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Manganese Exposure Induces Mild Behavioral and Histological Deficits in Wistar Rats: Ameliorative Effects of D-ribose-L-cysteine

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ABSTRACT

Heavy metal exposure is a global health problem and their presence in our environment has been greatly increased by industrial activities over the past century. Manganese is an essential trace metal in the body. However, exposure to elevated levels is neurotoxic, leading to manganism**,** a Parkinsonian-like syndrome. In the present study, we investigated the effects of D-ribose-L-cysteine on behavioral and histological changes in key brain regions (including, the hippocampus, cortex, striatum, and cerebellum) in rats following manganese treatment. Thirty-two adult rats were administered either saline (control), manganese (25 mg/kg intraperitoneally for 15 days at 48 hour intervals in 8 doses), D-ribose-L-cysteine (200 mg/kg, orally for 2 weeks), and manganese (Mn) and D-ribose-L-cysteine co-administration. At the end of the administration, behavioral and histological studies were performed. The Y-maze, elevated plus maze (EPM), and open field test (OFT) were used for neurobehavioral evaluation. Thereafter, brains were excised and processed for histological assessment via routine hematoxylin and eosin staining. Results revealed only mild behavioural change following manganese exposure documented by alterations in grooming frequency as seen in the Mn-only group. Nevertheless, the behavioural change noted here was attenuated by D-ribose-L-cysteine treatment evident in the manganese and D-ribose-L-cysteine co-administration group. Histological evaluation showed cytoarchitectural alterations following manganese exposure presented as pyknosis, vacuolations, and loss of cerebellar Purkinje cells. D-ribose-L-cysteine administration mitigated the observed manganese-induced distortions of neuronal architecture. These findings indicate that D-ribose-L-cysteine could improve anxiety-related behavioural deficits and maintain neuronal architecture thereby preventing potential physiological impairments in manganese-induced neurotoxicity.

Keywords: manganese, D-ribose-L-cysteine, neurotoxicity, behavior, histology

INTRODUCTION

Manganese (Mn) ranks as the 12th most prevalent element and the 5th most abundant metal on Earth. Human exposure to manganese is widespread due to its presence in the air, soil, and waterways, primarily stemming from natural erosion and industrial activities [1]. Nevertheless, the primary pathway humans are exposed to manganese is dietary intake. Elevated levels of this metal are commonly present in whole grains, vegetables, rice, and nuts. Additionally, manganese can be found in seafood, chocolate, tea, green leafy vegetables, spices, soybeans, and certain fruits such as pineapple and acai [2]. The variety of food sources containing manganese enables humans to effortlessly acquire sufficient levels of the metal, with recommended daily intake levels set at 2.3 mg/day for men and 1.8 mg/day for adult women [3]. Manganese plays crucial roles in various biological processes and is particularly significant in the regulation of metabolism [4]. It holds crucial importance in numerous widespread enzymatic reactions, contributing to the synthesis of amino acids (AA), lipids, proteins, and carbohydrates. Additionally, it plays a vital nutritional role in processes such as bone development, metabolism of fats and carbohydrates, regulation of blood sugar, absorption of calcium, urea metabolism, and the facilitation of autophagy [5]. Within the antioxidant defense system, manganese acts as a cofactor for superoxide dismutase (SOD) [4].

Environmental and occupational exposure to manganese is a significant concern due to its occurrence in various sectors, including battery manufacturing, mining, welding, and the application of fungicides containing the metal, such as maneb and mancozeb [6]. Exposure to manganese also happens through its inclusion as an additive in gasoline, known as methylcyclopentadienyl manganese tricarbonyl (MMT), as well as in fertilizers [7]. Additionally, manganese is present in paint and cosmetics, often in the form of Mn violet [8]. Environmental exposure can happen by either inhaling manganese particles or consumption from water sources with a high content of Mn. Although manganese is physiologically required, excessive exposure to the metal can have neurotoxic effects, causing it to accumulate in the brain. This buildup mostly occurs in the basal ganglia tissues, particularly in the striatum, globus pallidus, and substantia nigra [9]. Symptoms resulting from the buildup of Mn include dystonia, bradykinesia, and rigidity, attributed to the impairment of dopaminergic neurons and the occurrence of gliosis nigra [9,10]. Manganese exposure is associated with several adverse neurological effects [11]. One such neurologic disorder induced by the accumulation of Mn is called manganism. Manganism is a condition that has similarities with Parkinson's disease (PD). It is characterized by behavioural and cognitive abnormalities, as well as motor dysfunction [5]. The duration of occupational exposure that might result in manganism ranges from 6 months to 2 years. Motor and neuropsychiatric effects can persist for up to 14 years after the cessation of exposure to Mn [12].

