D-ribose-L-cysteine modulates paradoxical sleep deprivation-induced neurological impairments: anxiolytic and antioxidative study in rat model

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ABSTRACT
This study evaluated the anxiolytic and antioxidative potential of DRLC on behavioral deficits, and neuronal perturbations in the hippocampus following paradoxical sleep deprivation in adult Wistar rats. Animals were paradoxically sleep deprived for 7 days; DRLC (75 mg/kg b.w of Riboceine™) was pre- and post-administered for 21 days before and after paradoxical sleep deprivation. Thereafter, behavioral study was conducted to assess fear and anxiety, animals were sacrificed and biochemical analysis of oxidative stress markers and histomorphology of the hippocampus was performed. Behavioral assessments revealed that PSD elicited increased anxiety levels as demonstrated by reduced line crossing, reduced habituation time, and increased freezing time in OFT as well as increased time spent in closed arms of EPM. Also, oxidative stress levels were elevated by PSD with significantly decreased activities of catalase (CAT), superoxide dismutase (SOD) and glutathione (GSH) concentration as well as increased Malondialdehyde concentrations in the blood serum. However, pre- and post-treatment with DRLC significantly prevented and reduced anxiety levels and oxidative stress levels as well as prevented and repaired neuronal hippocampal damage associated with PSD respectively. In conclusion, DRLC was able to modulate oxidative stress-driven alterations and perturbed emotionality linked with PSD through its anxiolytic and antioxidative properties.

Abbreviations: D-ribose-L-cysteine(DRLC); Catalase(CAT); Glutathione transferase(GSH); Superoxide dismutase(SOD); Non-rapid eye movement-(NREM); Rapid eye movement(REM); Paradoxical sleep deprivation(PSD); Elevated plus maze(EPM); Open Field test(OFT)

Introduction
Sleep is a ubiquitous phenomenon and most species, including humans spend a significant time asleep. It is widely acknowledged that sleep is crucial for proper brain function. There are five phases of sleep: the wake phase, non-rapid eye movement (NREM) phase, which has 3 stages (N1 to N3), and the rapid eye movement (REM) phase. Wakefulness is characterized by more than 50% alpha waves and opening of the eyes. The N1 stage of the NREM phase is...
the most superficial stage of sleep characterized by skeletal muscle tone and regular breathing rate. The N2 stage, where the majority of sleep is spent, typifies a deeper stage of sleep characterized by lowered body temperature and heart rate. As deeper sleep develops, there is a transition to the N3 stage of NREM. This is the deepest stage of sleep when the body repairs worn-out tissues, builds bones and strengthens the immune system. REM sleep usually begins about 90 minutes after an individual falls asleep. Apart from the eye and diaphragmatic breathing muscles which remain active, all other skeletal muscles are inactive. The REM stage of sleep is characterized by dreaming and erratic breathing rate [1].

Each sleep phase is characterized by specific chemical, cellular and anatomic events of vital importance for normal neural functioning [2]. Different forms of sleep deprivation may lead to a decline of cognitive functions in individuals [1].

Sleep deprivation is the state of inadequate sleep which may either be acute or chronic. Chronic sleep deprivation is associated with fatigue, clumsiness, weight loss or gain, and daytime sleepiness [1]. Apart from causing physical health problems, sleep deprivation also has adverse effects on the brain [3] especially on hippocampal function [4].

Although several models of hippocampal function focus on its well-known role in cognitive functions, historically it has also been viewed as a neural mediator of emotion. Accumulating evidence that may accommodate some of this variability is that anxiety is functionally segregated within the hippocampus, with ventral subregions more involved in anxiety-related processes, and dorsal subregions more involved with cognitive processes. Hippocampal cellular and molecular processes critical for memory consolidation and emotionality are affected by the amount and quality of sleep attained. Chronic sleep deprivation triggers reduction of hippocampal cell proliferation and neurogenesis, which may eventually lead to hippocampal volume reduction. Hence, the impaired hippocampal plasticity and function contribute to cognitive disorders and psychiatric diseases [4].

