ORIGINAL RESEARCH ARTICLE

In vitro cytotoxic and inhibitory effect of tramadol, flunitrazepam and levonogestrel on neutrophils and myeloperoxidase

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ABSTRACT

The abuse of flunitrazepam, tramadol and levonorgestrel among adolescents is a major problem, with adverse effects on the immune system. Neutrophils serve as the host primary defense and employ the role of an essential enzyme, myeloperoxidase. This study investigated the in vitro effect of flunitrazepam, tramadol and levonorgestrel on the activity of the neutrophil. Neutrophils were isolated from blood samples of volunteers and seeded in culture plates, and then treated with flunitrazepam, tramadol and levonorgestrel. Trypan blue exclusion assay was used to assess the cytotoxic effect of flunitrazepam, (15, 150, 1500, 15,000, 150,000 ng/mL), tramadol (300, 3000, 30,000 µg/ml) and levonorgestrel (18.5, 185 and 1850 ng/mL) on the neutrophils, and the effect flunitrazepam (150,000 ng/mL), tramadol (30,000 µg/ml) and levonorgestrel (1850 ng/mL) on myeloperoxidase activity was assessed. The viability of neutrophils treated with flunitrazepam, (15,000, 150,000 ng/mL), tramadol (300, 3000, 30,000 µg/ml) and levonorgestrel (18.5, 185 and 1850 ng/mL) decreased significantly ($p < 0.05$) compared with control. Myeloperoxidase activity in neutrophils treated with flunitrazepam (150,000 ng/mL), tramadol (30,000 µg/ml) and levonorgestrel (1850 ng/mL) decreased significantly ($p < 0.05$) compared with background and positive control. This study revealed that flunitrazepam, tramadol and levonorgestrel altered the activity of myeloperoxidase and phagocytic activities of the neutrophil.

Keywords: neutrophils; myeloperoxidase; flunitrazepam; tramadol; levonogestrel

1. Introduction

Over the past decades, the increased susceptibility to infections among illicit drug users has become more widely acknowledged[1] and most drugs of abuse have been shown to alter certain functional aspects of the immune system[2–4]. Neutrophils are polymorphonuclear and phagocytic leukocytes that comprise the first line of host immune response against invading pathogens[5]. These cells employ different bactericidal pathways as a weapon to eliminate infectious agents[6]. Specifically, myeloperoxidase (MPO) which is the most abundant enzyme found in the azurophilic granules of the neutrophil is a key enzyme for the termination of bacteria through the generation of hypochlorous acid (HOCl)[7]. However, like every front-line defender, phagocytes are vulnerable and face the risk of injuries by toxins, oxidants, chemicals and especially drugs that might impair their

engulfing capacity[8].

Flunitrazepam, which is sold under the trade name Rohypnol, is a fast-acting sedative benzodiazepine that is prescribed for the short-term treatment of insomnia and as an oral premedication before surgery[9–11]. Paradoxically, although flunitrazepam is classified as a depressant, it can induce excitability or aggressive behaviour in some users[12]. Consequently, flunitrazepam is becoming increasingly abused by young people in Nigeria[13,14], and has been associated with several health effects[15], including significant immunological disorders[16].

Tramadol is an opioid analgesic used for management of acute and chronic pain in both hospitals and outpatient clinics[8,17]. Due to Tramadol rapidity to relieve pain, it is a controlled but readily available drug that is heavily patronized outside medical prescription[18]. Deaths due to tramadol overdose have been reported and are increasing in frequency across the globe with recognized risk factors such as depression, addiction, and seizures[19]. Recent studies suggest that opioids can have an adverse impact on the immune system and opioids use and abuse has been linked to significant immune-suppression, which renders individuals susceptible to infection.

Given the increasing adolescent sexual activity, unwanted pregnancies, and unsafe abortion in Nigeria[20], the use of emergency contraceptives has been encouraged[21]. Levonorgestrel is the most common emergency contraceptive pill that can be obtained over the counter from patent medicine and pharmacy shops in Nigeria[22]. Although levonorgestrel are not intended for repeated use[23], the repeated use of different contraceptives immediately before or after sex has been documented in several reports[24]. This has been associated with several side effects including tissue damage in human organs[25,26].

