Original Article

Repeated benzodiazepines ingestions affected behavioral and neurochemical profiles, with mild effect on histological integrities: modulatory efficacy of Nigella sativa oil

Abstract

Background: Benzodiazepines (BZDs) are a class of depressant drugs that have enjoyed widespread use in conventional clinical management of anxiety-related conditions such as panic disorders that require therapeutic central relaxation and sedation. Meanwhile, prolonged administration of benzodiazepines even at low doses has however been linked to variety of undesirable effects such as discontinuation relapse with the associated risk of abuse and dependency. **Aim:** This study investigated the behavioral, histological and biochemical outcomes of long-term low dose diazepam use and explored the potential role of nigella sativa oil (NSO) in the amelioration of the associated side effects. **Methods:** Adult Wistar rats (n=32) were randomized into four groups that received normal saline; diazepam; diazepam + NSO; or NSO only, respectively for 14 days. At the end of the period of the various exposures, the rats were taken through behavioral paradigms after which they were sacrificed for chemical and histological profiling. **Results:** diazepam-exposed rats exhibited stress-related manifestations with relatively poor performance in memory-related tasks. Repeated diazepam ingestion reduced brain antioxidant biomarkers while causing elevation of brain oxidative stress markers. On histological observation, mild degenerative changes were evident in the various brain regions of the diazepam-exposed rats. **Conclusion:** Interventional nigella sativa oil administration showed therapeutic potentials by mitigating and reversing the observed effects of diazepam, largely due to its antioxidant and anti-inflammatory effects as observed in the present study.

Keywords: Benzodiazepines, memory, behavior, oxidative stress, inflammation.

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Introduction

The pharmacologic benefits of benzodiazepines (BZDs) as psychoactive drugs in the clinical management of sleep disorders[1], epilepsy, and anxiety[3, 4] has contributed to their popularity. In the central nervous system, benzodiazepines work as positive allosteric modulators that bind to GABAA receptors, inducing conformational alterations that increase affinity for the GABA molecule, and enhancing GABA-induced neuronal hyperpolarization[2,5]. However, long-term use of BZDs presents concomitant major health risks such as addiction and dependence[1, 6], thereby increasing its potential for abuse and co-abuse with opioids and alcohol. BZD abuse has been associated with cognitive decline[7], hypothermia, respiratory suppression, coma, and death [4,8]. Typical of sedatives, they inhibit locomotor activity to varying levels leading to a higher incidence of falls and making the operation of machinery and vehicles potentially hazardous[8]. Benzodiazepines are frequently found in post-mortem blood samples of heroin users and in those related to overdose of other opioid substances where they appear co-administered to potentiate opioid effects[8]. Despite the level of awareness of the detrimental effects of BZD abuse, an estimated 3% of adults are still thought to have used BZDs for at least six months at some point in their lives[6], a significant portion of which may be without appropriate medical prescription. In rodents, BZD use has been associated with decreases in cerebral blood flow, decreases in caudate nucleus size, increases in lateral ventricular size[8], and decreases in dendritic spine density in pyramidal neurons. However, the mechanism by which BZDs exact these changes in the brain remains elusive[7]. The potential for benzodiazepine usage to cause certain detrimental effects is thus established, it, therefore, becomes imperative to discover and develop antidotes to counter these effects and save lives. Acting through GABA receptors, diazepam, like other BZDs, increases the levels of GABA in the brain through which it perpetuates its calming effect on the central nervous system. Therefore, prolonged diazepam use for longer than four weeks is not recommended. Following a strong caution by Penninx et al[7] against prolonged use of benzodiazepines as monotherapy in the management of anxiety and other related disorders with the subsequent recommendation that it be used only as a short-term adjunct, Nardi et al [8] claim that when carefully and judiciously prescribed, benzodiazepines are effective and well toler-

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ated. In a similar effort, Dubovsky and Marshall [9] criticized data presented in the literature as involving conflict of interest. According to Silberman et al [10], there is an overestimation of the rate of abuse and dependence among patients without previous record of substance abuse while also concluding that dose escalation is not necessary for long-term therapeutic benefits. Hirschtritt et al [11] also opined that benzodiazepines are a safe and effective option for the management of anxiety disorders following necessary screening of patient for a history of substance abuse, active opioid medications, cognitive impairment and age above 65 years, all of which contribute to negative outcomes. Likewise, Prashant et al [12], in contradistinction to findings by Penninx et al[7], suggests the possibility of bias reporting in favor of the risks against the therapeutic benefits of benzodiazepines. Nigella sativa oil (NSO) has been shown to prevent or reverse indications of neurological damage induced by various neurotoxins such as agricultural organophosphates in healthy rodent brains[13, 14]. NSO contains thymoquinones, riboflavin, and alkaloids as some of its bioactive ingredients which confer neuroprotective properties amongst a host of other pharmacological functions[13, 14]. The specific mechanism of action of NSO has not been fully elucidated but is thought to be linked to its antioxidant and anti-inflammatory properties[13, 14]. This study aims to determine the specific determinable histological, biochemical, and behavioural changes of prolonged BZD (diazepam) use in rats and the potential for NSO to prevent or reverse them. Here, we show that prolonged oral ingestions of diazepam surprisingly affected neurocognitive phenotypes with its central mechanism of toxicity being oxidative stress and inflammation. Meanwhile, a post-exposure intervention with the oil of nigella sativa markedly restored the affected psycho-cognitive behaviors induced by diazepam, majorly by preserving the intrinsic antioxidant and anti-inflammatory defense architectures in the exposed brains.

