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OXIDATIVE STRESS AND HISTOPATOLOGICAL CHANGES IN THE STOMACH OF ADULT MALE ALBINO RATS ADMINISTERED TRAMADOL

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ABSTRACT

The use of self-administered analgesics has become popular in recent times; this has resulted into a major global concern. This study was undertaken to investigate the oxidative stress and histopathological changes in the stomach of adult male albino rats administered with tramadol. Twenty albino male rats were randomly grouped into four (4) with n=5. Group 1 served as the control. The others group 2-4 were administered with tramadol orally for twenty-one days at a dose of 20 mg/kg body weight/day, 40mg/kg body weight/day and 80mg/kg body weight/day respectively. After the last dose, animals were fasted overnight and sacrificed by cervical dislocation. Stomach tissues were excised and taken to the laboratory for biochemical analysis using standard procedures. Results showed that, there was significant ($p<0.05$) reduction in the activities of endogenous antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), glutathione (GSH) and glutathione peroxidase (GPx) in groups 2, 3 and 4 when compared to control respectively. There was significant ($p>0.05$) increase in the level of malonaldehyde (MDA) in tramadol treated groups 2-4 when compared with control. The reduction of these enzymes was observed to be dose dependent. The concentration of interleukin (IL-1β) and tumor necrosis factor (TNF-α) were significantly ($p<0.05$) higher in groups 2-4 when compared to control. Histopathological changes in stomach tissues were only seen in groups administered tramadol. The results obtained suggested that tramadol increases lipid peroxidation and decreased the activity of antioxidant enzymes in the mucosal tissues of the rats as well as inflammation and oxidative stress.

KEYWORDS: Tramadol, oxidative stress, inflammation, histopathology, stomach ulcer.

INTRODUCTION

The use of self-administered analgesics has become popular in recent times, this has resulted into a major global concern, and self-administration has resulted to a significant increase in the rate of abuse, addiction and dependency on this class of drugs (Okwakpam et al., 2018). Analgesic is a class of drug used to achieve analgesia (pain relieve) and are among the most popular drugs which are being abused (Okwakpam *et al*., 2020). The abuse liability of naturally occur opiates (e.g., morphine, codeine) and synthetic opioids (e.g. tramadol, heroin, oxycodone, and buprenorphine) are well known (Moratti *et al*., 2010; Meyer *et al*., 2014).

Tramadol is a centrally acting opioid analgesic which is mainly used for the treatment of moderate to severe pain (Nossaman *et al*., 2010). Tramadol abuse, dependence as well as acute overdose -related deaths have been increasingly reported especially in young male adults (Lee *et al*., 2013). The prevalence of Tramadol abuse is 54% and over 65% of tramadol users within the age range of 18-37 years, while the average age of onset of use was 24 years (Liu *et al*., 2014). In Nigeria, the rate of tramadol abuse and misuse has been on a rapid increase among Nigerian youths in recent time (Owoade *et al*., 2019), and has been reported to cause multiple cases of toxicity and abuse (El-Ghawet, 2015). The main factor responsible for this could be link to off-label use of this drug (Kirby *et al*., 2015). Research has shown that persistent tramadol administration might lead to the accumulation of toxic metabolites in the body, increase the risk for its toxic kinetics effects and/or lower the clearance of tramadol, thus increasing its potential for dosage toxicity (Zaaijer *et al*., 2014). In response to its toxic kinetic effects, Oxidative stress have been shown to

be caused by a drug toxicity which then results to many diseases such a stomach ulcer (Okwakpam *et al*., 2020).