Several studies with diverging perspectives have revealed the effect of tramadol on immunological cells and markers[27–29] and the observation that anesthetics may affect the function of immune cells has been established[8]. Similarly, oral contraceptives have been associated with low and inflammatory status[30]. To the best of our knowledge, the specific impact of these drugs on the neutrophil as well as myeloperoxidase which play a significant role host immune response has not been well established. Therefore, this study investigated the in vitro effect of flunitrazepam, tramadol, and levonorgestrel phagocytic activity of the neutrophil using the percentage viability of neutrophils and myeloperoxidase release as an index.

2. Materials and methods

2.1. Drugs, reagents, kits, and chemicals

Flunitrazepam was manufactured by Swiss Pharma Ltd., Lagos, Nigeria. Tramadol and Levonorgestrel were products of Hovid Berhad, Ipoh, Malaysia. Neutrophil Myeloperoxidase Activity Assay Kit was a product of Cayman Chemical, 1180 East Ellsworth Road, Ann Arbour, Michigan 48108 USA. RPMI 1640, Bovine Serum Albumin (BSA), Trypan Blue Dye were all products of Sigma-Aldrich, PO Box 14508 Saint Louis, MO 63178 United States. All other chemicals such as $NaCl$, NaH_2PO_4, Ethanol, were also products of Sigma and were ensured to be of analytical grade

2.1.1. Preparation of phosphate buffer saline (PBS)

PBS was prepared by dissolving 0.795 g of Na_2HPO_4 to 9 g of $NaCl$ in 1 liter of distilled water. The pH of the solution was adjusted to 7.2, and then autoclaved and preserved at 4 °C.

2.1.2. Preparation of 0.4% trypan blue dye solution

Trypan Blue was prepared by dissolving 0.4 g of trypan blue powder in 100 ml of PBS. The solution was then filter using Millipore filter (0.20 µM) and kept in a dark container at 4 °C.

2.1.3. Preparation of culture medium RPMI-1640

Culture medium was prepared by dissolving 1 g of BSA in 100 ml of RPMI 1640 culture medium.

2.1.4. Preparation of flunitrazepam, tramadol and levonorgestrel

A stock solution (1 ml) of flunitrazepam, tramadol and levonorgestrel were prepared using appropriate solvents (ethanol and dimethyl sulfoxide) based on their solubilities. After that, varying concentrations of flunitrazepam (15, 150, 1500, 15,000, 150,000 ng/mL), tramadol (300, 3000, 30,000 µg/ml) and levonorgestrel (18.5, 185 and 1850 ng/mL) was prepared using RPMI-1640 medium. The choice of varying concentrations was based on the maximum plasma concentration (Cmax) of each drug.

2.2. Ethics statement

Ethical approval (BMS/AIEC/0147/21) was obtained from the Faculty of Basic Medical Sciences, Ladoke Akintola University of Technology.

2.2.1. Isolation of neutrophils

In this study, neutrophil isolation was done with reagents and according to the procedure in the neutrophil myeloperoxidase activity kit. About 15 ml of whole blood was obtained from 5 apparently healthy and drug free volunteers into an EDTA blood collection tube. The acquired blood was transferred into a 50 ml conical tube where 15 ml of filtered cell-based assay buffer was added to it.

10 ml of cell-based assay neutrophil isolation histopaque was then pipetted into another 50 ml conical tube where 30 ml of the diluted blood was slowly added to it and centrifuged at 500 rpm for 20–30 minutes at 18–26 °C. After centrifugation, the yellowish and clear top layers were carefully aspirated, while the reddish pellet containing neutrophils and red blood cells were left in the tube.