Materials and methods

Ethics committee approval: Ethical review and approval for this study was granted by the Ethical Review Committee of the Faculty of Basic Medical Sciences on 21.04.2017, University of Ilorin, with reference number UIL/UERC/AN2074.

Study subjects

This study used adult male Wistar rats (N = 32) (100 g – 120 g) obtained from the murine breeding centre in Ogbomosho, Oyo State, Nigeria, and housed in the animal facility of the Faculty of Basic Medical Sciences, University of Ilorin in accordance with the university's Guide for the Care and Use of Laboratory Animals. There was an acclimatization period of seven days during which animals were allowed free access to food and water.

Experimental Design

Experimental animals were assigned randomly as follows; n = 8 animals each in four groups (I - IV) with each group treated as accordingly. Group I received 1 ml of normal saline /kg/day; Group II received diazepam at 2 mg/kg/day only; Group III received diazepam at 2 mg/kg/day with NSO at 1 ml/kg/day; and Group IV received NSO at 1 ml/kg/day only. Oral administration was done once in a day for fourteen consecutive days.

Assessment of behavior and memory

Morris water maze procedure

The Morris water maze (MWM) was used to assess the spatial, long-term (LTP) and short-term (STM) memories in a standardized black pool filled with 23 ˚C – 24 ˚C water with dimensions 60 cm depth X 136 cm diameter. The pool was divided into quadrants and had a submerged circular platform. Standard MWM protocols were followed[10] and trials were recorded by video system. Trial sessions were given on days 11, 12 and 13. On day 14, the LTM was assessed using the escape latency, duration in seconds to navigate to the hidden platform while the STM was recorded as the average escape latencies of two subsequent trials. After removal of the hidden platform, the percentage time spent in the platform quadrant constituted reference memory (RM).

Open field test procedure

All rats were placed in a standardized well-lit box whose floor was divided into 4 X 4 squares and observed for exploratory, locomotor and anxiety-related behaviors. Time spent in the centre before the commencement of motion (freezing time), the number of lines crossed within the testing period (line crossing frequency). Anxious rats are less mobile and will also tend to avoid the central squares.

Biochemical evaluations

Brain tissues harvested from each group were treated in 30% sucrose solution after which they were homogenized (100 Mm Tris-HCl [Ph 7.6]) in 0.1 M DDT) and centrifuged at 2500 revolutions per minute for 10 min, and the resuting supernatant was collected into labelled tubes for various assays.

Glutathione (GSH) Assay

Supernatant from homogenate of previously frozen brain samples was used for the assay following instructions from the reagent kit while the concentrations of reduced and oxidized glutathione in fixed quantity of the brain tissue were calculated and expressed as µmol of GSH/mg protein.

Quantification of Malondialdehyde (MDA)

Malondialdehyde as marker of lipid peroxidation shows the rate of degradation of polyunsaturated fatty acids that are typical of neuronal membranes. Through its reaction with thiobarbituric acid (TBA) in the form of thiobarbituric acid reactive substance (TBARS), the quantity of MDA is derived from the amount of TBARS due to their MDA-TBA (1:2) adduct product of their reaction. The concentration of TBARS was determined according to a method of Mihara and Uchiyama and was expressed as nmol/mg of protein.

Quantification of Superoxide dismutase activity (SOD)

Superoxide dismutase activity is increased in situations of exposure to oxidative stress in the cells which induces rapid synthesis of the enzyme. The antioxidant enzyme breaks down (dismutates) reactive oxygen species-derived superoxide radicals into hydrogen peroxide and molecular oxygen which is its mechanism of defense against cellular toxicity of superoxide radicals. This SOD activity assay is based on the rate of inhibi-

tion of nitroblue tetrazolium (NBT) in the biochemical reaction where SOD competes with NBT for the superoxide radical generated by the xanthine-xanthine oxidase.

Immunohistochemical evaluations

Tumor necrosis factor alpha (TNF-α) quantification assay

The principle of TNF- α assay is based on an immunoassay technique that uses TNF-α–specific (anti-TNF-α) antibody. The presence of TNF-α is then determined by its binding to the anti-TNF-α antibody which has been mobilized in a microplate. TNF-α is a marker of inflammation which assay is used to determine the presence or progression of inflammation and proliferation of tumor cells.

Interleukin-10 (IL-10) assay

IL-10 is an anti-inflammatory cytokine that is used as a marker for inflammatory and autoimmune pathologies due to its role in the prevention of these cellular events. Hippocampal tissue homogenate was centrifuged at 14,000 x g at 4˚C for 20 minutes and sandwich ELISA was performed (BioSource International, Camarillo, CA) with the limits of detection at 25 pg/ml, following manufacturer's instructions.

Nitric Oxide (NO) assay

The level of NO in the brain homogenate was measured with Griess reagent in the form of nitrite. The Griess protocol which was performed per manufacturer's instruction is a two-step reaction during which nitrite is reduced to nitrogen oxide which then reacts with a second reagent that ends in a stable product detectable at 540 nm absorbance.

Tissue processing and Histology

Alcohol dehydrated brain tissues were processed through xylene to provide tissue transparency and subsequently paraffin embedded. Serial sectioning of the whole brain at 5 µm was done followed by staining with Cresyl violet for Nissl substance.

