

LOPERAMIDE INDUCED NEUROTOXICITY IN RATS: BIOCHEMICAL AND HISTOLOGICAL EVIDENCE

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ABSTRACT: *Loperamide is a safe over-the-counter antidiarrheal drug. However, at supratherapeutic doses, it produces opioid effects. Here, we evaluated the neurotoxic effects of loperamide in rats brain. 20 rats were randomized into 4 groups (A-D) of 5 rats each. Rats in groups A (control) and B received vehicle for 7-day while rats in groups B, C and D were orally gavaged with 1.5, 3 and 6 mg/kg body weight (BW) of loperamide hydrochloride. The results revealed a dose dependent decrease in acetylcholine. Reactive oxygen species increased significantly while antioxidant enzymes were significantly ($p < 0.05$) lowered in the brain. Loperamide induces necrotic related morphological changes in rat brain with significant increase in malondialdehyde, protein carbonyl and fragmented DNA. Loperamide deplete acetylcholine thus causing the accumulation of reactive oxygen species and oxidation of cellular macromolecules. This study provides biochemical evidence supporting the neurotoxicity associated with supra-therapeutic dose consumed for euphoria effect.*

KEYWORDS: Loperamide, Neurotoxicity, Oxidative stress, Neurotransmitters, Lipid peroxidation, Antioxidant enzymes

INTRODUCTION

Loperamide is a safe over-the-counter antidiarrheal drug (Lasoff et al. 2017). However, there are reported cases of its toxicity at supra-therapeutic doses (Eggleston et al. 2016; Idris and Kaye, 2018; Salama et al. 2017; Swank et al. 2017; Wightman et al. 2016). It modulates the binding of calcium-activated calmodulin complex with its receptor (Daly, 2006), leading to an increased non-propulsive and decreased propulsive activity at the myenteric plexus (Regnard et al. 2011). Its opioid agonist activities has led to its abuse and misuse 1, 9-12 (Bishop-Freeman et al. 2016; Dierksen et al. 2015; Enakpene et al. 2015; Miller et al. 2017). Indeed, the abuse and misuse has led to some associated neurotoxicity. The agonist property of loperamide at the μ -receptor is responsible for the opioid-like euphoria activity (Baker, 2007; Daniulaityte et al. 2013). At therapeutic dose, it is excluded from the central nervous system (CNS) by P-glycoprotein at the blood-brain barrier (Gibbs et al. 2017; Mercer and Coop, 2011). However, it penetrates the CNS by inhibiting P-glycoprotein at supra-therapeutic dose encountered in the case of abuse and misuse to produce opioid effects (Wightman et al. 2016). Catatonic features such as seizures, somnolence, weakness motor retardation and loss of consciousness are some of the neurological symptoms that

are associated with its abuse and misuse (Di Rosa and Di Rosa, 2014; Miller et al. 2017). Moreover, some of these neurological dysfunctions, via autonomic nervous system, can induce malfunctions of peripheral organs including the heart (Wightman et al. 2016). Interestingly, its overdose has been reported to induce catatonia (Di Rosa and Di Rosa, 2014). Indeed, 15% of the reported toxicities categorized by organ system are neurological related (Eggleston et al. 2016; Idris and Kaye, 2018; Salama et al. 2017; Swank et al. 2017; Wightman et al. 2016). In a recent metabolomics and proteomics study, loperamide was considered a weak neurotoxic agent (Schultz et al. 2015). To achieve euphoria, 400 mg of loperamide are consumed (Swank et al. 2017). In addition to the neurotoxic effect, there are reported cases of cardiotoxic effect of loperamide (Miller et al. 2017). Despite the reported neurotoxic effect of loperamide, there is no biochemical evidence in support of the supra-therapeutic dose of this pharmacological agent. In this study, we present the biochemical changes resulting from loperamide-induced neurotoxicity.

MATERIALS AND METHODS:

Experimental animals

Male albino rats of Wistar strain (112.60 ± 4.30 g) were purchased from the Animal Holding Unit, Department of Biochemistry, University of Ilorin, Ilorin, Nigeria. They were kept in clean plastic cages, placed in a well-ventilated house conditions and supplied with feeds (Capefeed Ltd., Osogbo, Nigeria), and water *ad libitum*. This study was approved by Nile University of Nigeria Committee on the Use of Laboratory Animals (NUN/BCH/ECULA/018/003).