Oxidative stress is viewed as an imbalance between the production of reactive oxygen species (ROS) and their elimination by protective mechanism (Hussain *et al*., 2016). This ROS are normally produced within the body in limited quantity and are important compounds involved in cell regulation (Pan *et al.*, 2008). Excessive production of this ROS in the cell and tissues causes higher production of ROS, immune response, inflammation, and susceptibility to infection (Durackova, 2010), such that antioxidant system cannot be able to neutralize them, which then leads to chronic inflammation in the stomach (Ďuračková, 2010). It has been reported that oxidative stress can induce inflammation (Hussain *et al*., 2016). Gastric inflammation is a highly complex biochemical protective response to cellular/tissue injury (Mansour *et al*., 2022). Oxidative stress activates a variety of inflammatory mediators involved in several chronic diseases (Hussain *et al*., 2016). Clinical evidence suggests that oxidative stress and inflammation are linked to overproduction of ROS, and are likely to represent an important component for the development of several diseases including inflammation-associated with chronic diseases (Hussain *et al*., 2016). Various inflammatory stimuli such as excessive ROS production have been important to initiate inflammatory process resulting in synthesis and secretion of pro-inflammatory cytokine. Production of tumor necrosis factor alpha (TNF-α), and interleukins 1-beta (IL-1β) play a critical role in inflammation resulting in several chronic disease. The aim of this study is to investigate the effect of chronic administration of tramadol on oxidative stress markers and histological changes which may result to gastric mucosal oxidative injury known as inflammation in the stomach of male albino rats.

MATERIALS AND METHOD

Drugs and Reagents

Tramadol hydrochloride (Pharmacy, UMC Groningen, Netherlands) was prepared from tablets obtained from a local pharmacy. All other reagents were commercially available analytical-grade chemicals.

Experimental Animals

For the study, 20 adult rats weighing 140-160g obtained from the animal house of the Biochemistry department of University of Port Harcourt and transferred to the animal house of the department of Biochemistry Rivers State University, Port Harcourt. The rats were randomly assigned to 4 groups of five rats each in separate cages and allowed to acclimatize within a period of 14 days. They were housed under specific pathogen free (SPF) conditions and were provided standard feed (Vital feed, Nigeria) and water *ad libitum*, but starved for 12hours prior to commencement of experiment. All animal experiments were carried out in compliance with guidelines for Care and Use of Laboratory Animals (OECD, 2001).

Table 1: Experimental Design.

Group	No. of Animals	Administered Dose
1	5	Distilled water vehicle
2	5	20 mg/kg body weight
3	5	40 mg/kg body weight
4	5	80 mg/kg body weight

Drug Administration

Different doses of tramadol were freshly prepared and administered to the animals orally by body weight Treatment for twenty-one (21) days. At the end of the period, the animals were sacrificed by cervical dislocation. Stomach samples were also harvested into sample bottles containing phosphate buffer solution for the preparation of homogenates. Separate portions of the stomach sample were also preserved in 10% formalin for histopathological examination.

Preparation of Stomach Homogenates

One gram of the stomach was homogenized in 5ml of phosphate buffer solution using a laboratory mortar and pistil. The homogenized sample was then centrifuged at 3000rpm in a bench centrifuge for 15 minutes. The clear supernatant was then transferred into a clean plane bottle and labeled appropriately. The supernatants from stomach homogenate were used for antioxidant assay and interleukin and tumor necrotic factor tests.

Biochemical and Antioxidant Assessment on the Stomach Homogenates

The methods used by Kanu *et al*. (2016) were adopted.

Superoxide Dismutase (SOD) activity

Principle: This assay was based on the inhibition of nitroblue Tetrazolium (NBT) reduction. Illumination of riboflavin in the presence of O_2 and electron donor like methionine generates superoxide ions. One unit of SOD activity is defined as that amount of enzyme required to inhibit the reduction of NBT by 50% under the specified conditions.

Procedure: The reaction mixture contained 1.9ml phosphate buffer (pH 7.8), methionine NBT and riboflavin with serum in a total volume of 3ml. illumination of the solution taken in 10ml beaker was carried out in an aluminum foil lined box, with a 15W florescent lamp for 10 minutes. The control which lacked the enzyme source was also included. Absorbance was measured at 560nm and value expressed in units/GmHb.

Catalase (CAT) activity

Principle: This method is based on the measurement of a decrease in absorbance of the sample by the induced decomposition of H_2O_2.

Procedure: The rate was recorded by measuring the reduction in absorbance during 3 minutes at 240nm in 1.5ml of reaction mixture. The reaction mixture contained 13.2nM H_2O_2 in 50nM phosphate buffer (pH 7.0) and 0.1ml of the cell homogenate. The control mixture contained 50mM phosphate buffer (pH7.0) and 0.1ml of cell homogenate. Catalase activity was expressed in micromoles of H_2O_2 separated within one minute with one gram of weight cells used.

