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Flunitrazepam Misuse Causes Purkinje Cell Degeneration: An Experimental Study in Rats

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Abstract

Flunitrazepam (FPAM), a sedative and anxiolytic drug, is abused as a recreational substance. However, it is known to be neurotoxic, affecting diverse brain regions. This study examined its histological effect on the cerebellar cortex. Twenty male Wistar rats, divided into four groups of five, consisting of a control and three FPAM-treated groups, were employed. After 14 days of administration of 2 mg, 4 mg, and 8 mg per kg body weight, respectively, of FPAM. Histological results showed a dose-dependent degeneration of Purkinje cells (Pn) characterised by vacuolation and nuclei shrinkage. With a Pn-based scale, a semi-quantitative evaluation revealed the proportion of degenerating Pn using a microscope with 15 mm (eyepiece number) and an objective of x 40, per 10^{-1}mm ^{2} were 4, 6, and 9 respectively, which were significantly different from control ($p< 0.05$, $P< 0.001$, and $p< 0.001$, respectively). The findings revealed that FPAM use had a twofold degenerative tendency in the cerebellar cortex between low and high dosages, indicating potentially harmful implications in FPAM addiction.

Keywords: Flunitrazepam, Cerebellum, Purkinje Cells, Wistar Rats, Histological Alterations.

INTRODUCTION

The cerebellum is an important part of the brain in all vertebrates. Proper functioning of the cerebellum rests largely on the health of the Purkinje cells (Pn) [1, 2]. Their cyton form a single layer between the granular and molecular layers and are the second largest cells in the CNS, characterised by elaborate dendritic trees [3-5]. The cells are essential in regulating motor impulse and constitute the singular output from the cerebellum [6]. Although commonly associated with movement, the cerebellum also plays a significant role in cognition and emotion [7, 8]. Contemporary studies suggest a high use of FPAM among alcohol and drug addicts [9]. It has been tagged "a date rape drug," and a report also suggested that it can precipitate violent behaviour in predisposed subjects [9, 10]. FPAM's association with criminality had earned it a bad reputation, causing its reclassification as a dangerous drug [11].

Despite this history, there is a slough in FPAM usage amongst undergraduate students, and it has become a popular recreational substance taken in various cocktails [12, 13]. FPAM misuse is associated with dissociation or automatism, in which an individual finds it difficult to recall events that occurred while under the influence after the drug wears off [9-14]. The report also suggested that abrupt withdrawal of FPAM therapy in clinical situations may result in benzodiazepine withdrawal syndrome characterised by the following: insomnia, psychosis, seizures, and anxiety [9]. FPAM causes necrosis to cells of the prefrontal cortex [15], and degeneration in the hippocampus [16]. Several other works have highlighted the neurotoxicity potential of FPAM [15-17], however, studies on its effects on the cerebellar cortex (CC) are rare. The primary endeavour of this study is to evaluate the histological changes observed in the CC of FPAM-treated rats.

MATERIALS AND METHODS

Experimental Animals

For this study, twenty [20] adult male Wistar rats having body weight between 150–210 g were used. They were acclimatised in our well-ventilated research laboratory for a period of 3 weeks in cages. Illumination

was 12 hours of daylight and 12 hours of darkness rhythmically daily throughout the experiment. Rats had free access to water and diet ad libitum. The room temperature was 24°C ± 2°C and the humidity 80%. The ethics committee of FBMS, Niger Delta University, authorised the protocols used in this investigation (FBMS2024/049).

Experimental Design

The rats were randomised into four equal groups (gp) (n = 5 each) and treated as indicated. The dose of FPAM was chosen based on a previous study [18]. The duration of treatments was 14 days. FPAM (Roche) was obtained from a reputable pharmacy: Denson Pharmacy Limited, Yenegoa 569101, Bayelsa State, Nigeria.

1. Group 1 (control). The rats received food and water.
2. Group 2. Rats received FPAM 2 mg/kg body weight /day dissolved in distilled water orally.
3. Group 3. Rats received FPAM 4 mg/kg body weight/day dissolved in distilled water orally.
4. Group 4. Rats received FPAM 8 mg/kg body weight/day dissolved in distilled water orally.