Chemicals and solvents

Loperamide (Purity $\geq 97\%$), ethanol, epinephrine, 5, 5'-dithio-bis (2- nitrobenzoic acid), hydrogen peroxide (H_2O_2), 2, 4-dinitrochlorobenzene (CDNB) and thiobarbituric acid were procured from Sigma-Aldrich Inc., St. Louis, USA. All other reagents used were of analytical grade and supplied by Sigma-Aldrich Inc., St. Louis, USA.

Animal groupings and treatments

Rats (20) were randomized into 4 groups of five rats each. Rats in groups A (control) received vehicle for 7-day while rats in groups B, C and D were orally gavaged with 1.5, 3 and 6 mg/kg body weight (BW) of loperamide hydrochloride. These doses were chosen corresponding to 400 mg of loperamide taken by 60 kg adult for euphoria (Swank et al. 2017). Rats were sacrificed under light diethyl-ether anaesthetization 24 h after last treatment. Brain homogenate was prepared as described by Ajiboye et al. (2014). Protein content of the brain was estimated as described by Olson and Markwell (2007).

Biochemical assays

Acetylcholine (Cat. No. MAK056) was estimated in the brain homogenate using the procedure in the commercial assay kit. The activity of acetylcholinesterase was determined as described by Wilson and Henderson (2007). Dopamine in the brain of rats was determined as described by Reza Shishehbore et al. (2013). Monoamine oxidases (MAO-A and MAO-B) activity was determined as described by Chand Basha et al. (2014). Superoxide anion radical level ($\bullet O_2^-$) as described by Ajiboye et al. (2015). Concentration of H_2O_2 was estimated as outlined in the H_2O_2 assay kit

(Sigma-Aldrich; MAK 311). Activities of superoxide dismutase (SOD) and catalase (CAT) were determined as described by Misra and Fridovich (1972) and Shangari and O'Brien (2006) respectively. Glutathione S-transferase (GST) and reduced glutathione (GSH) were quantified as described by Habig et al. (1974) and Ellman (1959) respectively. Extent of lipid peroxidation, protein oxidation and DNA fragmentation were estimated by quantifying the end product of the reactions; malondialdehyde (Reilly and Aust, 2001), protein carbonyl (Levine et al. 1990) and fragmented DNA (Burton, 1956) respectively. Protein content of both liver and serum were estimated as described by Olson and Markwell (2007).

Histopathology

Brain of rats was fixed in 10% formalin solution for 48 h, grossed, dehydrated through different grades of ethanol, xylene and embedded in paraffin. Section (3 - 4 μ m) of the liver was stained with hematoxylin and eosin stains, and mounted on microscope (TP1020, USA) for photomicrography as described by Ajiboye (2012).

Statistical analysis

Data were expressed as the mean for five rats \pm standard error of mean (SEM). GraphPad Prism 6 for windows, version 6.01 (GraphPad Software Inc, CA, USA) was used for Analysis of Variance (ANOVA). Turkey-Kramer test was used to detect significance differences between means within the treatment groups. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Loperamide dose dependently depletes the concentration of acetylcholine in the brain of rats following 7 days repeated oral administration of loperamide (Fig. 1a). The highest concentration investigated in this study (6 mg/kg BW) produced 4.5-fold decrease. Contrastingly, the activities of AchE increased significantly ($p < 0.05$) in the brain of rats treated with loperamide after 7 days (Fig. 1b). There was no significant ($p < 0.05$) difference in the concentration of dopamine and, the activities of MAO - A and MAO - B after 7 days repeated oral administration of loperamide (Figs.1c-d).