After aspiration, 30 ml of cell-based assay red blood cell lysis buffer was added into the tube. The cells and buffer were mixed by mixing for about 10–15 minutes to lyse the cells and then centrifuged at 1200 rpm for 10 minutes to pellet the neutrophils. After centrifugation, the reddish supernatant was carefully removed and 5 ml of RPMI containing 1% BSA was added to the tube and mixed.

Centrifugation at 1200 rpm was repeated for 5 minutes to pellet the neutrophil. 5 ml of RPMI containing 1% BSA was added to the tube again, mixed and centrifuge again @ 1200 rpm for 5 minutes. The cells were then resuspended in 20 ml RPMI containing 1% BSA and mixed well to ensure cell separation. The isolated cells were seeded in a 96-well cultured plate at a density of 1×10^5 cells/well.

2.2.2. Cytotoxicity assay by trypan blue dye exclusion technique

Cytotoxicity Assay by Trypan Blue Dye Exclusion Technique was conducted according to the method of Strober[31]. A cell suspension was made in a fixed volume (1–2 ml) of cells. Neutrophils (1×10^5 cells/well) were then incubated in RPMI 1640 culture medium (100 µl) in 96-well microplate for 1 hour at 37 °C, with an aliquot of the solutions (50 µl) of the varying concentrations of flunitrazepam (15, 150, 1500, 15,000, 150,000 ng/mL), tramadol (300, 3000, 30,000 µg/ml) and levonorgestrel (18.5, 185 and 1850 ng/mL).

Cells were then washed and resuspended in RPMI-1640 medium. 50 µl of the cell suspension was taken and mixed with an equivalent volume of trypan blue dye, and then incubated for 3 minutes at 37 °C. This mixture was transferred to a hemocytometer to count viable and non-viable cells. The hemocytometer was place on the stage of a binocular microscope and the cells were focused on. The number of cells was counted/ml.

% viability was calculated using the formula:

$$Viable\ cells\ (\%) = \frac{total\ no.\ of\ viable\ cells\ per\ ml\ aliqot}{total\ no\ of\ cells\ per\ ml\ aliqot} \times 100 \qquad (1)$$

The assay was performed in triplicate for each test group and for control cells, only RPMI 1640 medium

and solvent was added without test compound.

2.2.3. Determination of myeloperoxidase activity

Neutrophils (1×10^5 cell/well) were seeded in a 96-well plate at 100 µl. Two wells which contained culture medium only were included to serve as background control. Then, the cells were treated with flunitrazepam (150,000 ng/mL), tramadol (30,000 µg/ml) and levonorgestrel (1850 ng/mL) for 2–4 hours in a cell culture hood and phorbol myristate acetate (PMA) was added at 1:10000 to activate MPO release. At the end of the treatment, the plate was centrifuged at 1200 rpm for 10 minutes at 18–25 °C. After centrifugation, the culture supernatant (25 ml) was obtained and transferred to corresponding plates in the experimental plate. Positive control was also included, and these wells contain 50 µl of positive control.

To the 25 ml of culture supernatant, diluted assay buffer was added. 25 µl of the diluted culture supernatant was the transferred to corresponding wells in another experimental plate. Then, 25 µl of the cell-based Assay MPO inhibitor solution was added. 50 µl of 3,3',5,5'-tetramethyl-benzidine (TMB) substrate was then added to each well and covered, this was incubated for 5–10 minutes. Absorbance was read at 650 nm.

2.2.4. Statistical analysis

This research was a completely randomized design (CRD). Results were expressed as mean ± standard error of mean (SEM). Data generated were subjected to one way analysis of variance (ANOVA), after which Tukey test was conducted in order to identify the variation within the treatment group. *P*-value <0.05 was regarded as statistically significant and denoted by alphabets.