Determination of Glutathione Peroxidase (GPx) activity

Principle: This method is based on the oxidation of NADPH to $NADP^+$ which is accompanied by a decrease in absorbance at 340nm.

Procedure: 120µl of assay buffer and 50µl of co-substrate mixture was added to three wells (non-enzymatic wells). In positive control well, 100µl of assay buffer, 50µl of co-substrate mixture and 20µl of dilute glutathione peroxidase (control) were added to three wells. In the sample wells, 100µl of assay buffer, 50µl of co-substrate mixture and 20µl of sample were added to three wells. The reactions were initiated by the addition of 20µl cumene hydroperoxide to all the used wells. The plates were carefully shaken for few seconds to mix; then at 340nm using a plate reader to obtain at least 5 points. Glutathione peroxidase activity was calculated using.

$$GR\ activity = \frac{\Delta A_{340}/min}{0.00373\mu M^{-1}} \times \frac{Volume\ of\ Sample}{Volume\ of\ Reaction} \times Sample\ dilution$$

Glutathione peroxidase activity was expressed in u/mg protein

Determination of Lipid Peroxidation (Malondialdehyde)

Principle: Malondialdehyde (MDA) reacts with thiobarbituric acid (TBA) to form a red or pink colored complex which in acid solution, absorbs maximally at 532nm. Lipid degradation occurs forming such products as MDD (from fatty acids with three or more double bonds), ethane and pentane (from the n-terminal carbons of 3 and 6 fatty respectively). MDA appears in the blood and urine and is used as an indicator of free radical damage to functional molecules. MDA is a sign of lipid peroxidation.

Procedure: A volume (0.1ml) of the serum was mixed with 0.9 ml of water in a beaker. After that, 0.5ml of 25% TCA (trichloroacetic acid) and 0.5ml of 1% TBA (thiobarbituric acid) in 0.3% NaOH were also added to the mixture. The mixture was boiled for 40min at 95°C in a water-bath and then cooled in cold water. Then, 0.1ml of 20% sodium dodecylsulphate (SDS) was added to the cooled solution and mixed properly. The absorbance was taken at 532nm and 600nm against the blank.

Determination of Glutathione (GSH) Level

The glutathione was established using the high sensitivity Rat GSH enzyme-linked immunosorbent assay (ELISA) kit CSB-E11987r (CUSABIO, Biotechnology Company Sweden) according to the manufacturer's instructions. The assay employs the quantitative sandwich enzyme immunoassay technique. The optical density of each well was determined within 5minutes, using a microplate reader set to 450nm.

Determination of Tissue Level

Tissue level of inflammatory markers TNF-α and IL-1β was measured using the high sensitivity Rat enzyme-linked immunosorbent assay (ELISA) kit CSB-E11987r (CUSABIO, Biotechnology Company Sweden) according to the manufacturer's instructions. The assay employs the quantitative sandwich enzyme immunoassay technique.

Histopathological Examination

Microscopic observation of the heart of all experimental animals was conducted. The stomach of each animal will be dissected out, then fixed in 10% formalin for 48 h and processed for histopathological examination. Four micrometer-thick paraffin sections was stained with haematoxylin and eosin for light microscope examination using conventional protocol as according to Bancroft and Stevens (1982). The histopathological finding of the test groups will be compared to the control group.

Statistical Analysis

Statistical package for social science (SPSS), version 23.0 was used for statistical analysis. Results were expressed as mean ± standard error of mean (SEM), (n=5) and statistically analyzed by a one-way analysis of variance (ANOVA) followed by a Tukey's multiple comparison test as a post-test. Analysis at $p \leq 0.05$ was considered to indicate statistical significance.