D-ribose-L-cysteine (RibCys) is a derivative of cysteine that has been demonstrated to increase the antioxidant capacity of cells by promoting the production of glutathione (GSH) within cells [13]. D-ribose-L-cysteine has also been reported to be responsible for the detoxification of cellular oxidative stress [14]. Deficit in GSH results has been associated with oxidative damage, cancer, cystic fibrosis, cardiovascular diseases and neurological disorders [15]. Insufficient levels of GSH have also been linked to the development of Parkinson's disease (PD), a neurodegenerative condition characterized by movement abnormalities. Thus, the exploration of antioxidants to prevent or slow down the advancement of movement abnormalities in individuals with Parkinsonian syndrome has garnered significant attention [16].

Our study postulates that D-ribose-L-cysteine administration modulates motor, cognitive, and anxiety-related behaviors while also ameliorating histological damage caused by Mn-induced neurotoxicity. Hence this study was aimed at investigating the effects of D-ribose-L-cysteine on cognitive and motor behaviors, anxiety, and histological changes in manganese-induced neurotoxicity.

MATERIALS AND METHODS

Animal procurement, management and treatment

Thirty-two (32) adult albino strain male Wistar rats (*Rattus norvergicus*) weighing between 150 and 200g were used for the experiment. Animals were obtained from the Animal House, Alex Ekwueme Federal University, Ndufu-Alike Ikwo, Nigeria and were randomly assigned into four (4) groups with eight (8) rats in each group. The rats were housed at room temperature in clean plastic cages in a clean environment with a 12-hour day/light cycle. Thereafter, the rats were allowed to acclimatize to the animal house condition for two weeks with free access to feed and water and the grouping was maintained throughout the experiment. All experimental protocols were in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and approved by the local institutional animal care and use committee. The animals were randomly divided into 4 groups and were administered Manganese (Mn) and/or D-ribose-L-Cysteine (RibCys) as shown: Group I (Control): received normal saline intraperitoneally (i.p) for 15 days; Group II (Mn only): received 25mg/kg Mn i.p for 15 days; Group III (RibCys only): received oral 200 mg/kg RibCys for 15 days; Group V (Mn+RibCys): received both oral 200 mg/kg daily and 25 mg/kg Mn i.p for 15 days.

The selected dose of manganese is determined by a review of existing research that has demonstrated a significant increase in Mn accumulation within regions of the brain and alterations in biochemical characteristics in rats [17,18]. The dosage of D-Ribose-L-Cysteine is determined using data derived from prior study [19].

Ethical approval

Ethical approval was sought and obtained from the research and ethics committee of the Faculty of Basic Medical Sciences, University of Cross River State (UNICROSS), with number (FBMS/UNICROSS/23/17). All animals were treated according to the Guidelines prepared by the National Research Council for the Care and Use of Laboratory Animals.

Neurobehavioral assessment

Behavioral tests were conducted at the end of the administration in a quiet room. Before evaluating a fresh animal, the apparatus was cleaned with 5%

ethanol to eliminate any potential bias caused by the previous animal's odours. The tests were videotaped and later graded by trained observers unaware of the experimental design. The following tests were carried out;

Assessment of motor activity

Motor activity was assessed using the open field test (OFT). The test was performed as previously described by Ijomone *et al.* [20] using protocols adapted from Brown *et al.* [21]. The open field test, developed by Hall and Ballachey [22] is a widely employed method for evaluating general locomotor activity, anxiety, and exploratory behaviours in experimental rats and mice. The apparatus comprises a box measuring 72×72×36 cm, with the floor divided into 18×18 cm square units. The interior of the apparatus is painted white, and the floor is covered with Plexiglas. Animals are placed in the centre of the box and allowed to move freely for 5 minutes. Various parameters are recorded during the test, including locomotion frequency (number of crossings between squares), rearing frequency (number of times animals stand on their hind paws), rearing against the wall (the number of times animals stand on their hind paws against the wall), hinding (calculated by adding the rearing frequency and rearing against the wall), and groomimg frequency (number of times the animal scratches the sides of its head with its forepaws).