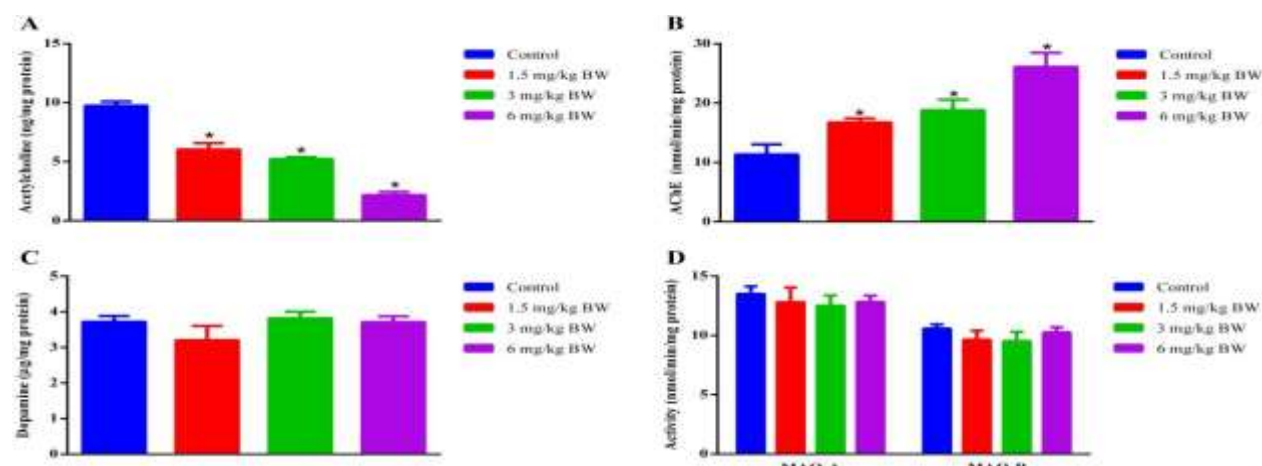


Figure 1: The effect of Loperamide on the level of neurotransmitters in the brain of rats.

Loperamide dose dependently raised ($p < 0.05$) the level of ($\bullet\text{O}_2^-$) with a concomitant decrease in the activities of SOD in the brain of rats (Figs. 2a-b). Furthermore, the level of H_2O_2 in the brain of rats increased significantly ($p < 0.05$) following 7-days repeated administration of loperamide when compared to the control rats (Fig. 2c). Contrastingly, the activity of CAT decreased significantly ($p < 0.05$) when compared to the control rats (Fig. 2d). Also, the level of GSH and the activity of GST were significantly ($p < 0.05$) lowered in the brain of loperamide-treated rats compared to the control rats (Figs. 3a-b).

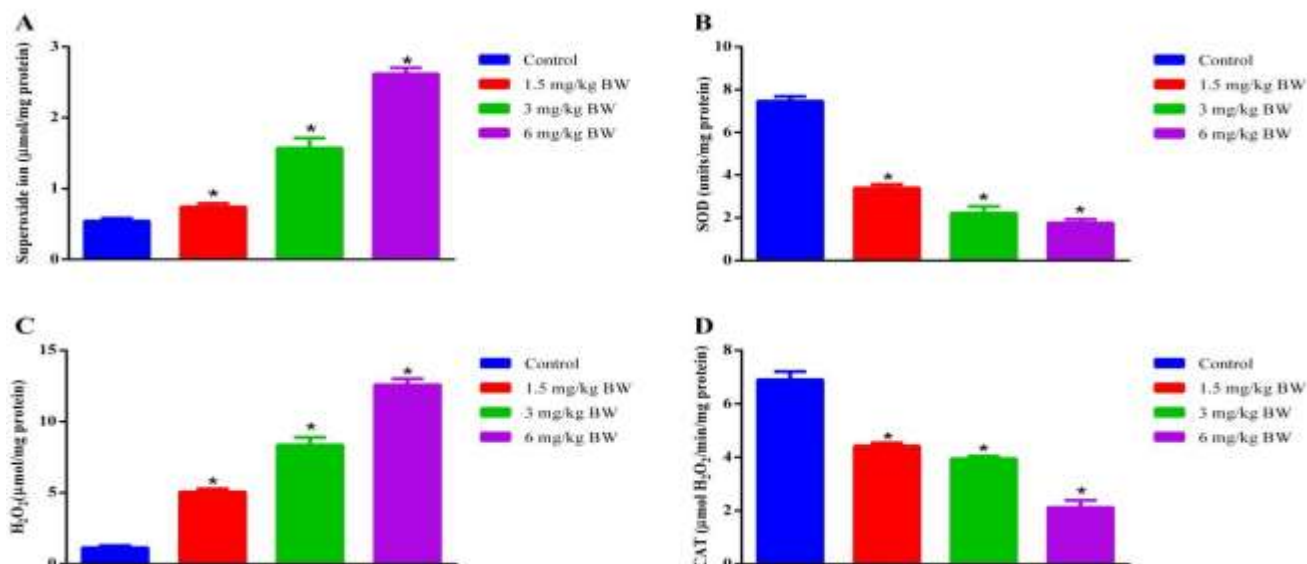


Figure 2: The effect of Loperamide on reactive oxygen species and antioxidant enzymes (catalase and superoxide dismutase)