Statistical Analysis

Behavioural and biochemical data was analysed using one-way analysis of variance (ANOVA) followed by a *post hoc* Bonferroni's multiple comparison test. Graphpad Prism software (version 5.0, La Jolla, CA) was used to obtain results expressed as mean ± standard error with $p < 0.05$ considered statistically significant.

Results

Diazepam led to poor cognitive and motor behaviors

Marked reduction in line crossing frequency in the open field assay was recorded in animals that were given BZD. Meanwhile, this trend was significantly reversed by treatment with NSO as higher line crossing frequencies was observed in the intervention group compared to the BZD exposed rats (Figure 1A). The freezing period of the BZD rats was increased beyond that observed in the control rats but co-administration with NSO marginally reduced it to levels comparable with the controls (Figure 1C).

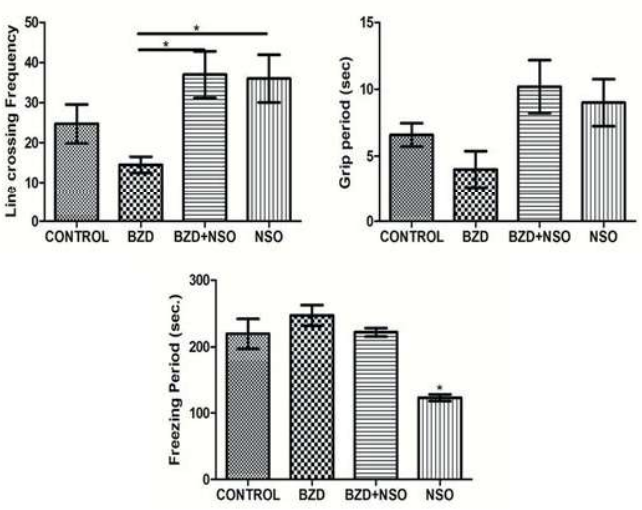

Figure 1: Motor and Anxiety-like behaviors in rats after exposure to benzodiazepines and Nigella sativa oil. Control: Normal Saline, BZD: Benzodiazepine, BZD+NSO: Benzodiazepine + Nigella Sativa Oil and NSO: Nigella Sativa Oil. Single asterisk (*) indicates significant (p≤0.05) increase or decrease from the control and or other groups

Short- and long- term memory (STM and LTM) behavioural assay as quantified by escape latency in the MWM showed delayed escape latencies for the BZD exposed rats while NSO treatment in co-exposed and sole ingestion rats caused reduction in the latency to find the hidden platform, when compared with the BZD exposed rats (Figure 2A). Reference memory (RM) was quantified by the amount of time spent in the platform quadrant after the platform was removed. We observed that BZD exposure does not affect the time that the rats spent around the quadrant region compared to control. Nonetheless, reference memory was evidently improved by the ingestions of NSO, either in co-exposure or NSO only, as the rats in these groups had higher platform latency relative to the BZD and control (Figure 2C).

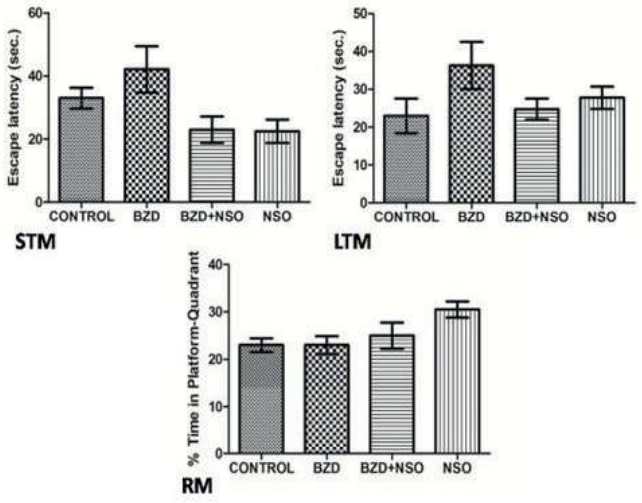

Figure 2: Memory indices in rats after exposure to benzodiazepines and Nigella sativa oil. Control: Normal Saline, BZD: Benzodiazepine, BZD+NSO: Benzodiazepine + Nigella Sativa Oil and NSO: Nigella Sativa Oil.

Benzodiazepine uptake reduces glutathione redox activity

Exposures to BZD did not affect the brain concentrations of reduced glutathione (GSH) which is a trend observed across all the test and control rats. However, in contrast to what is observed in GSH, the brain concentrations of other glutathione isoforms (glutathione peroxidase, glutathione S-transferase, and glutathione reductase) were depleted following BZD exposure, although only GST was significant (Figure 3). Interventions with NSO shows a statistically significant potential for enhanced antioxidant ability by boosting the concentrations of GPX, GST and GR in the brains of the treated rats ($p<0.05$) (Figure 3).