D-ribose-L-cysteine (DRLC), a precursor to the antioxidant glutathione [5], is a very simple and unique molecule that is produced naturally all the time in the body. It is a combination of three simple building blocks of protein or amino acids-cysteine, glycine, and glutamine. It is believed to enhance wound healing [5], attenuates testicular damage [6], attenuates memory deficit induced by lipopolysaccharide [7]. DR LC primarily protects and detoxifies the cell and studies have shown that glutathione production declines with age from age 20, at a rate of about 1% per year [8]. Depletion of the body’s glutathione level is triggered by air toxicants, food, stress, radiation, and pharmaceutical medications [9–12]. DR LC is a synthetic antioxidant that is effective in combating oxidative stress. Studies have shown that DR LC supplementation has both protective and ameliorative impacts on tissue damage that results from oxidative stressors [13–15]. There is evidence linking oxidative stress to the occurrence of depression and anxiety. Since, brain lipid peroxidation cannot be directly assessed in living subjects, the need to measure oxidative stress markers in other biological fluids such as the serum is paramount. An increased concentration of oxygen reactive species (ROS) in cells and tissues and the inability of a biological system to detoxify these reactive products often result in harmful effects on important cellular structures like proteins, nucleic acids, and lipids. Sleep deprivation tends to cause increased generation of reactive oxygen species which leads to the buildup of oxidative stress in different brain regions that can predispose a person to various neurodegenerative disorders. Obtaining adequate sleep is challenging in a society that values ‘work around the clock’. Hence, the development of interventions to combat the negative cognitive effects of
sleep deprivation is key. Studies have shown that sleep deprivation is a potent oxidative stressor [16,17] that leads to various neurodegenerative disorders. Therefore, this study intends to investigate the impact of D-ribose-L-cysteine (DRLC) on behavioral deficits, and neuronal perturbations in the hippocampus triggered by increased oxidative stress parameters in the serum, induced by paradoxical sleep deprivation.

**Materials and Methods**

**Animal Care**

Thirty-five (35) adult male Wistar rats weighing between 180 and 200 g were procured, acclimatized, and housed in the animal house of the Faculty of Basic Medical Sciences, Osun State University. The rats had liberal access to rat chow and water. Animal care and experimental procedures were performed according to the ARRIVE guidelines, and the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

**Animal Grouping and Treatment**

Thirty-five (35) Wistar rats weighing between 180 and 200 g were randomly divided into 5 groups with n = 7.

- Group I: Control group.
- Group II: PSD group paradoxically sleep deprived for 7 days
- Group III: PSD + DRLC group paradoxically sleep deprived for 7 days and post-treated with DRLC (75 mg/kg b.w) [7,15] for 21 days.
- Group IV: DRLC + PSD group pretreated with DRLC (75 mg/kg b.w) for 21 days to evaluate the protective impact of DRLC and then paradoxically sleep deprived for 7 days;
- Group V: DRLC group treated with DRLC (75 mg/kg b.w) only for 21 days. This serves as positive control for groups III & IV.

DRLC was procured from Max International, dissolved in distilled water, and administered orally using oral gavage. Pre and post-treatment with DRLC were to access the protective and ameliorative potentials of D-Ribose-L-Cysteine.

**Paradoxical Sleep Deprivation (PSD) Protocol**

The multiple platform technique was used to induce PSD according to the method described by Nunes et al., 1994 [18]. The water tank with dimensions 42 cm x 35 cm x 17 cm) contained 12 platforms (3 cm in diameter) surrounded by water up to 1 cm beneath the surface. Each group was placed on the 6 platforms with the animals being able to move from one platform to another. Animals were sleep-deprived both day and night. They were only taken out of the tank and fed in conventional cages for 3 hours daily after which they were returned to the tank. PSD lasted for 7 days.

**Neurobehavioural Paradigms**

**Open field test (OFT)**

The locomotor activity and anxiety-like behavior were measured in the open field test [19]. Rats were gently placed into a corner of the box and allowed to explore the apparatus for 3 minutes. Exploratory motor activity (EMA) which includes horizontal locomotion (the number of squares crossed) and vertical activity (rearing) were recorded. At the end of the test, the rat was gently picked from the maze, returned to home cage and fecal boli removed and urination spots cleaned. The maze was cleaned with 95% ethanol before the commencement of the next test period.

**Elevated plus maze test (EPM)**

The elevated plus maze is used for assessing anxiety responses of rodents [20]. At the commencement of this test, animals were centrally
placed between the open and closed arms, and the behavior of each animal was consistently recorded using a video recorder mounted in the room for 5 min. The training session lasted for 5 min before the test session. The number of times the rats entered the open arm or closed arm and the time spent in each arm were recorded.