Assessment of anxiety-related behavior

Anxiety-like behavior in laboratory animals is frequently evaluated with the help of the EPM [23,24]. It may be utilised to acquire an understanding of disorders such as post-traumatic stress disorder (PTSD), Parkinsonism, and other conditions characterised by anxious behaviour. This model hinges on the aversion to open spaces, as evident in the animal's inclination to spend more time in the enclosed arms of the maze [25]. The elevated plus maze is designed with four raised arms extending from a central platform, creating a plus-shaped configuration. Two opposing arms are enclosed, except for the ceiling, entrance, and exit points, while the other two opposing arms are open, except for the central platform [26]. During the test, a mouse or rat is placed in the central area and allowed to explore the maze for a predetermined short duration. The comparison of the time spent in the enclosed arms to the time spent in the open arms serves as an indicator of anxiety or fear. This test capitalizes on rodents' natural inclination to avoid open or elevated spaces, balanced by their inherent curiosity to explore novel areas. In essence, a less anxious mouse is expected to frequent the open, more exposed arms, while a mouse exhibiting heightened anxiety tends to spend more time in the enclosed arms [27].

Assessment of cognitive performance

The Y-maze task assesses short-term spatial memory as a measure of cognitive functions. Behavioural analysis on the Y-maze was performed as previously described by Ijomone and Nwoha [28] using protocols adapted from [29,30]. The apparatus is made of wood, having three arms in the shape of a Y. Each arm is 40 cm long, 30 cm high and 10 cm wide. The rats were placed in the Y-maze, at the end of a pre-determined start arm and allowed to move freely for 8 min. Arm entry is defined as when the hind paws of the rats are completely within the arm. Spontaneous alternation is defined as rats entering all three arms in the overlapping triplet sets. The percentage of spontaneous alternation is calculated as (spontaneous alternation/ (total number of arm entries – 2) ×100.

Sample collection

At the termination of the experiment, rats were euthanized via diethyl-ether inhalation, followed by rapid cervical dislocation. Each experimental group's hippocampus, cerebellum, striatum, and frontal cortex were rapidly removed after the brain was excised and fixed in a 10% neutral buffered formalin solution.

Histological studies

After behavioral studies, rats were euthanized and brain rapidly excised. Brain tissues were fixed in a 10% neutral buffered formalin solution. Following fixation, the tissues were processed using standard paraffin embedding methods, and 5 µm thick serial sections were obtained with a rotary microtome. The sections were stained with routine Hematoxylin and Eosin (H&E) techniques to assess the overall histological structure as previously described by (Bancroft and Gamble, 2008; Ijomone *et al.*, 2018) [31,32]. Sections were observed under a digital brightfield microscope, and photomicrographs of the hippocampus, striatum, cerebellum and cortex were obtained at x400 magnification.

Statistical analysis

Quantitative data was expressed as mean ±SEM. The significant difference between means for different groups was determined using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc tests with GraphPad Prism Version 8 (GraphPad Inc, San Diego, USA) statistical software. A P value <0.05 was considered statistically significant.

RESULTS

Effects of co-exposure with RibCys and Mn on cognitive, motor and anxiety-related behaviors in Mn-treated rats

Locomotor and exploratory activities were assessed and no statistically significant difference between groups was observed in locomotion frequency (line crossing) and hinding. Results using one-way ANOVA test showed a significant difference in grooming frequency in the experimental groups (p = 0.002; $F_{(3,25)}$ = 10.07). Further multiple comparisons with Turkey's test showed Mn treatment significantly (p < 0.01) increased grooming activities compared to control and RibCys only treatment. However, RibCys co-treatment with Mn significantly (p < 0.001) attenuated Mn-induced heightened grooming activity (Fig. 1).