RESULTS

Table 2 shows the effect of Tramadol on antioxidant enzyme activities. The result obtained shows that there was a significant decrease in the activity of CAT, SOD, GPx in the stomach tissue of groups 2(20mg/kg), 3(40mg/kg) and 4(80mg/kg) animals that were treated with different doses of Tramadol when compared to control in a dose dependent manner. Figure 1 and 2 shows the effect of Tramadol Administration on oxidative stress damage markers (glutathione and malonaldehyde). The result obtained shows that there was a significant increase in the tissue concentration of MDA in group 2 (20mg/kg), group 3 (40mg/kg) and 4(80mg/kg) when compared to control while there was significant decrease in the tissue level of GSH level in group 2, 3 and 4 animals that were treated with different doses of Tramadol when compared to control. Figure 3 and 4 shows effect of Tramadol on inflammatory markers (tumor necrosis factor-alpha and Interleukin-1beta). The result obtained shows that there was no

significant difference in the tissue level of TNF-α and IL-1β in all test groups when compared to the control.

Table 2: Effect of Tramadol Administration on antioxidant enzyme activities.

Group	CAT (U/mg protein)	SOD (U/mg protein)	GPx (U/mg protein)
Control	54.50 ± 1.95^{a}	24.71 ± 0.56^{a}	123.68 ± 2.08^{a}
20mg/kg	51.79 ± 0.77^{b}	23.49 ± 0.56^{b}	118.61 ± 1.15^{b}
40mg/kg	51.51 ± 0.94^{b}	22.50 ± 0.79^{b}	114.23 ± 2.20^{b}
80mg/kg	49.41 ± 0.35^{b}	21.75 ± 0.89^{b}	113.04 ± 0.98^{b}

Values are expressed as Mean ± Standard error of mean (SEM) n=5. Values with the same superscript within a column are not significantly different at ($p<0.05$).

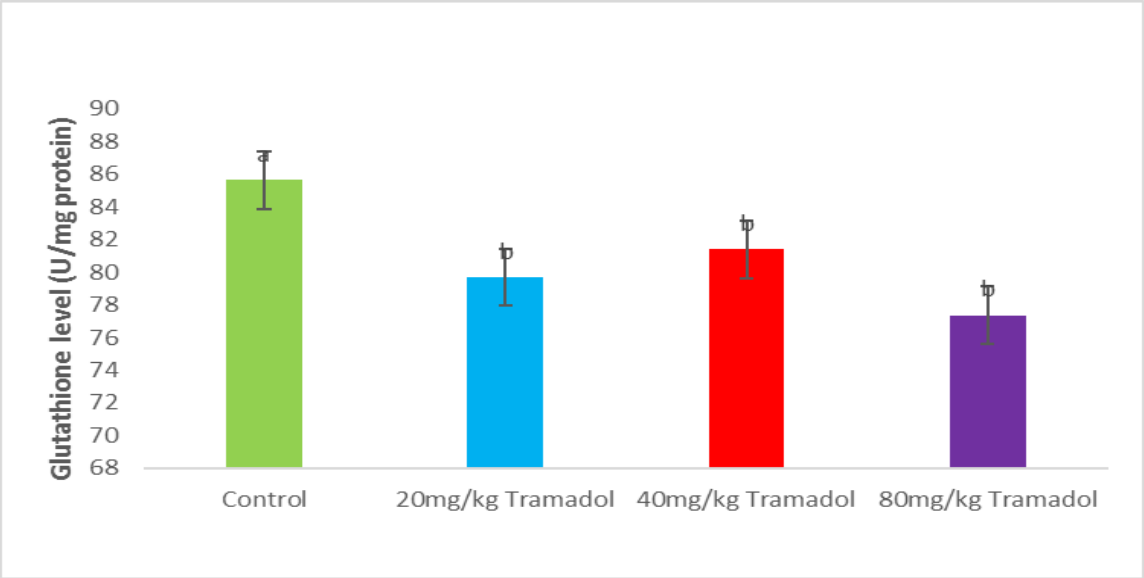

Figure 1: Effect of Tramadol Administration on stomach homogenate glutathione level of adult male Wistar rats.

Values are expressed as Mean ± Standard error of mean (SEM) n=5. Values with the same superscript within a column are not significantly different at ($p<0.05$).