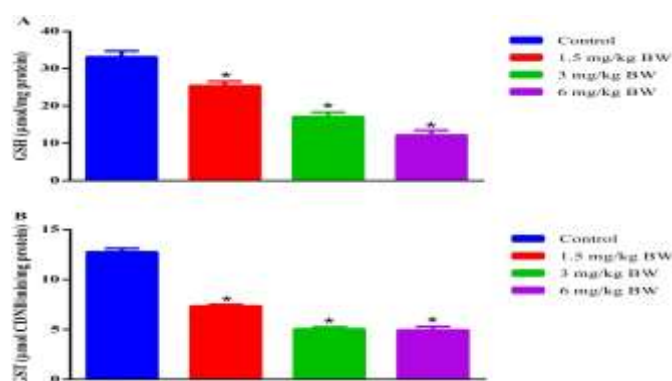


Figure 3: The effect of Loperamide on glutathione level and glutathione S-transferase activity in the brain of rats

Level of MDA, a product of lipid peroxidation, in the brain of rats increased significantly following 7-days repeated administration of loperamide when compared to the control rats (Fig. 4a). Similar, increase ($p < 0.05$) in the biomarker of protein oxidation, protein carbonyl, was observed after 7-days repeated administration of loperamide (Fig. 4b). In addition, fragmented DNA increased significantly ($p < 0.05$) in the brain of loperamide-treated rats when compared to the control rats. The increase observed for the highest dose investigated was $> 100\%$ (Fig.4c).

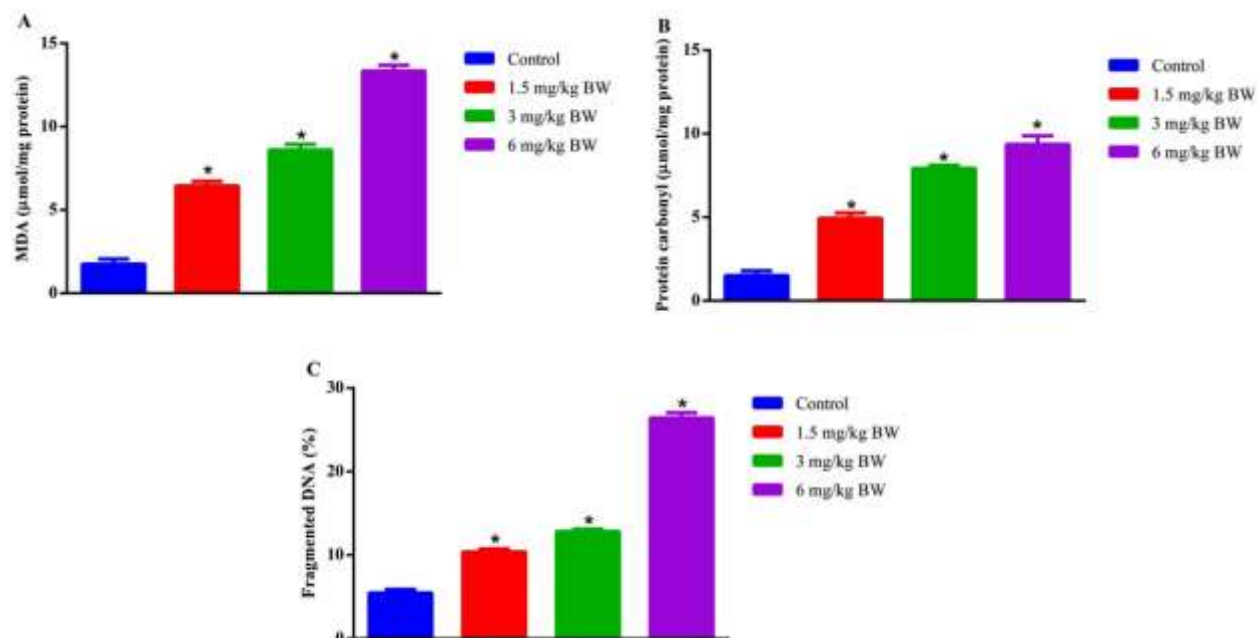


Figure 4: The effect of Loperamide on cellular macromolecules (protein carbonyl and fragmented DNA) in the brain of rats.