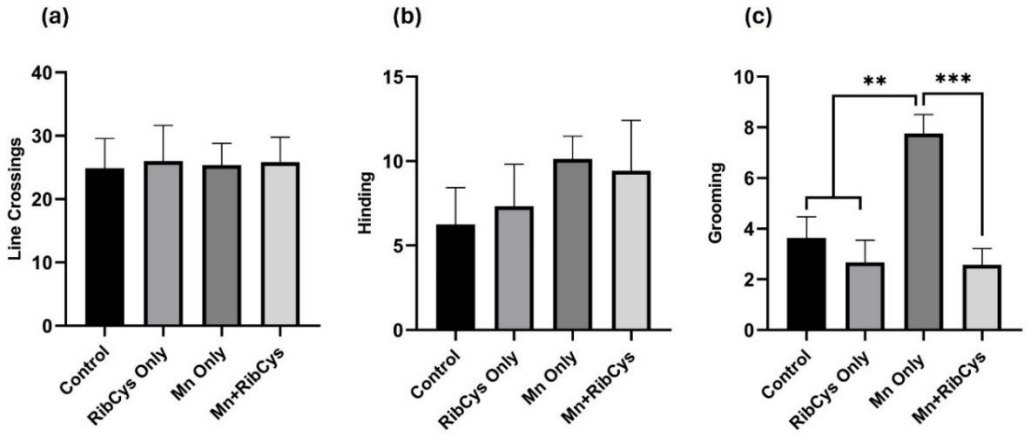

Figure 1: Effects of co-exposure with RibCys and Mn on locomotion and exploration on the OFT **(a)** line crossings, **(b)** hinding. **(c)** grooming. Data were analyzed using one way- ANOVA followed by Turkey's post hoc test for multiple comparisons. **p<0.01, ***p<0.001.

Assessment of cognition using the one-way ANOVA test showed no significant difference in percentage of spontaneous alternation in the experimental groups on the Y-maze (p = 0.9907; $F_{(3,25)}$ = 0.0359). Turkey's test for multiple comparisons showed no significant difference between groups (Fig. 2).

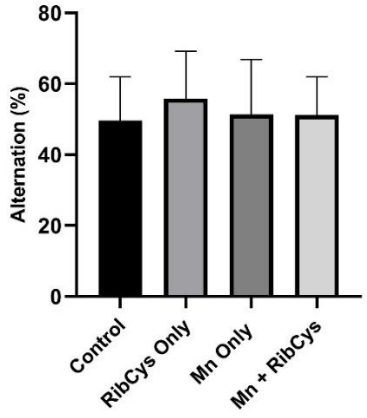

Figure 2: Effects of co-exposure with RibCys and Mn on percentage of spontaneous alternation on the Y-maze. Data were analyzed using one way- ANOVA followed by Turkey's post hoc test for multiple comparisons.

Anxiety-like behavior in rats was assessed and result revealed no statistically significant difference in the frequency of open-arm entries (p = 0.2989; $F_{(3,15)}$ = 1.340) and frequency of closed-arm entries (p = 0.5227; $F_{(3,15)}$ = 0.7716) in the experimental groups. Similarly, Turkey's test for multiple comparison showed no significant difference between control and treatment groups (Fig. 3).

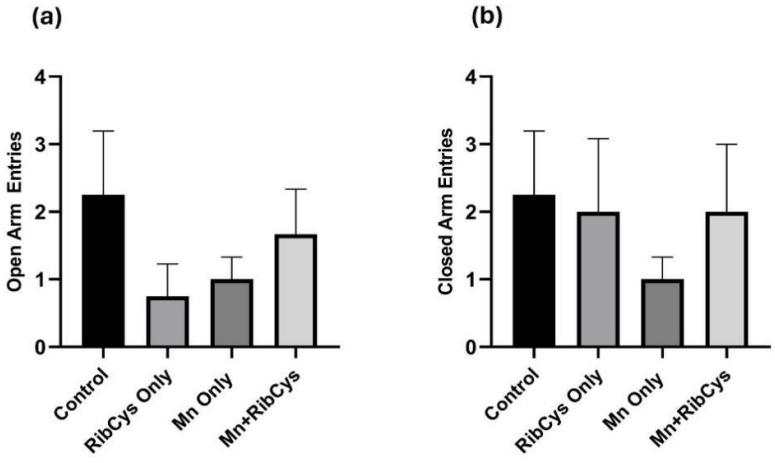

Figure 3: Effects of co-exposure with RibCys and Mn on anxiety-like behavior on the EPM **(a)** open-arm entries, **(b)** closed-arm entries. Data were analyzed using one way- ANOVA followed by Turkey's post hoc test for multiple comparisons.

RibCys mitigated Mn-induced cytoarchitectural distortions in brain regions

In the hippocampus, the control group presents the normal histology of the hippocampal CA1, which is made up of three distinctive layers; stratum oriens, pyramidalis and radiatum. The pyramidalis layer contains homogeneously distributed large pyramidal neurons with acidophilic nuclei and prominent nucleoli. In addition, there are numerous glial cells dispersed through the hippocampal layers. Mn exposure in the CA1 elicited pyknosis, characterized by deeply stained eosinophilic cells, in the pyramidalis layer of the CA1. In addition, there are presence of more than a few vacuoles which indicates the disintegration of CA1 parenchyma. All other treated groups show intact histological appearance with no obvious alteration of tissue morphology (Fig. 4).