3. Results

3.1. Percentage viability of neutrophils after treatment with flunitrazepam, tramadol and levonorgestrel

The result in **Figure 1** indicated a significant decrease ($p < 0.05$) in the percentage viability of neutrophils treated with flunitrazepam at concentrations higher than the maximum plasma concentration (Cmax) (15,000 ng/mL and 150,000 ng/mL) when compared with the control cells.
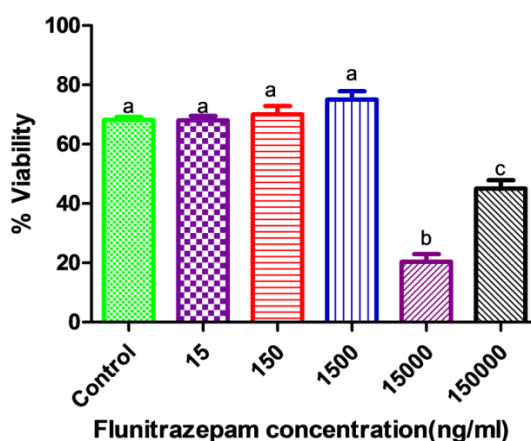

Figure 1. Viability Percentage (%) of neutrophils after treatment with different concentrations of Flunitrazepam. Values are mean ± SD; *n* = 4 and mean values bearing different alphabets are significantly different ($p < 0.05$).

Similarly, there was a significant decrease ($p < 0.05$) in the percentage viability of neutrophils treated with tramadol at the Cmax (300 µg/ml), and higher concentrations (3000 µg/ml and 30,000 µg/ml) when compared with the control (**Figure 2**).
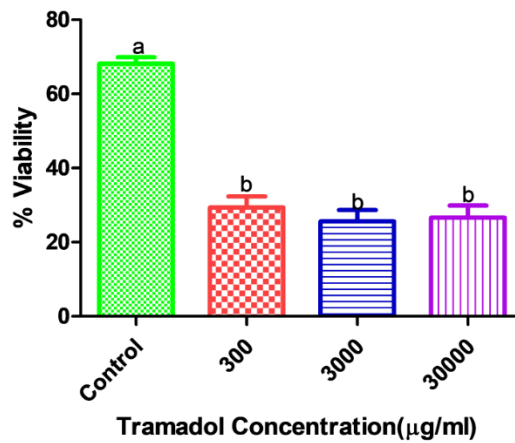
4

Figure 2. Viability Percentage (%) of neutrophils after treatment with different concentrations of Tramadol. Values are mean ± SD; n=4 and mean values bearing different alphabets are significantly different ($p < 0.05$).

Also, in **Figure 3**, there was a significant decrease ($p < 0.05$) in the percentage viability of cells treated with levonorgestrel at the Cmax (18.5 ng/mL), and higher concentrations (185 ng/mL, 1850 ng/mL) when compared with the control.
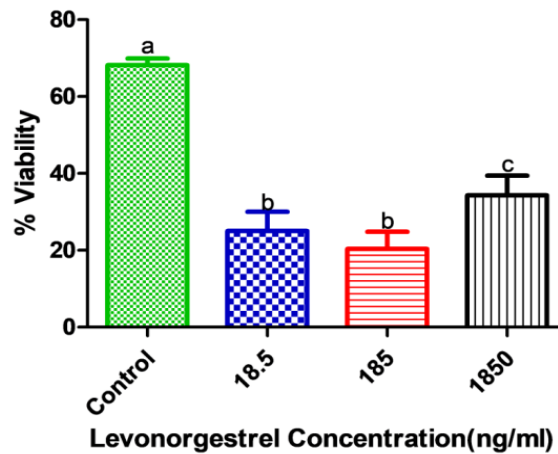

Figure 3. Viability Percentage (%) of neutrophils after treatment with different concentrations of Levonorgestrel. Values are mean ± SD; n=4 and mean values bearing different alphabets are significantly different ($p < 0.05$).