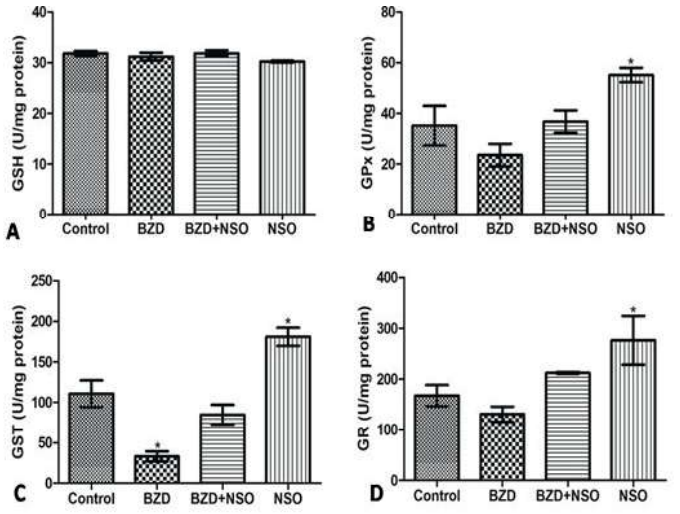

Figure 3: Glutathione redox activities in rats exposed to diazepam and Nigella sativa oil. Control: Normal Saline, BZD: Benzodiazepine, BZD+NSO: Benzodiazepine + Nigella Sativa Oil and NSO: Nigella Sativa Oil. Single asterisk (*) indicates significant ($p\leq0.05$) increase or reduction from control and or other groups.

Benzodiazepines elevate levels of oxidant and pro-inflammatory markers

Significant reduction in total antioxidant capacity was evident in BZD treated rats but which was elevated NSO exposed rats, albeit to non-statistically significant levels. This is supported by observations of elevated levels of ROS, MDA and SOD accompanied by reduced levels of catalase in BZD treated rats (Figure 4). The levels of ROS were rather indistinguishable in the BZD+NSO and NSO groups (Figure 4B), however additional parameter comparison between the two groups showed lower levels of MDA and SOD (Figures 4C and 4D) and higher levels of catalase in the NSO group (Figure 4E). NSO clearly improved the total antioxidant capacity and marginally lowered ROS and MDA relative to the BZD group. The levels of some pro- and anti-neuroinflammatory markers, cytokines and chemokines released by activated macrophages and astrocytes, were investigated. Elevated concentrations of TNF-α in the BZD treated group ($p < 0.05$) were consistent with increased likelihood of ongoing inflammatory processes. Nitric oxide (NO) levels were maintained at near control rat levels as contrasted with the drastically lowered levels observed in the rats exposed to NSO in both the NSO and BZD+NSO groups providing credence to the NO reduction effect of NSO. Interleukin-10 (IL-10) levels were however lowered in the BZD group and markedly elevated ($p\leq0.05$) in the NSO group, indicative of greater anti-inflammatory potential in the latter group (Figure 5).

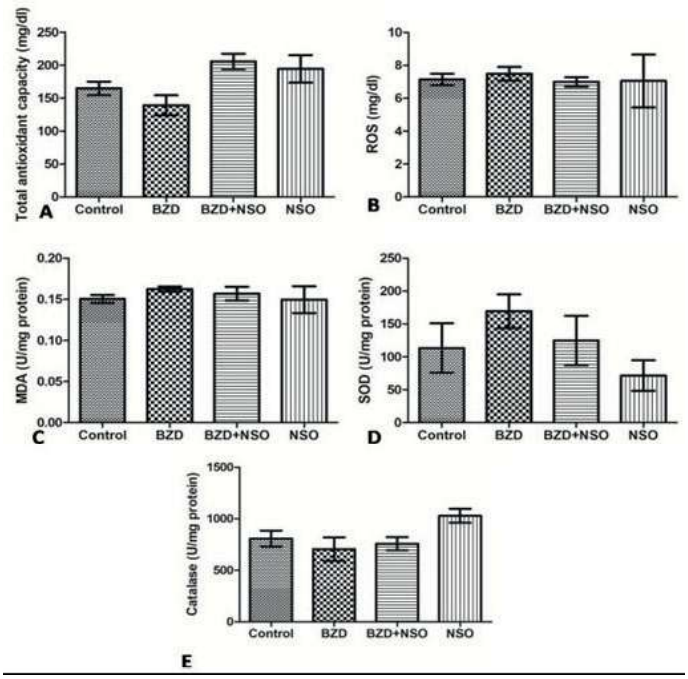

Figure 4: Antioxidant, oxidative and lipid peroxidation markers in rats exposed to benzodiazepines and Nigella sativa oil. Control: Normal Saline, BZD: Benzodiazepine, BZD+NSO: Benzodiazepine + Nigella Sativa Oil and NSO: Nigella Sativa Oil.

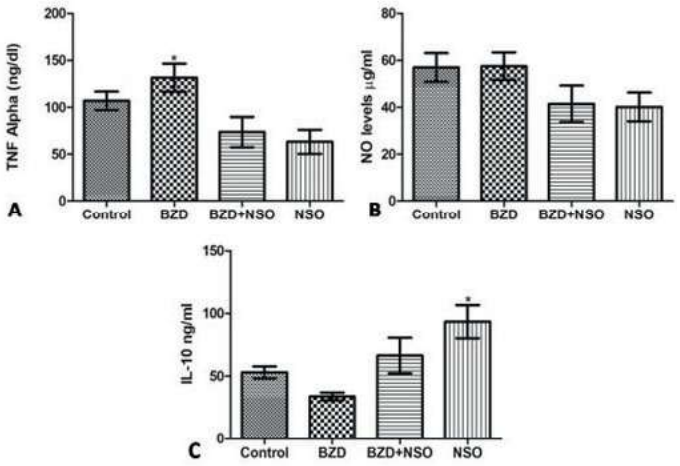

Figure 5: Pro and anti-inflammatory markers in rats exposed to benzodiazepines and Nigella sativa oil. Control: Normal Saline, BZD: Benzodiazepine, BZD+NSO: Benzodiazepine + Nigella Sativa Oil and NSO: Nigella Sativa Oil. Single asterisk (*) indicates significant ($p\leq0.05$) increase from the control and or other groups.