Brain Isolation

At the end of the experiment, rats were deeply anaesthetised with ketamine [19], and decapitation in a painless manner. Brains in respective cranial cavities were fixed *in-situ*, in 10% phosphate buffer formalin for 48 hours. Afterword, the brains were carefully taken out of the cavities, and the cerebelli was isolated so that it could be processed as histology tissue.

Tissue Processing

Isolated cerebelli were routinely processed for haematoxylin and eosin light microscope sections. Cerebellar tissue was excised, dehydrated through ascending alcohol gradients, cleared with xylene, and embedded in paraffin wax. 5-µm sections were cut using a rotary microtome, mounted on slides, and stained with hematoxylin and eosin [18].

Histological Study

A semi-quantitative scale based on Purkinje cell population was used to estimate the extent of neurodegeneration (20). Briefly, the representative section (n = 3 per slide) was assessed for degenerating neurons. Degenerating cells were identified by any of these criteria: intense eosinophilic cytoplasm, swelling of the cell body, and voidness [21].

Analysis of Histological Sections

The processed H&E sections were analysed using a microscope with 15 mm (eyepiece number) and an objective of x 40.
Consequently,
Diameter of field of view=F/M

Where F is the number of the field of view (FOV) of the eye piece, and M, magnification of the objective lens.
Diameter = 15/40 = 0.375mm
Hence, area of view is 1.0×10^{-1}mm

Statistical Analysis

One-way ANOVA with Tukey's post hoc test was used to identify the significant differences between groups. Data were expressed as mean ± standard deviation. Statistical significance was assigned at $p < 0.05$. Statistical analysis was performed using GraphPad Prism 5 software.

RESULTS

Histological Changes in the Cerebellum

The number of injured or degenerating neurones in the representative sections (n = 3 per slide) were counted. They were identified by well-documented criteria [21]. A modified Purkinje cell-based quantitative scale was adapted to estimate the extent of neurodegeneration [20]. All assessments were done at 15mm eyepiece number with x40 objective magnification. Areas of count were randomly selected (n-3) per slide. Pn are prone to assault irrespective of location within the cerebellum [22].

Cerebellar cortex of control (Fig. 1A) showed normal histology of the cerebellum with molecular layer, Purkinje cell layer, and granular layer. It also revealed large, piriform-shaped Purkinje cells and numerous populated cells in the granular layer. Rats administered 2 mg/kg of FLAM showed fairly normal cerebellar architecture with few vacuolations (Fig. 1B), while those administered 4 mg/kg of FPAM showed mild disruptions across the Purkinje and granular layer (Fig. 1C). The group treated with 8 mg/kg of FPAM (Fig. 1D) showed severe disruptions of the Purkinje layer and formations of vacuoles along the granular layer, focal area oedema, and disintegration of granule cells.