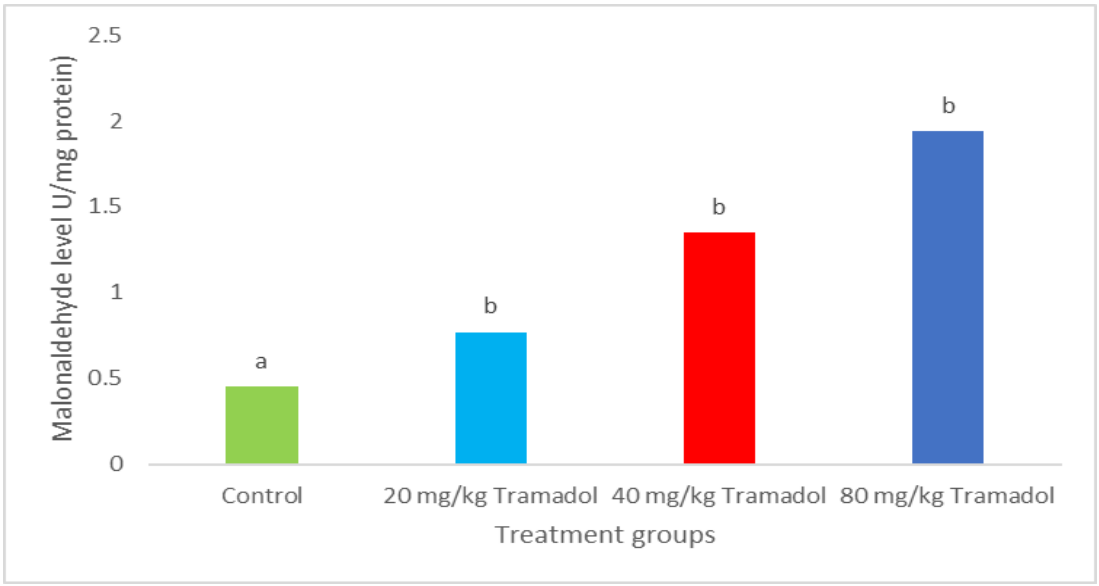

Figure 2: Effect of Tramadol Administration on stomach homogenate malonaldehyde level of adult male Wistar rats.

Values are expressed as Mean ± Standard error of mean (SEM) n=5. Values with the same superscript within a column are not significantly different at ($p<0.05$).

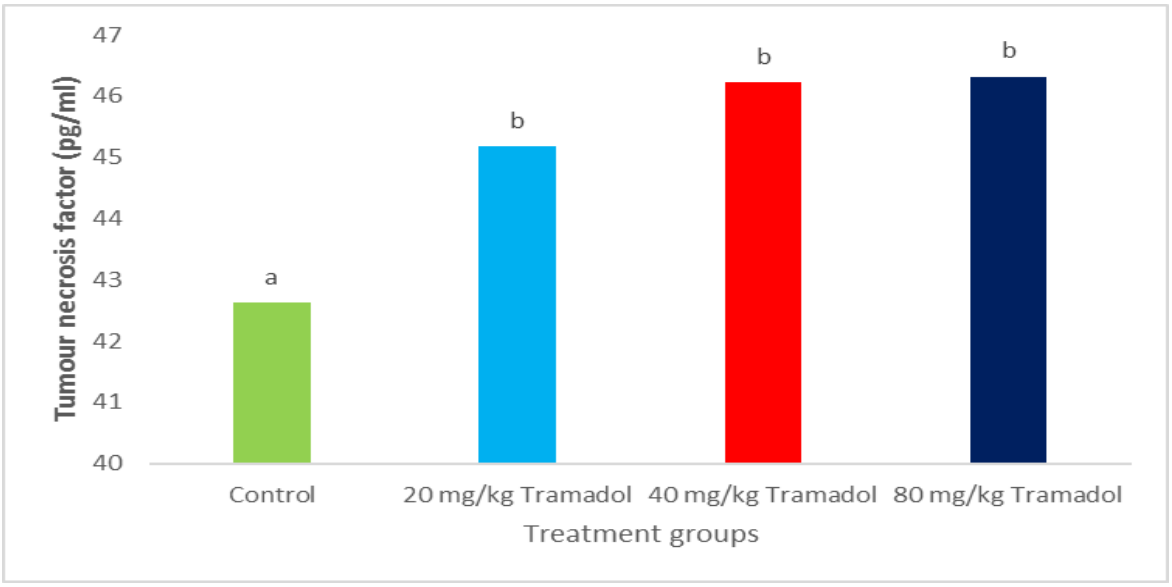

Figure 3: Effect of Tramadol Administration on stomach homogenate tumor necrosis level of adult male Wistar rats.