Repeated administration of loperamide for 7-days induced cystic degenerative changes, degenerated neuronal cell bodies and extensive nuclear depletion in the brain of rats (Fig. 5).

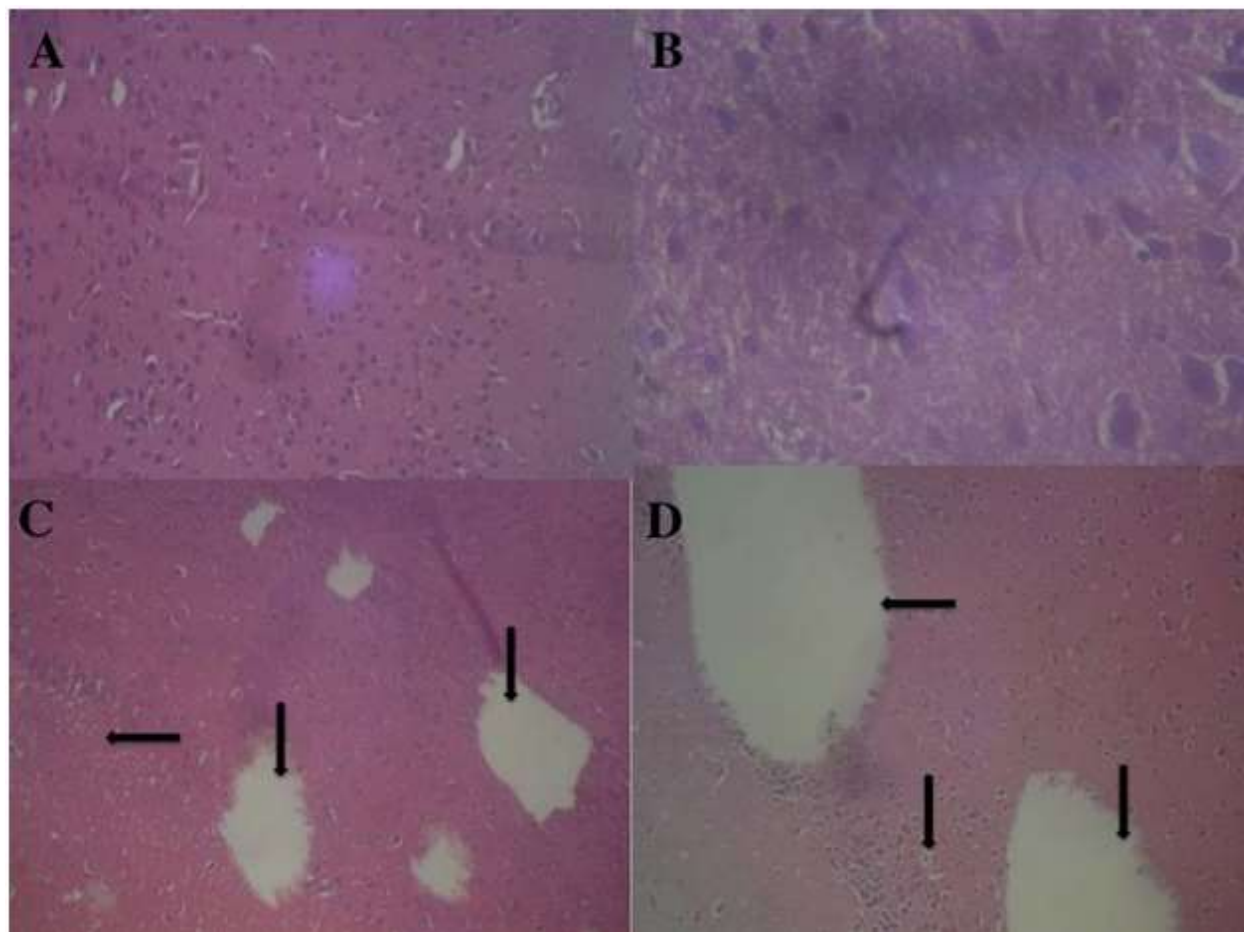


Figure 5: The effect of Loperamide of the Morphology of the brain of rats.