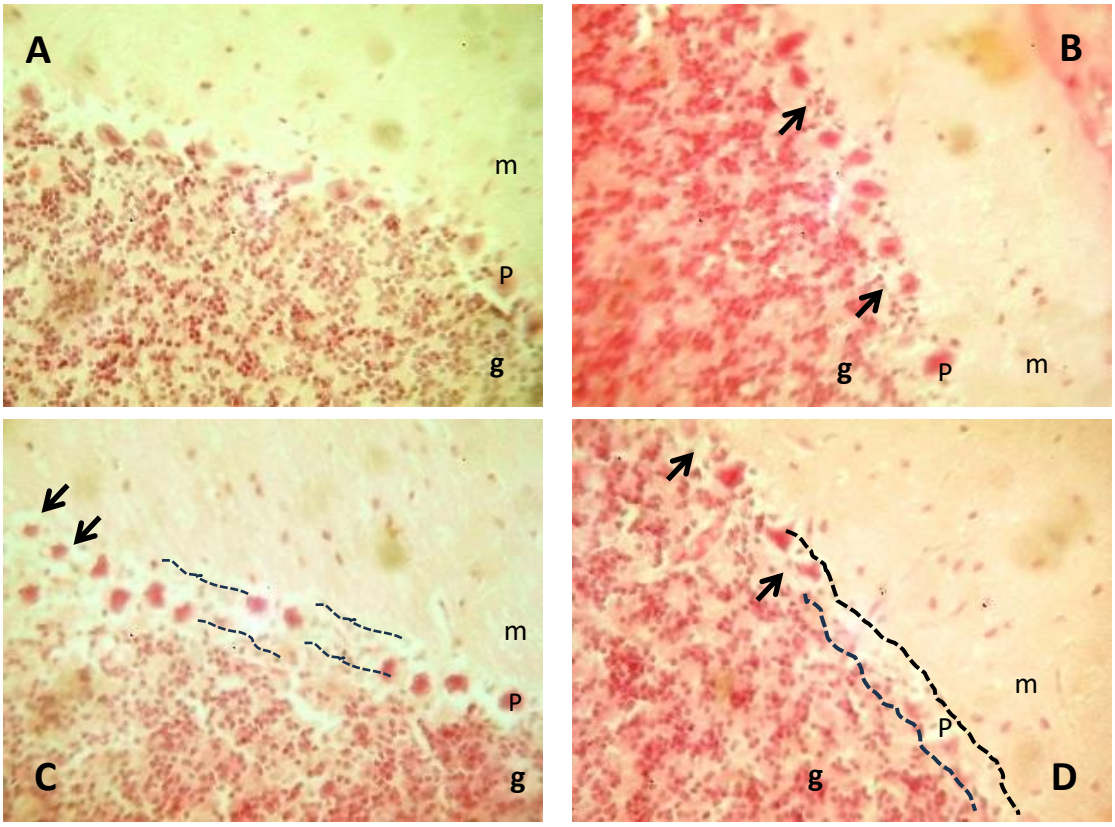

Figure 1: Histological micrographs, control (A) showing normal histology with molecular (m), Purkinje (p) and granule (g) layers. Rat fed 2 mg FPAM (B) showing fairly normal cerebellar architecture. CC of 4 mg FPAM (C) showed mild disruptions & disintegration of the Purkinje cells (delineated dash) and mild vacuolation within g-layer. The CC of 8 mg FPAM (D) showed high disruptions & degeneration (delineated dash) of the Purkinje layer and vacuolar disintegration of granule cells. Shrinkage of Pn (black arrows) were also observed in the CC of groups 3 & 4 rats H and E stain. Mag. X 400.

Semi-Quantitative Analysis Result

Table 1: Showing the neurodegenerative index per group

Groups	Dose of FPAM (mg/kg body weight/day)	Degenerative cells /1.0 x 10^{-1}mm
1	Nil	1.7 ± 1.4
2	2	4.0 ± 0.89
3	4	6.3 ± 1.4
4	8	8.8 ± 1.2

Values are mean ± SD, $P < 0.05$, n = 6.

Post-hoc analysis using Tukey's HSD test revealed significant differences in Pn degeneration among the different treatment groups. The proportion of degenerating Pn per 0.01 mm square of CC was significantly different between control ($p < 0.05$ vs. gp2; $p < 0.001$ vs. gp3 & gp4) and the FPAM treatment groups. Each of the 3 treatment groups was also significantly different from each other: gp-2 vs gp-3 ($p < 0.05$) and gp-4 ($p < 0.001$); and gp-3 vs gp-4 ($p < 0.01$). The proportion of Pn in the 8 mg FPAM treatment group was about 2-fold of what we observed in the 2 mg gp (Table 1, Fig. 1).