Values are expressed as Mean ± Standard error of mean (SEM) n=5. Values with the same superscript within a column are not significantly different at ($p<0.05$).

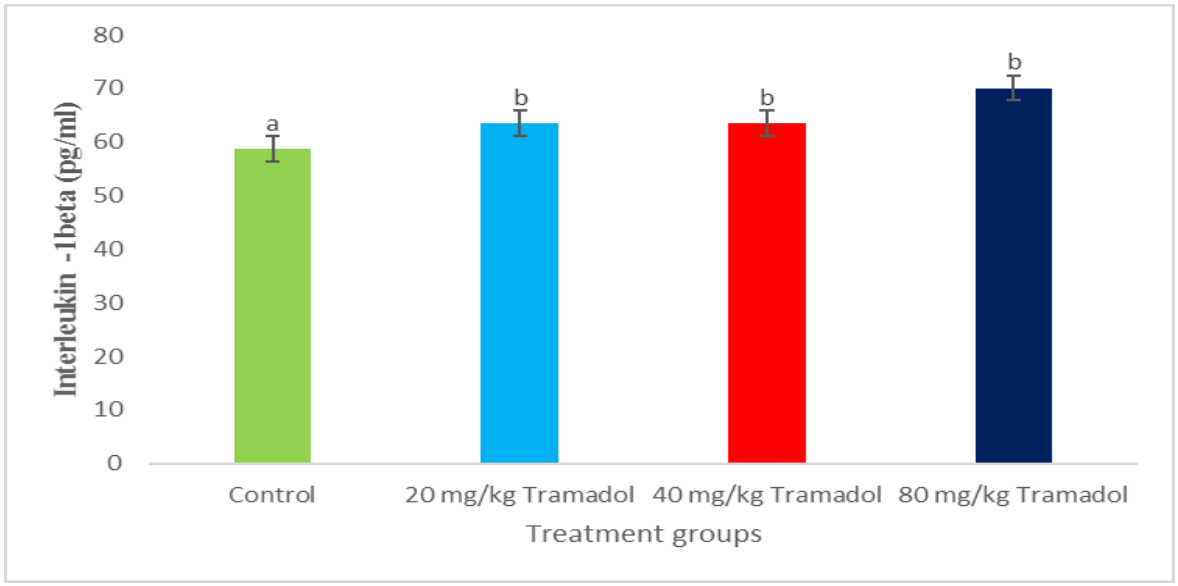

Figure 4: Effect of Tramadol Administration on stomach homogenate Interleukin-1beta level of adult male Wistar rats.

Values are expressed as Mean ± Standard error of mean (SEM) n=5. Values with the same superscript within a column are not significantly different at ($p<0.05$).

Effect of Tramadol Administration on Stomach histology

Photomicrographs of group 1 (control) show gastric tissue cardiac-type mucosa consisting of tightly packed glands occupying approximately 50% of the mucosal thickness and 50% superficial consisting of foveolar cells that are tall and columnar with mucous cells. The intervening stroma is thinly fibrocollagenous and is sparsely infiltrated by mononuclear inflammatory cells, predominantly lymphocytes. The muscularis mucosa is of normal thickness. Group 2 administered with 20mg/kg of tramadol showed a minimal change histomorphology when compared to group 1. While group 3 administered with 40mg/kg of tramadol showed moderate denudation of the surface epithelium (SE) and the muscularis mucosa (MM) is also moderately thickened. Group 4 which was administered with the highest dose of tramadol (80mg/kg) shows that there is near complete erosion of the surface epithelium (SE), the muscularis mucosa (MM) is severely thickened. There is marked chronic inflammatory (CI) exudate into the epithelium with extension into the subepithelial structures up to the muscularis propria (MP).