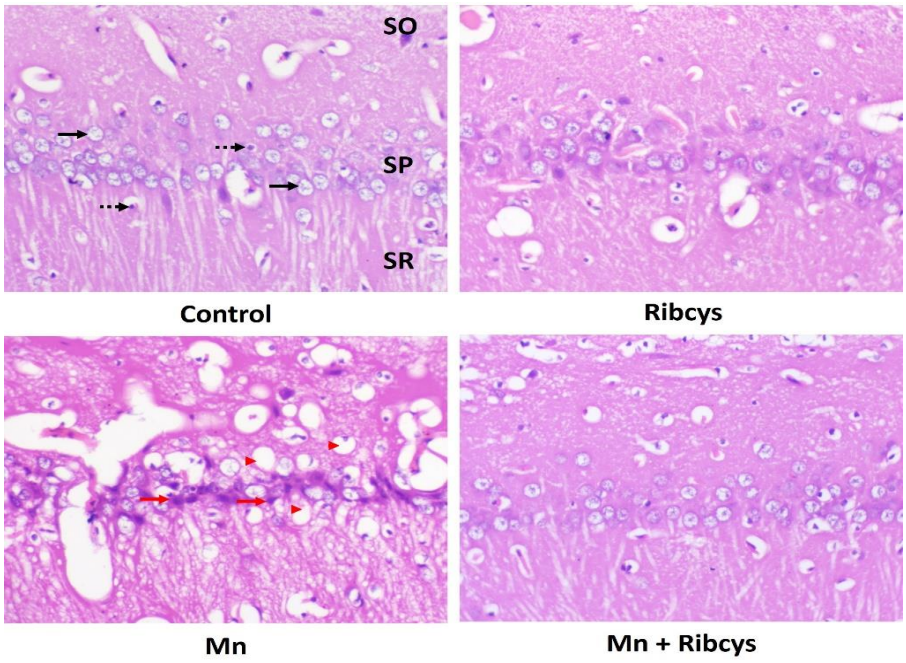

Figure 4: Histology of hippocampal CA1 in control and experimental groups. H&E x400 magnification. SO – Stratum oriens; SP – Stratum pyramidalis; SR – Stratum radiatum; Black arrows – intact pyramidal neurons; Dashed arrows – glial cells; Red arrows – Pyknotic cells; Arrowhead – Vacuoles.

The cortical histology depicted in the control group shows distinct pyramidal neurons characterized by large round nuclei and prominent nucleoli. Interspersed within the cortical neurons are the glial cells. Mn-treated cortex shows several pyknotic cells characterized by deeply stained eosinophilic cells. In addition, there was also the presence of several vacuoles indicative of disintegrating cortical parenchyma. All other groups show intact cortex histology with no obvious tissue damage (Fig. 5).