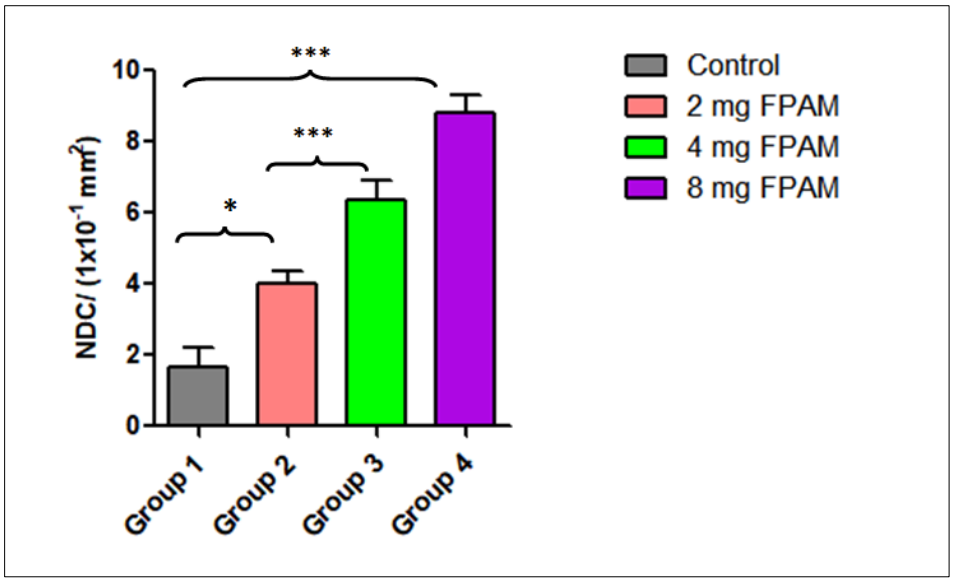

Figure 2: Showing the graphical representation of the effect of different doses of FPAM on the CC of an adult Wistar rats. * P< 0.05 & *** P< 0.001

DISCUSSION

This study investigated the histological effects of FPAM on the CC of adult Wistar rats. Our findings revealed a significant dose-dependent impact on Purkinje cell (Pn) integrity. The observed increase in the proportion of degenerating Pns across all FPAM treatment groups, compared to the control, strongly suggests a cytotoxic effect of FPAM on the cerebellar neuronal population. This is consistent with a growing report suggesting the potential for benzodiazepines to induce neurotoxicity [15]. While the precise mechanisms may require further investigation, the dose-dependent harmful effects of FPAM, as demonstrated in our study are noteworthy, particularly with the sudden rise of FPAM misuse among university students and young adults [9-23], often in combination with other substances, notably alcohol and tramadol [24, 25]. Unfortunately, the negative effect of FPAM exposure is also seen during organogenesis; studies show that it impacts the development of many brain regions, including the cerebellum [26].

Our findings somewhat confirmed that of Tewari and Co., who found that the adult cerebellar white matter is also vulnerable to FPAM neurotoxicity [27]. The present data (Table 1 and Fig. 1) suggested that FPAM dose-dependent disruptions in the cytoarchitecture of CC are more evident in the Pn, perhaps due to its significantly larger size. Opanashuk *et al*., [28], reported that increasing the dosage of FPAM causes a progressive loss of white matter in the rat, which correlates with the work of Tewari and Co [27]. This indirectly correlates with the present study that shows the effect of flunitrazepam on the cerebellar cortex is dose-dependent.

The detrimental effect of FPAM on Purkinje cells was recorded in several studies during the late 1980s [29, 30]. Consequently, the present study is by no means novel; yet, it is imperative to emphasize the vulnerability of Pn cells to FPAM exposure, especially in the contemporary Z-generation, where substances such as FPAM are increasingly utilized for recreational purposes [31, 32]. Though debatable, the potential danger of FPAM misuse compromising neuronal and neuropil integrity or functions needs more emphasis. Buttressing this fear, studies show that FPAM may potentially contribute to cerebellar motor and cognitive function decline [33, 34]. Mechanisms, such as oxidative stress, apoptosis, or impaired neurotrophic support, have been identified as crucial FPAM outcomes in the cerebellum [15]. Future research into the behavioural consequences of FPAM-induced cerebellum injury is required to determine the functional ramifications of these histological abnormalities.

In conclusion, our study demonstrated that FPAM use had a twofold degenerative tendency in the cerebellar cortex between low and high dosages, implying potentially detrimental consequences in FPAM addiction. Additionally, this study provides compelling evidence for FPAM-induced cerebellar damage, which may serve as a basis for certain cerebellar lesions that could result in clinical conditions such as cerebellar oedema, vasculitis, or even ataxia.

Conflict of Interest: None

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