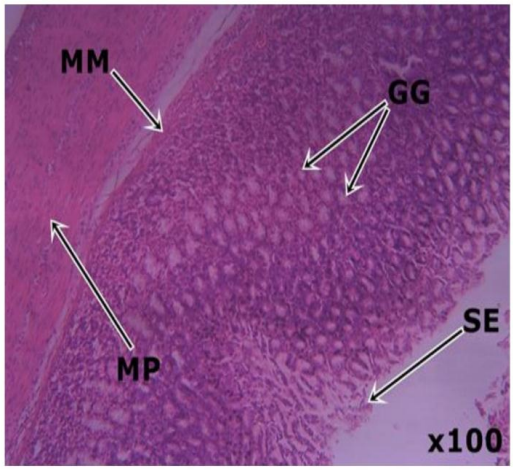

Plate 1: Photomicrograph of the gastric tissue of rats group 1 (control) showing that, muscularis mucosa (MM) is of normal thickness (arrows).

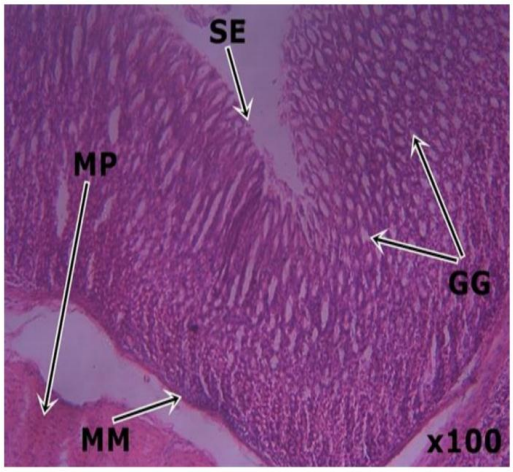

Plate 2: Photomicrograph of the gastric tissue of rats treated with 20 mg/kg body weight showing that, muscularis mucosa (MM) is of normal thickness (arrows).

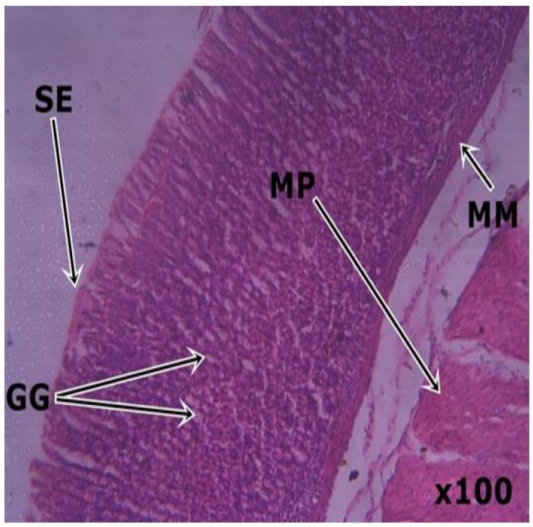

Plate 3: Photomicrograph of the gastric tissue of rats treated with 40 mg/kg body weight, shows that the muscularis mucosa (MM) is also moderately thickened (arrows).

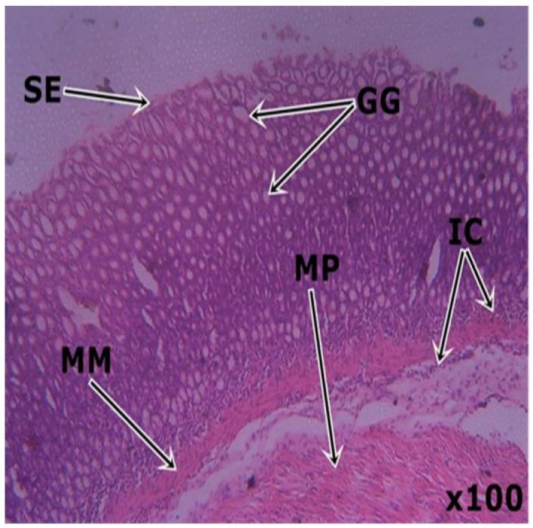

Plate 4: Photomicrograph of the gastric tissue of the rats treated with 80 mg/kg body weight shows that the muscularis mucosa (MM) is severely thickened (arrows).