3.2. Myeloperoxidase activity in neutrophils treated with flunitrazepam, tramadol and levonorgestrel

In **Figure 4**, a significant decrease ($p < 0.05$) in the activity of myeloperoxidase was recorded following treatment of neutrophils with flunitrazepam (150,000 ng/mL) when compared with the background and positive control.
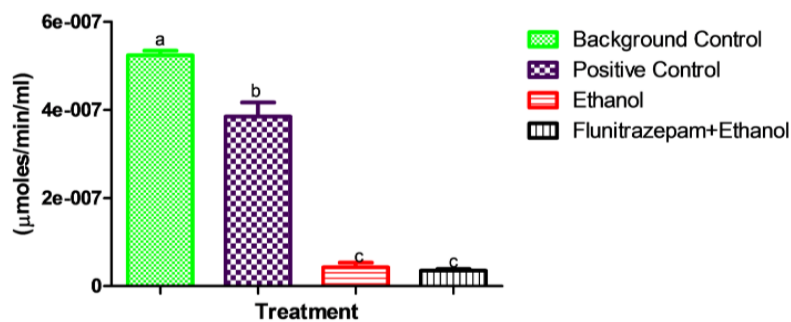

Figure 4. Effect of Flunitrazepam (150,000 ng/mL) on neutrophil myeloperoxidase activity. Values are mean ± SEM; $n = 4$ and mean values bearing different alphabets are significantly different ($p < 0.05$).

Similarly, there was a significant decrease ($p < 0.05$) in the activity of myeloperoxidase enzyme in neutrophils treated with tramadol (30,000 µg/ml) when compared with the positive and background control (**Figure 5**).

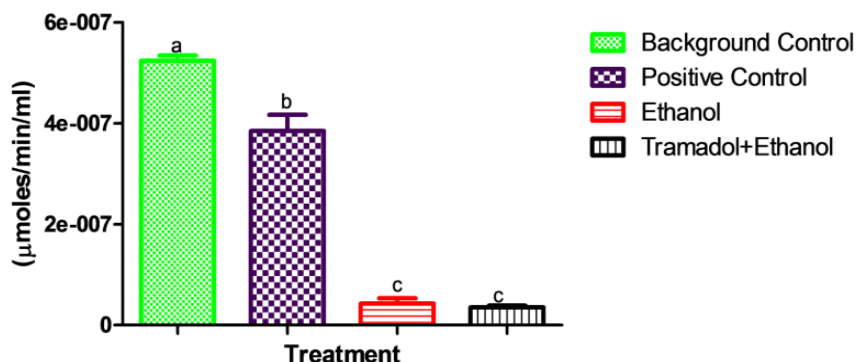**Figure 5.** Effect of tramadol (30,000 µg/mL) on neutrophil myeloperoxidase activity.
Values are mean ± SEM; $n = 4$ and mean values bearing different alphabets are significantly different ($p < 0.05$).

In addition, **Figure 6** showed a significant decrease ($p < 0.05$) in myeloperoxidase enzyme activity following treatment with the highest concentration of levonogestrel (1850 ng/mL) when compared with the background control and positive control.
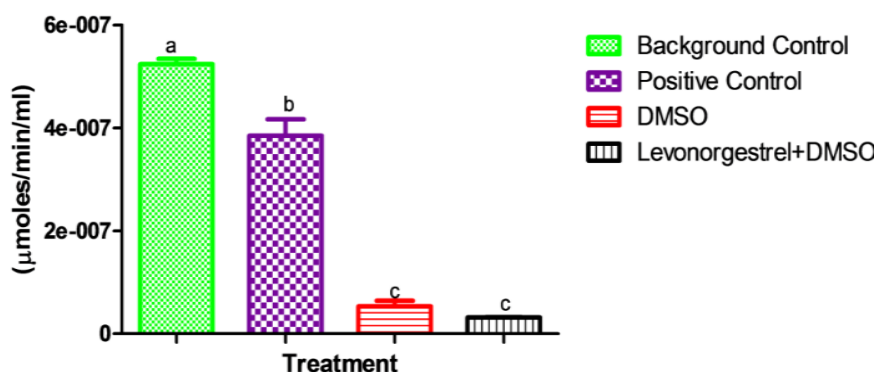

Figure 6. Effect of Levonorgestrel (1850 ng/mL) on neutrophil myeloperoxidase activity.
Values are mean ± SEM; $n = 4$ and mean values bearing different alphabets are significantly different ($p < 0.05$).