Sample Collection

After the completion of treatments and neuro-behavioral testing, animals were euthanized by intraperitoneal anesthetic injection of ketamine hydrochloride (50 mg/kg) and a mid-abdominal incision was made on the anterior abdominal wall toward the thoracic region to expose the heart. Blood was collected via cardiac puncture using a sterile needle and 5 ml syringe into plain bottles. The blood sample was allowed to clot for 30 mins, centrifuged at 2000 g for 10 mins and serum was pipetted into polypropylene tubes for immediate biochemical analysis. The animal’s brain tissue was excised from the skull, rinsed in 0.25 M sucrose 3 times for 5 minutes each, and thereafter fixed in buffered neutral formalin (BNF). Sacrifice of the animals was done in batches based on the duration and treatments of animals in each group.

Determination of Oxidative stress markers

Superoxide dismutase (SOD), Catalase (CAT), Glutathione (GSH), and Malondialdehyde (MDA) assay kits procured from Bio Legend Inc., San Diego, CA, USA were used to determine the activities of SOD and CAT, and the concentrations of GSH and MDA in the blood serum of the experimental animals for the quantification of oxidative damage in the experimental animals.

Superoxide dismutase activity

Superoxide dismutase scavenges superoxide anion, converting it into hydrogen peroxide and oxygen thus preventing peroxynitrite production. The activities of Superoxide dismutase in the serum was determined according to the method of Misra and Fridovich [21] based on the ability of SOD to inhibit the autoxidation of adrenaline to adrenochrome in a basic medium. Summarily, 75 mM tris-HCl, 30 mM EDTA, 2 mM pyrogallol (pH 8.2) was added to 50 microliter of the serum. Absorbance was read at 420 nm using a microplate reader.

Glutathione assay

The glutathione (GSH) concentration was estimated following the procedure developed by Ellman [22] and described by Beutler et al. [23], based on the ability of the Ellman reagent, 5, 5-dithiobis-2-nitrobenzoic acid (DTNB), to react with compounds containing sulfhydryl groups, yielding a mixed disulfide (GS-TNB) and 2-nitro-5-thiobenzoic acid (TNB) with the absorbance of the anion (TNB^-) read at 412 nm.

Catalase activity

Determination of catalase activity in the serum was performed according to the dichromate method described by Sinha [24] based on the utilization of hydrogen peroxide by catalase using the potassium dichromate K_2Cr_2O_7/acetic acid reagent. Briefly, when heated in the presence of hydrogen peroxide, the dichromate in acetic acid reduces to chromic acetate, which is measured spectrophotometrically at 610 nm.

Malondialdehyde assay

MDA concentration was determined by measuring thiobarbituric acid reactive substances present in the blood serum according to the modified method of Buege and Aust [25], 1 ml of the serum was added to 2 ml of the reagent comprised of trichloroacetic acid, thiobarbituric acid, and hydrochloric acid (TCA/TBA/HCl) at ratio 1:1:1. After vigorously shaking, it was boiled for 15 min, cooled on ice, centrifuged at 3000 rpm for 10 min, and the absorbance was read at 532 nm against a blank.
**Histology and histochemistry**

Fixed brain tissues in BNF were dehydrated with ascending grades of alcohol, clear in xylene, infiltrated and embedded with paraffin wax, and mounted on a paraffin cassette. Thereafter, hippocampal sections of 6 microns were cut using a microtome, mounted on a clean slide, and stained with Hematoxylin and eosin for general histology according to the method of Bancroft & Layton [26] and Cresyl fast violet for histochemical demonstration of Nissl substances according to the method of Pilati et al. [27], Photomicrographs were captured using a Biobase binocular research microscope (China) connected to a 5.0 MP Amscope.

**Statistical Analysis**

Quantitative outcomes of neurobehavioral and biochemical examinations were analyzed using GraphPad Prism® (version 8) software. The outcomes were plotted in One-way ANOVA followed by Turkey’s multiple comparisons test. Significance was set at p < 0.05*. The staining polarity of the Cresyl fast violet stain was measured using the Image J software.

**Results**

**Sleep Deprivation was Associated with Increased Emotionality in the Open Field Test**

Obvious signs of anxiety and emotionality displayed following PSD were significantly reduced with pre- and post-treatment with DRLC as observed in Figure 1. PSD group exhibited significantly (p < 0.05) reduced time of habituation with a mean value of 55.13 ± 3.262 when compared to the control group as well as the DRLC treated groups which showed significantly increased habituation time with p-value <0.05 as seen in Figure 1A. In addition, the PSD group displayed significantly reduced number of crossing (15.60 ± 1.217) with a p-value <0.05 in contrast to the control group having a mean value of 31.40 ± 1.230. However, DRLC treated animals (PSD+DRLC, DRLC+PSD groups showed a significantly increased no of crossings with a p-value <0.05 when compared with the PSD group as seen in Figure 1B. Furthermore, PSD animals spent significantly increased time freezing with a mean value of 8.24 ± 2.130 when compared to the control and DRLC treated groups as observed in Figure 1C.