Benzodiazepine uptake diminished neurotransmitter levels to varying extents

The levels of the inhibitory neurotransmitter, gamma aminobutyric acid (GABA), the receptor of which is acted upon by BZDs, were depleted in the BZD exposed group (Figure 6A) but markedly elevated above normal levels in the NSO group. Similarly, acetylcholine (ACh) levels though depressed in BZD exposed animals were significantly increased in the BZD+NSO and NSO groups (Figure 6B). Co-administration of BZD and NSO had a more profound recoil effect on the ACh levels than on GABA. The levels of catecholamines and monoamines were reduced in the BZD groups but consistently elevated in the NSO only groups (Figure 7). Concentrations of serotonin

and dopamine in the BZD+NSO group were higher than in the control rats whereas lower levels of noradrenaline were recorded ($p>0.05$).

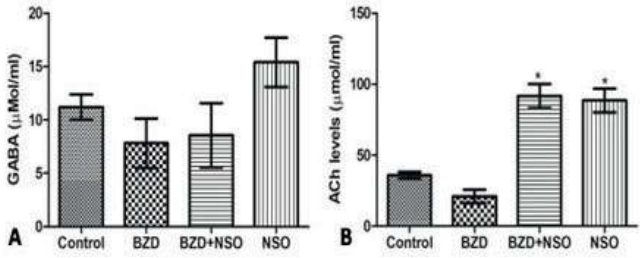

Figure 6: GABA and ACh levels in rats after exposure to benzodiazepines and Nigella sativa oil. Control: Normal Saline, BZD: Benzodiazepine, BZD+NSO: Benzodiazepine + Nigella Sativa Oil and NSO: Nigella Sativa Oil. Single asterisk (*) indicates significant ($p\leq0.05$) increase from the control and or other groups.

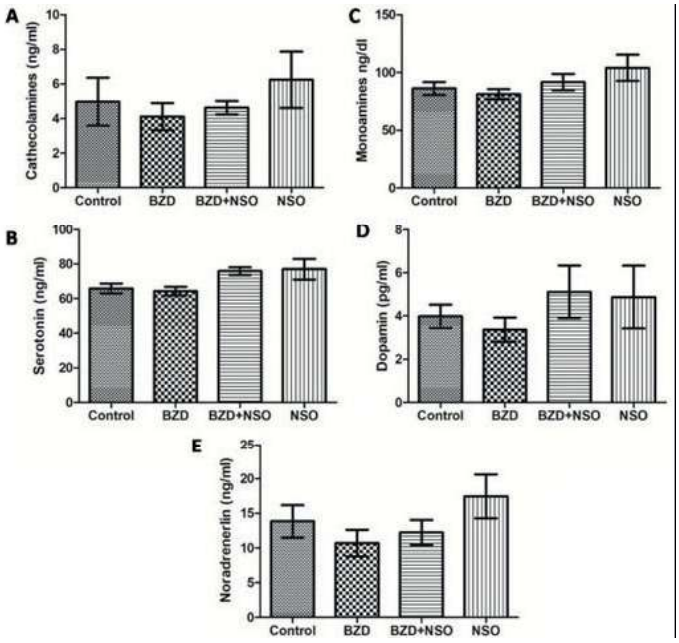

Figure 7: Catecholaminergic neuromodulators in rats following exposure to benzodiazepine and Nigella sativa oil. Control: Normal Saline, BZD: Benzodiazepine, BZD+NSO: Benzodiazepine + Nigella Sativa Oil and NSO: Nigella Sativa Oil.

Benzodiazepine administration increases neuronal metabolism and apoptosis

Caspase 3 and GLUT 4 levels were relatively elevated in the BZD administered group which contrasted with those observed in the NSO group that demonstrated lower levels than those observed in the controls (Figure 8). The co-administration of NSO with BZD lowered caspase 3 levels to a much larger extent than observed with GLUT 4.

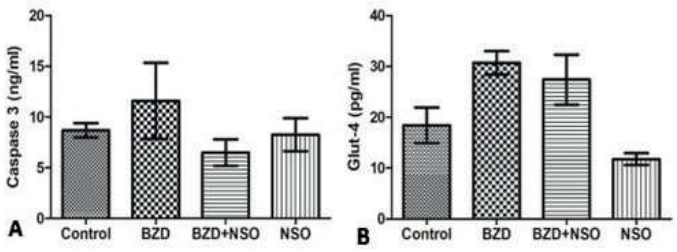

Figure 8: Caspase 3 and Glut-4 levels in rats exposed to benzodiazepine and Nigella sativa oil. Control: Normal Saline, BZD: Benzodiazepine, BZD+NSO: Benzodiazepine + Nigella Sativa Oil and NSO: Nigella Sativa Oil.

Histoarchitecture of various brain regions

Degenerative changes, although mild, were evident in all parts of rat brains exposed to BZD (Figures 9 - 11). Cells in the motor cortex and putamen appear lighter stained and larger with apparent intracellular vacuoles after treatment with BZD, compared to the controls (Figure 9). The stain intensity in the brain regions show a qualitative increase after the ingestion of NSO. Purkinje cells were fewer and more dispersed in the cerebella of BZD rats but were clearly more numerous and closely packed in the control rats but more loosely packed in the BZD+NSO rats. Individual cell boundaries of the hippocampal regions were less distinct in the BZD group as well as fewer cells in the hilar region than any of the other groups.