4. Discussion

An elevated or decreased count of leukocytes and its sub-types are very crucial in assessing the immune status of the body to infection, infestation, cancer and/or toxicity[32]. In this study, the depletion observed in the percentage of viable neutrophils after incubation with flunitrazepam (15,000 ng/mL and 150,000 ng/mL) (**Figure 1**) suggested that flunitrazepam is cytotoxic especially at high doses and could alter the phagocytic activity of the neutrophil by depleting the population of cells capable of phagocytosis. In addition, the observed decrease in percentage viability of neutrophils incubated with flunitrazepam also suggested that the abuse of flunitrazepam could result in neutropenia. This agrees with previous studies where showed that incubation of polymorphonuclear neutrophils with flunitrazepam inhibited their phagocytic and killing capacity of human pathogens[33].

The immunological status of the body upon exposure to acute or chronic tramadol intoxication or administration is still unclear[34]. Some of the studies posited that tramadol intoxication has significant effects on some immunological parameters, whereas others are of contrary opinion[34]. However, in this present study, the depletion in the percentage of viable neutrophils after incubation with tramadol (300 µg/ml, 3000 µg/ml

and 30,000 µg/ml) (**Figure 2**) suggested that abuse of tramadol could also alter the phagocytic activity of the neutrophils and result in neutropenia. In contrast, a previous study showed that incubation of human peripheral blood phagocytic cells with increasing doses of tramadol (5 µg/ml) affected neither the percentage of cells capable of phagocytosis, nor their phagocytic index[8]. This discrepancy might be due to variation in concentrations.

The trend observed in the percentage viability of neutrophils treated with levonorgestrel (18.5 ng/mL, 185 ng/mL, 1850 ng/mL) is similar to that of flunitrazepam and tramadol (**Figure 3**), and suggested that levonorgestrel could alter the phagocytic activities of neutrophils and also result in neutropenia. This is consistent with the findings of who reported low neutrophil function and inflammatory status of women using oral contraceptives[30], and that of who observed an increase in the proportion of neutropenic women after initiating oral contraceptives[35]. Neutropenia is the abnormal low count of neutrophils[36] and can be caused by drugs, infections, chemotherapy agents, and autoimmune diseases[37]. As a consequence, people with neutropenia are more susceptible to bacterial infections and without prompt medical attention, the condition may become life threatenin[38]. Hence, the observation in this study might explain the increased susceptibility to infections among illicit drug users.

Myeloperoxidase (MPO) is the most abundant enzyme found in human polymorphonuclear neutrophil[39] and is released primarily by neutrophils to provide defense against invading pathogens[5]. MPO is usually used as a marker of neutrophil accumulation in tissues and a marker of neutrophil activity when it is measured in plasma[40]. In this study, the significant decrease recorded in myeloperoxidase released after incubation with flunitrazepam (150,000 ng/mL), tramadol (30,000 µg/ml) and levonorgestrel (1850 ng/mL) (Figure 4–6) reflected a positive correlation between the percentage of viable cells and myeloperoxidase activity. Hence the decreased myeloperoxidase activity could be associated with the depletion in percentage of cells capable of phagocytosis and suggested an inhibitory effect of the drugs on myeloperoxidase release. The depletion in myeloperoxidase release observed in this study is similar the findings of who found out that patient suffering from neutropenia have significant decrease in myeloperoxidase up to being undetectable[6].

5. Conclusion

In conclusion, this study revealed that flunitrazepam, tramadol and levonorgestrel exhibited cytotoxic effect on neutrophils, inhibited the activity of myeloperoxidase, and ultimately altered the phagocytic activities of neutrophil.

Authors contributions

Conceptualization, EBO; methodology, EBO; software, JOA; validation, EBO and ASA; formal analysis, JOA; resources, EBO, PTI, BJO and PAA; writing—original draft preparation, JOA; writing—review and editing, EBO; supervision, EBO and ASA. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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