**Sleep Deprivation Increases Anxiety**

The elevated plus maze test was done to evaluate anxiety and fear. PSD+DRLC, DRLC+PSD, DRLC treated animals, as well as the control group, showed reduced time spent in the closed arm (68.00 ± 1.412, 65.80 ± 0.851, 51.60 ± 1.920, and 53.040 ± 1.213 respectively) whereas PSD animals significantly spent more time (113 ± 1.406) in the close arm of the apparatus as seen in Figure 2.

**SOD, GSH, and CAT Profiles increases following D-Ribose-L-Cysteine Administration**

Differential expression of superoxide dismutase and catalase activities, as well as glutathione concentrations, were assessed in the experimental rats as seen in Figures 3A, B, and C respectively. Results showed significant increase (p < 0.05) in the activities of catalase (CAT) and superoxide dismutase (SOD), as well as the levels of glutathione (GSH) in DRLC, treated animals (PSD+DRLC, DRLC+PSD, DRLC) when compared with PSD animals (group B). PSD rats expressed significantly decreased levels in the activities of these enzymes (CAT & SOD) and concentration of glutathione (GSH).

**DRLC Reduced Lipid Peroxidation**

MDA profiles as shown in Figure 3D, increased significantly in the PSD animals (7.200 ± 2.131) as against the reduced MDA mean value
(2.400 ± 1.208) of the control group, as well as the DRLC treated groups. There was no observable significant difference in the malondialdehyde levels of animals pre and post-treated with DRLC when compared with the control.

**Histological and Histochemical Evaluation**

In Figures 4 and 5, the control group showed a well-outlined array of cells within the hippocampus as seen from the cornu ammonus (CA) to the dentate gyrus (DG). Normal cellular densities were observed in the different hippocampal layers characterized by normal dendritic spines (yellow arrows). Group B animals, however, showed fragmentation in the pyramidal and granular cell layer with the presence of pyknotic cells. The pre-and post-treated DRLC animals showed regenerative changes (black arrows) mildly similar to the morphologic appearance of control treatments; their cortical layers appeared better structured and delineated with a distinct layering when compared with PSD animals (group B). The DRLC group showed similar morphological observation to that of the control group.

Also, in Figures 6 and 7, normal cytoplasmic Nissl substance staining was observed in the control (A) and pre-and post-treated DRLC animals characterized with well-stained neurons (yellow arrows) whereas, the PSD group showed chromatolytic changes in the pyramidal and granular cell layers with reduced cytoplasmic Nissl staining intensity (black arrow).

**Discussion**

This current study was designed to investigate the anxiolytic and antioxidative potential of D-Ribose-L-Cysteine following paradoxical
sleep deprivation in male Wistar rats. Laboratory rats normally sleep for about 12 hrs/day of which 15–20% of the sleep time corresponds to the REM sleep stage. This study confirmed REM sleep deprivation as a stressor, which may have deleterious effects on learning and memory sub-served by the hippocampus via its anxiogenic and oxidative mechanisms.

In an attempt to establish the anxiety-related behavior associated with PSD and DRLC treatment, the EPM and OFT were used to assess fear and anxiety in the experimental groups. Our findings confirmed previous results of behavioral deficits in sleep-deprived animals [28,29]. While sleep deprivation was associated with increased exploratory activities in open field test following tread-mill sleep deprivation [30], in our study, sleep-deprived rats exhibited reduced motor activity characterized by decreased crossings, decreased time of habituation and increased time spent freezing. This difference in exploratory response may be due to the difference in the technique and duration employed to induce sleep deprivation. In addition, although some studies showed that sleep deprivation reduced anxiety-like behavior [31,32], however, in this study, paradoxical sleep deprivation was associated with increased anxiogenic effect as reflected by increased time spent in the closed arm of the maze. This finding was similar to the report of Silva et al. [33], and Omotoso et al. [34]. However, treatment with DRLC before and after PSD attenuated the anxiety-like behaviors characterized by increased crossings, decreased freezing time, increased time of habituation as well as reduced time spent in the closed arm. Hence, DRLC was confirmed as an anxiolytic agent in this study. Though the mechanism by which DRLC exerts its anxiolytic property is yet to be fully elucidated, our study suggests that this is likely to be related to the antioxidative property of DRLC to reduce the oxidative stress attenuated with PSD.