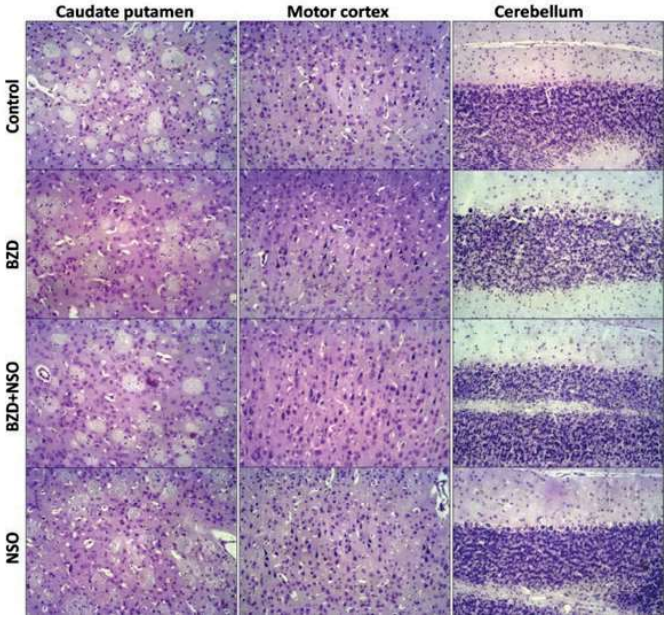

Figure 9: Representative photomicrographs of the caudate putamen, primary motor cortex and cerebellum of rats exposed to benzodiazepines and Nigella sativa oil. Control: Normal Saline, BZD: Benzodiazepine, BZD+NSO: Benzodiazepine + Nigella Sativa Oil and NSO: Nigella Sativa Oil.

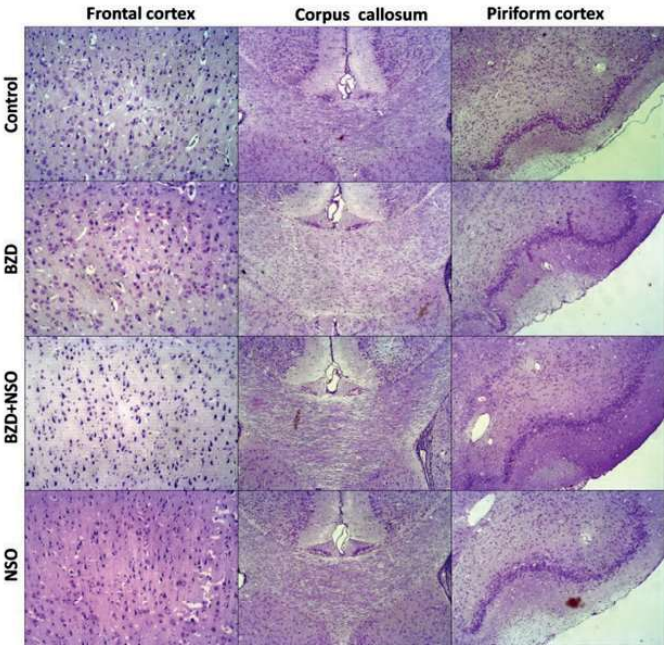

Figure 10: Representative photomicrographs of the frontal cortices, corpus callosum and the piriform cortices of rats exposed to benzodiazepines and Nigella sativa oil. Control: Normal Saline, BZD: Benzodiazepine, BZD+NSO: Benzodiazepine + Nigella Sativa Oil and NSO: Nigella Sativa Oil.

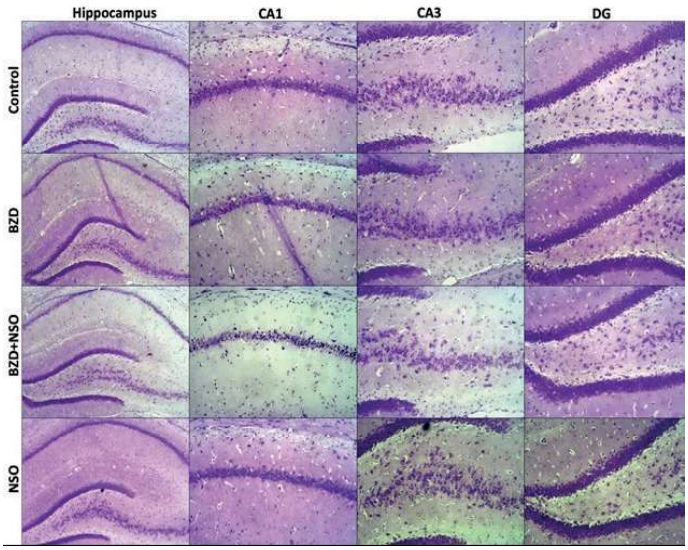

Figure 11: Representative photomicrographs of the hippocampus of rats exposed to benzodiazepine and Nigella sativa oil. Control: Normal Saline, BZD: Benzodiazepine, BZD+NSO: Benzodiazepine + Nigella Sativa Oil and NSO: Nigella Sativa Oil.