DISCUSSION

Tramadol is a synthetic analogue of which is centrally acting analgesic for treatment of moderate to severe, acute or chronic pain (Nossaman *et al*., 2010). In this study, tramadol was studied as a drug, not as analgesics because there is alarming increase in tramadol abuse among Nigerian youths due to believe that tramadol could lengthen the duration of intercourse before ejaculation in men whom have problems with premature ejaculation and causes mood changes (Owoade et al., 2019). This study evaluated the toxicity of tramadol on oxidative stress, inflammation and histopathological changes in the stomach because of people addiction to the drug. The role of the stomach in tramadol metabolism predisposes it to toxic injury (Nossaman *et al*., 2010). In the present study, the obtained results showed that, after 3 weeks (21 days) of tramadol administration on tissue activities shows the effect of tramadol in the stomach.

A large amount of polyunsaturated fatty acids found in all the biological membranes is susceptible to peroxidative attacks by oxidants resulting in lipid peroxidation. So, Lipid peroxidation production was used as a marker of oxidant-induced tissue injury. It was reported that elevated MDA is indicating an increase of free radical generation and it is considered as a useful measure of oxidative stress status. In this study, it was recorded that, administration of tramadol significantly ($p<0.05$) increased the level of malonaldehyde (MDA) in

tramadol treated groups when compared with control group, indicating oxidative injury in a dose dependent manner.

The toxic effect of tramadol administration leads to a large population of unquenched free radicals leading to the state of oxidative stress. This is evidence in inhibition in the activities of antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), glutathione peroxide (GPx) and reduced level of glutathione (GSH) in the stomach of rats. The activity of SOD, CAT GSH and GPx are important antioxidant enzymes which plays a pivotal role of scavenging in oxidative free radicals (Owoade *et al*., 2019). These antioxidants enzymes do not only play fundamental, but indispensable role in the in the antioxidant protective capacity of biological systems against free radical attack (Ighodaro *et al*., 2017). The inhibition of these antioxidant enzymes observed in this study could be linked to exhaustion of these enzymes as a result of oxidative stress caused by tramadol administration. Result obtained in table 2 and Figure 1 showed that, administration of tramadol significantly ($p<0.05$) decreased the activity of SOD, CAT, GSH, and GPx in a dose dependent manner.

Interleukin (IL-1β) and Tumor Necrosis Factor (TNF-α) are important cytokine that is crucial for host defense responses to infection and injury. Both of these molecules are involved in a process that destroys cartilage and bone, driving even more inflammation and leading to the symptoms of many autoimmune diseases, such as gastric ulcer that is formed in the lining of the stomach. From table 4, there was a significant ($p<0.05$) increase in the level of IL-1β and TNF-α when administered tramadol. In the presence of ROS, there is an increase production of these cytokines and in turn, signaling accentuates oxidative stress (Zhang *et al*., 2009). In healthy person, they help the body to fight off infections. However, high levels of TNF-α and IL-1β in the blood can cause unnecessary inflammation, resulting in painful symptoms. Excessive production of these cytokines drives the damaging inflammation which begins to circulate in the blood. They arrive at the target area to trigger the inflammation process (Zelova *et al*., 2013). TNF alpha does this by triggering the production of several immune system molecules, including Interleukin-1 Beta (IL-1β). The test groups showed changes in histomorphology when compared to group in a dose dependent manner. Group 4 which was administered with the highest dose of tramadol (80mg/kg) shows that there is near complete erosion of the surface epithelium (SE), the muscularis mucosa (MM) is severely thickened. There is marked chronic inflammatory (CI) exudate into the epithelium with extension into the subepithelial structures up to the muscularis propria (MP).

CONCLUSION

Oxidative stress is one of the major contributors to the development of stomach disease. Gastric mucosa can be exposed to severe oxidative stress with considered levels of inflammatory cell accumulation, which leads to the development of gastric mucosal. Tramadol increases lipid peroxidation and decreased the activity of antioxidant defense enzymes in the mucosal. Increase in the dose level of tramadol increases the levels of IL-1β and TNF-α. This study provides evidence that chronic tramadol consumption induces oxidative stress and inflammation in the gastric mucosal. Its use should therefore be limited to prescription only. Our findings underlined the need to avoid indiscriminately prolong and use of tramadol, since prolonged daily use of the drug may lead to damage accumulation.

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