Sleep has been confirmed to limit metabolic requirements whereas sleep deprivation, on the other hand, enhances metabolic rate and in turn increases oxidative stress. Previous research has shown that sleep deprivation elicits psychotic episodes by induction of increased oxidative stress levels [35], triggered by increased metabolic activities. Lipid peroxidation is a known degenerative process of oxidation that leads to destruction of cell membranes, lipoproteins, and other lipid-containing structures [36]. Lipid peroxidation has been linked to a variety of neuropsychiatric disorders, including depression, schizophrenia, bipolar mood disorders, attention deficit hyperactivity disorder, and Alzheimer’s disease [37,38]. Previous studies have shown that increased serum lipid by-products, MDA and Lipid hydroperoxide (LOOH), are linked with patients with anxiety disorders [39,40]. This conforms to the findings in our present study,

Figure 2. Elevated plus maze test. the PSD group exhibited low time of habituation and spent more time in the closed arm when compared with the control group (p < 0.05). animals pre and post-treated with DRLC in this study showed little or no emotional impairment as indicated by the significantly reduced time spent in the closed arm when compared with the PSD group (p < 0.05). the values are expressed as mean ± SEM. P < 0.05 is considered to be statistically significant; * indicate significant level of difference when compared with control; + indicate significant level of difference in comparison with PSD group.
with increased serum MDA level observed in PSD which elicits lipid peroxidation and eventually elevated anxiety levels as seen in Figure 2. Whilst high anxiety levels have been established to significantly increase oxidative stress [41], it has also been shown that oxidative stress could trigger anxiety-related behavior [42]. However, D-Ribose-L-cysteine supplementation in this study revealed decreased serum lipid peroxidation evidenced by reduced MDA levels. This could be associated with its ability to prevent anxiogenic-like effects of sleep deprivation and its role as a bioactive source of antioxidants, therefore, reducing oxidative stress elicited by PSD.

In addition, reductions in glutathione level, and catalase and superoxide dismutase activities are commonly used as markers of oxidative stress [43–45]. Sleep deprivation has been established to induce reduced antioxidant levels in different brain regions [16,46] and this conforms to our findings which revealed reduced serum glutathione level and catalase and SOD activities following paradoxical sleep deprivation which may be due to increased free radical production triggered by PSD. However, DRLC treatment in this study was associated with an increased antioxidant level characterized by elevated GSH levels as well as increased catalase and SOD activities. It is suggested that
DRLC supplementation augments the synthesis of glutathione [10] which in turn scavenges the free radicals produced thereby reducing oxidative stress. Hence oral supplementation with DRLC increased glutathione level by making available the raw nutritional materials such as cysteine which can be used in glutathione synthesis. Furthermore, positive correlation in glutathione and other antioxidant markers assayed for in this study were observed in animals pre- and post-treated with DRLC. Superoxide dismutase (SOD) and catalase (CAT) are two very important enzymes capable of protecting the brain from oxidative damage by ROS [47]. This study has shown that DRLC supplementation can enhance the activities of SOD and CAT which may subsequently lead to a reduction in oxidized lipid content buildup, thus providing a defense against cell damage caused by ROS.

Figure 4. Representative photomicrographs showing the general morphology of the hippocampus of the Wistar rats across the various groups stained with H & E. (Scale bars: 50 µm). the Dentate Gyrus (DG) composed of granule cells, Cornu amanos (CA1-3) containing pyramidal cells, are well demonstrated across the experimental groups.

Figure 5. Representative photomicrographs showing the granule and pyramidal cells (neurons) of the hippocampus of the wistar rats across the experimental groups stained with H & E stain (scale bars: 25 µm).
The morphological demonstration of the hippocampus of PSD animals in this study showed pathological changes characterized by necrotic and chromatolytic neurons (Figure 7–9). Consistent with findings from this study, decreased nissl staining intensity is often seen in pathological conditions \[48\] (fig 10). Uncontrolled generation of ROS is a known cause of necrotic neuronal death which is the hallmark of neurodegenerative diseases. Rats treated with DRLC presented with normal hippocampal morphology. The findings of this study confirm the neuroprotective and ameliorative potentials of DRLC against PSD-induced neuronal damage.

**Conclusion**

Though numerous studies emphasize the possible chronic impact of long-term sleep deprivation or chronic sleep restriction on the occurrence of neurodegenerative diseases such as Alzheimer’s disease and dementia \[49–51\]; however, our present study suggests that D-Ribose-L-Cysteine (DRLC) supplementation possibly attenuates and ameliorates behavioral deficits and neuronal damage of the hippocampus caused by paradoxical sleep deprivation via its anxiolytic and antioxidative properties.

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