Discussion

As highlighted by Griffin and colleagues[2] in their review on the beneficial pharmacological effects and clinical uses of BZDs in various pathological and postoperative states, the effects of BZDs are not always detrimental and undesired with some benzodiazepine derivatives exhibiting anti-inflammatory properties[17]. However, this study shows that prolonged intake of benzodiazepines even at a relatively low dosage may affect psycho-cognitive behaviors and related functions, and these effects may be associated to the marked reduction in antioxidant capacities, which was accompanied by non-proportional elevation of oxidative stress and neuro-inflammatory biomarkers coupled with imbalance in neurotransmitters concentrations in the brains of the exposed rats. Oxidation related stress and neuro-inflammation are associated with myriad of neurologic sequelae and are positively identified where neurotoxicity is suspected [18, 19, 20,15]. This study also showed that co-administration of NSO potentially alleviates or reverses the neurotoxic events generated by the long-term use of BZD.

The Morris water maze was used as a standardized assay of hippocampal-dependent learning memory in rodents, whereas, the open field test was used to assess motor activity and anxiety-like behaviors. The major observed behavioral changes in this study were the registration of significantly lowered memory indices quantified as short time, long time, and reference memory, as well as the drastic reduction in normal locomotor activity in the rats chronically subjected to BZD ingestion. These changes were like those observed in other experiments from our lab, where we exposed rats to low dosages of potentially neurotoxic chemicals [15,16], or those in which the antioxidant capacity of rodents was experimentally reduced [21, 19, 22]. These neurological, cognitive and motor findings were worrisome considering the ubiquity of BZD prescription and the further unregulated abuse of the same by people that have developed dependence and addiction.

The direct effects of BZD use could account for some of these observations, as BZD activity characterised by binding to the BZ1 is associated with anterograde amnesia and possibly poor performance where memory tests are concerned [2]. Central inflammatory responses also lead to "sickness behavior" characterised in part by reduced locomotor and social activities. Paradoxically, BZD in short-term use is useful as an anxiolytic in the management of anxiety and social avoidance post social distress in rodents [3]. Here, rats exposed BZD were observed with fear or anxiolytic-like behaviors and reduced exploratory/locomotor abilities, as evidenced in the prolonged freezing periods and low line crossing frequencies in the open field test. These effects on motor and anxiety-like behaviors may in part be associated the recruitment of circulating peripheral monocytes into the CNS following chronic inflammation as reported above. Further credence to impaired behaviors of BZD-exposure is the reduced antioxidant activity, increased oxidative stress markers, increased inflammatory markers, altered neurotransmitters release and brain histoarchitecture in the exposed rats.

Repeated oral ingestions of BZDs reduces the brain's capacity for antioxidant activity as demonstrated by a reduction in GPx, GST and GR concentrations coupled with reduced total antioxidant capacity and catalase activity. Previous studies have confirmed the tendency for lowered antioxidant capacity to lead to inflammatory response and oxidative stress before the body's response mechanisms have had an adequate chance to engage compensatory changes [23]. Upregulation of genes associated with glutathione metabolism has been observed in the short-term post glutathione depletion event although it was not linked to an increase in GSH levels[23]. An actual compensatory elevation of GSH was previously reported 48 hours post depletion showing evidence of robust anti-inflammatory compensatory mechanisms in the brain[19, 24], however this was most probably due to their use of a once off depletion event rather than multiple repeated insults used in our study. GSH levels in our study remained rather low, yet it is a crucial element in the preservation and recovery of neurons faced by oxidative stress[25, 26, 27]. Furthermore, marginal increases in the levels of ROS and MDA in the BZD exposed rats provided further credence to the concept of suppressed antioxidant activity.

Surprisingly, a key antioxidant enzyme, SOD recorded elevated activity in BZD exposed rat's brain, probably as the body's compensatory mechanism in enhancing the depleted antioxidant activity from other chemical systems. This result may be buttressed with the finding obtained in Sprague-dawley rats 24 – 48 hours post single event depletion of GSH [19]. However, in our case this compensation could not re-establish balance as it may have led to the production of H_2O_2 while the downstream enzymes, GPx and catalase, for its neutralisation remained suppressed in activity. Zhang et al., 2018 [22] also observed reduced catalase activity in stressed rats although in their case, it was also coupled with lowered SOD activity. The lowered GPx and GST observed in this study thus rendered the BZD rats vulnerable to chronic oxidative stress and accompanying pathological changes. Synaptic dysfunction and depletion of neural connections associated with lowered antioxidant capacity are known to impair learning memory[19], as was observed in the behavioral manifestation of impaired memory and reduced locomotor activity of rats exposed to BZD in this study.

We have shown that prolonged use of benzodiazepines leads to sustained neurological insult that depresses the brain's protection against exaggerated neuroinflammation and oxidative stress.

This study also observed sustained high levels of pro-inflammatory markers. Elevation in inflammatory cytokines/chemokines has been widely associated with cognitive dysfunction, and as an integral event in the genesis of neurotoxicity, neurodegenerative diseases, and psychosis. TNF-α, a potent proinflammatory cytokine often used as an indicator of inflammation [28] was markedly elevated in BZD exposed rats. TNF-α binds to TNF receptor 1, initiating a cascade of chemical processes, part of which is cell death through the death domain in the tail end of this receptor. Nitric oxide which ordinarily is a vasodilator and anti-inflammatory molecule, is pro-inflammatory when produced in excess amounts. The production of such cytokines and secondary messengers by activated microglia and astrocytes function to initiate protective mechanisms for CNS tissue under normal physiological conditions but lead to undesirable chemical, pathological and physical changes when prolonged. Interleukin 10 (IL-10) being an anti-inflammatory cytokine plays a crucial, and often essential role in the prevention and remediation of inflammation and pathologies of autoimmunity post inflammatory insult [29]. The right amounts of IL-10 have to be present to exert delicate control moderating the extent neuroinflammation [30,31,32]. The suppressed levels of IL-10 coupled with depressed anti-inflammatory markers in the BZD exposed brains of this study provided a plausible cause for the relatively unchecked inflammatory response and resultant neurodegeneration.