DISCUSSION

Neurotoxicity accounts for 15% of organ-related toxicity associated with loperamide abuse and misuse. However, there is no biochemical evidence supporting this toxicity. In this study, we present the effects of loperamide on neurotransmitters, cholinergic and monoaminergic enzymes, and oxidative stress biomarkers in the brain of rats. The inhibitory activity of loperamide on the cholinergic neurotransmission has been demonstrated in human colon (Burleigh, 1988), bronchial smooth muscle (Tamaoki et al. 1992), guinea pig ileum (Yagasaki et al. 1978) and colon (Kojima et al. 2005). Consistently, we noted acetylcholine depletion in the brain of loperamide-treated rats. This could have resulted from the inhibition of its release by interacting with opiate receptor sites of the rat brain. The increase in activities of acetylcholinesterase seen in this study may also account for the depleted acetylcholine level in the brain. Contrastingly, Shi et al. (2015) noted a decrease in the activity of AchE in zebra fish exposed to loperamide. The depletion of Ach possibly mediated by the increased AchE activity and its release from cholinergic nerve cells could have accounted for impaired learning and memory function of patients presenting with loperamide

abuse and misuse (Miller et al. 2017). This suggests impaired cholinergic neurons. Contrary to the reported depletion of dopamine by opioid agonist, loperamide had no significant effect on the level of dopamine. The non-significant change in dopamine suggests that loperamide is not likely to produce any Parkinson's disease - like pathology (Hauser and Hastings, 2013) or motor deficit. The non-significant change in MAO-A and MAO-B further confirms that loperamide does not have dopaminergic effect. It is apparent that dopamine and the monoamine oxidases (MAO-A and MAO-B) are not associated with the neurotoxic effect of loperamide. Interestingly, depression has not been reported in cases of loperamide abuse and misuse. Oxidative stress has been implicated in the pathogenesis of neurodegenerative disorder (Hauser and Hastings, 2013). We noted increased levels of reactive oxygen species ($\bullet\text{O}_2^-$ and H_2O_2) and decreased activities of antioxidant enzymes (SOD, CAT, GST and GSH) in the brain of rats. This suggest overwhelmed antioxidant arsenal and could impair nervous system by triggering peroxidation of lipids (Ajiboye, 2018). The elevated H_2O_2 , observed here, is not connected to MAO-A and MAO-B activities reported to raise H_2O_2 levels (Bortolato et al. 2008). The depleted GSH further corroborate the involvement of oxidative stress, since this protein scavenges H_2O_2 . The reduced level of GSH could be responsible for the decrease in the activity of GST. Our findings on these antioxidant enzymes are consistent with the reported effects of loperamide in the intestine and jejunum of rats (Jabri et al. 2017; Rtibi et al 2017). Oxidation of cellular macromolecules (carbohydrates, DNA, lipids and protein) results from overwhelmed antioxidants system (Ajiboye et al. 2015). The increased MDA, protein carbonyl and fragmented DNA suggest that loperamide provokes oxidative stress. Consistent with these findings is the reported increase in MDA, protein carbonyl and fragmented DNA in the brain of loperamide treated rats (Olofinisan et al. 2018). The apparent lipid peroxidation, protein oxidation and DNA fragmentation might have resulted from the imbalance in ROS production and antioxidants in favour of former. This event (oxidation) could affect several cellular functions such as membrane organization (Nikki, 2009) and protein Cystic degenerative changes, degenerated neuronal cell bodies and extensive nuclear depletion were observed in the brain of loperamide-treated rats. The observed degenerations are marks of cell death arising from apoptosis and cell death. Conclusively, we have demonstrated with biochemical parameters that loperamide induced neurotoxicity by depleting acetylcholine through the increased AchE activity. The increased ROS production could have accounted for the overwhelmed antioxidant enzymes, oxidative stress and the oxidation of cellular macromolecules in the brain of rats.

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