical and immunological parameters in albino male rats; evaluation of possible reversal following its withdrawal. *Benha veterinary medical journal*, *33*(2), 418-429.
34. Ali, H. A., Afifi, M., Saber, T. M., Makki, A. A., Keshta, A. T., Baeshen, M., Al-Farga, A. (2020). Neurotoxic, hepatotoxic and nephrotoxic effects of tramadol administration in rats. *Journal of Molecular Neuroscience*. *70*, 1934-42.

35. Awadalla, E. A., & Salah-Eldin, A. E. (2016). Molecular and histological changes in cerebral cortex and lung tissues under the effect of tramadol treatment. *Biomedicine & Pharmacotherapy*, *82*, 269-280.
36. Faria, J., Barbosa, J., Leal, S., Afonso, L. P., Lobo, J., Moreira, R., & Dinis-Oliveira, R. J. (2017). Effective analgesic doses of tramadol or tapentadol induce brain, lung and heart toxicity in Wistar rats. *Toxicology*, *385*, 38-47.
37. Barbosa, J., Faria, J., Garcez, F., Leal, S., Afonso, L. P., Nascimento, A. V., & Dinis-Oliveira, R. J. (2021). Repeated administration of clinically relevant doses of the prescription opioids tramadol and tapentadol causes lung, cardiac, and brain toxicity in wistar rats. *Pharmaceuticals*, *14*(2), 97.