Continuous use of BZD led to a reduction in the brain levels of GABA, an inhibitory neurotransmitter and neuromodulator that has a calming effect on the brain overall and has also been linked to improved memory and relief of mental stress[33,2,3,4]. A decrease in GABA levels is associated with the display of anxiety- and stress-like behaviors exemplified by fear to explore new surroundings, avoiding the central squares, keeping to the walls and generalised reduced locomotor activity. The concentrations of dopamine, serotonin and monoamines were reduced in all BZD rats, albeit not to statistically significant levels. The "biogenic amine hypothesis of depression" links low amine levels to depression and anxiety, and forms the basis of amine elevation in antidepressant management. Noradrenaline deficiency too is linked to certain forms of cognitive and memory impairments that involve projections from the locus coeruleus[35,36]. Lowered amines would thus disrupt the rats' ability to learn and recall resulting in poor memory-linked behavioural performances. Previous studies have demonstrated that anxious and neuroinflamed rats demonstrate suppressed exploratory behaviour [35, 15, 16], corresponding to lower line-crossing frequencies observed in the current study. Our study showed lowered levels of acetycholine in the BZD exposed rats, which was subsequently restored following interventional ingestions of NSO. Acetycholine (Ach) has been identified as essential in the learning memory processes being related to the cognitive deficits of dementias such as Alzheimer's disease (AD), with an increase in ACh levels has been shown to reverse some of the observed deficits [22].

Caspase 3 is a recognised marker that mediates programmed neuronal death (apoptosis) during embryological brain development and delays neuronal death following neurological insult such as during ischemic injury or traumatic brain injury [37; 38]. The levels of caspase 3 in the brains of BZD exposed rats were reasonably higher than those of controls. This depicts events during neuronal cell death in rats exposed to extended duration treatment with BZD. GLUT-1 is one of the transporters of glucose in capillaries supplying brain cells and thereby satisfying the energy demand of neurons in brain[39]. Depletion of this transporter results in the death of neurons due to starvation. In this study result, BZD significantly reduced the level of GLUT-1 in exposed rats, thereby leading to cell death. High TNF-α level as observed in the inflammatory result, is also associated with excitotoxicity, which is one of the potential mechanisms that can contribute to neuronal cell death.

The reduction in the levels of inflammation in rodent models after induction of brain injury is often associated with better functional recovery outcomes. The functional and behavioral outcomes investigated in this study were STM, LTM and RM in the MWM, and locomotor activity as determined by the open field test. It is evident that co-administration with NSO sub-served this role as rats in the BDZ+NSO group outperformed the BZD group in both the STM and LTM tasks. It can be concluded that administration of NSO which as an anti-oxidant and anti-inflammatory agent to the injured brain prevented development of psychological and behavioral phenotypes associated with CNS insult. The rats treated with NSO alone further excelled at the memory, locomotion and anxiety tasks compared to the control animals, and demonstrated lower oxidative stress and pro-inflammatory marker levels, further confirming the efficiency of NSO as an anti-oxidant and anti-inflammatory agent. This result corresponds with that of other studies showing the potency of NSO against oxidative stress and neuroinflammation-driven impaired memory and motor dysfunction[40; 16, 41, 42]. NSO also improved the levels of apoptosis markers; Caspase 3 and GLUT-1, thereby preserving the histoarchitecture of the brains treated with it. Various studies have reported the improvement of memory and locomotion with increased neurotransmitters activities[43,44,45]. NSO increased GABA, ACh, Catecholamine, Monoamine, Serotonin, and Dopamine neurotransmitters, when administered together with/without BZD, modulating the depleting effect of BZD on neurotransmitters levels, and generally improving their activity in exposed rats.

Conclusion

In conclusion, prolonged ingestion of low dose benzodiazepines leads to oxidative stress, neuro-inflammation with altered neurotransmitters levels and degenerative changes in the brain as evidenced by the reduction of antioxidant and anti-inflammatory biomarkers. These elevation of inflammatory markers of oxidative stress, and brain neurotransmitter level imbalance as well as various region-specific cyto-architectural alterations were observed across the brain. This leads to various manifestations of behavioral impairment, including impaired memory, impaired locomotion, and anxiety. Finally, interventional NSO demonstrated therapeutic potentials against BZD dependency-linked neurotoxicity affecting behavioural and psycho-cognitive abilities by offering improvements in the areas studied.

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Author contribution subject and rate:

Imam Aminu (20%): Concept and design of the study, definition of intellectual content, experimental studies, literature search, collection of data, analysis and interpretation of data, manuscript preparation, editing and submission of manuscript.

Kudirat Funmi Lambe-Oladeji (10%): Concept and design of the study, experimental studies, literature search, collection of data and analysis, analysis and interpretation of data, manuscript editing and review and final approval of the version to be published.

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