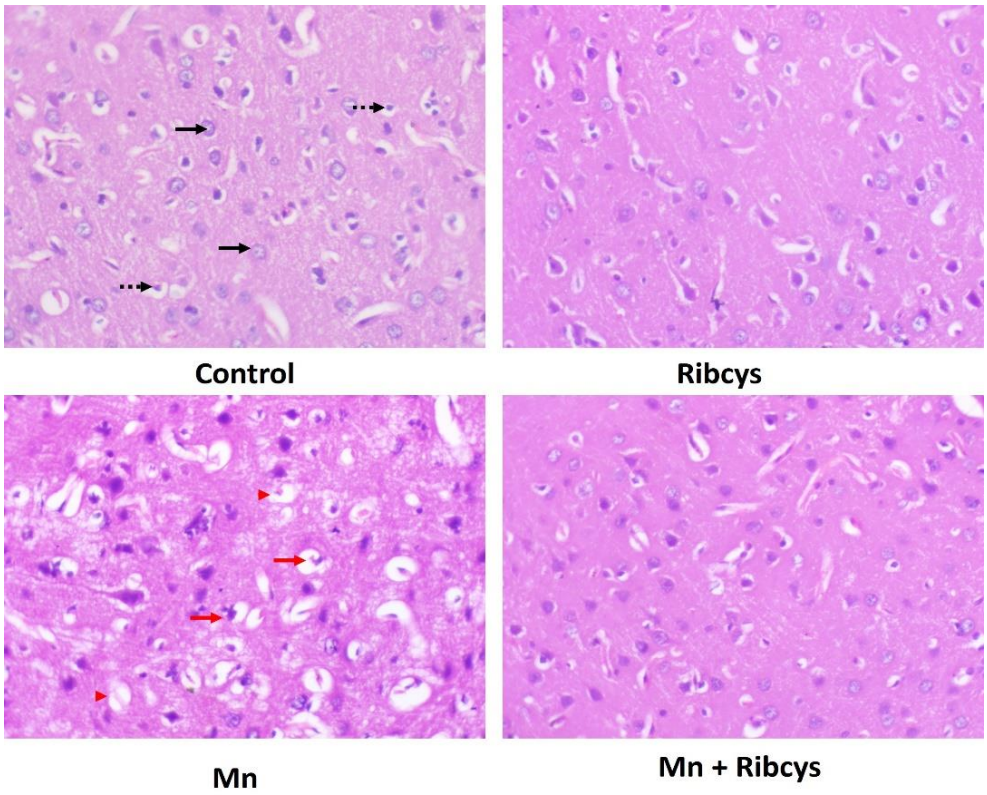

Figure 5: Histological changes in the cortex of experimental groups. H&E x400 magnification. black arrows – intact pyramidal neurons; dashed arrows – glial cells. red arrows – pyknotic cells; arrowheads – vacuoles.

The typical striatal histology is depicted in the control group. There are visible striations composed of white and grey matter. Interspersed within the striatal neurons are the glial cells. The striatum of Mn-exposed rats displays several pyknotic cells characterized by deeply stained eosinophilic cells. In addition, there is the presence of more than a few vacuoles which is a tell-tale sign of disintegrating striatal parenchyma. All other groups show intact cortex histology with no obvious tissue damage (Fig. 6).

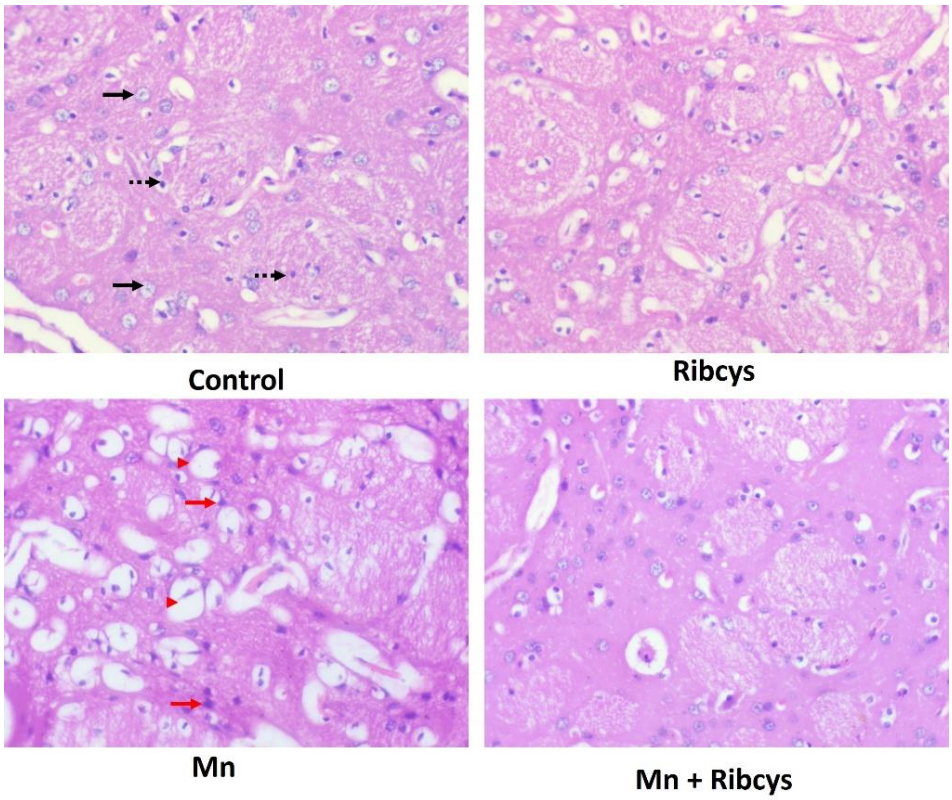

Figure 6: Histological changes in the striatum of experimental groups. H&E x400 magnification. Black arrows – Striatal neurons; Dashed arrows – glial cells; Red arrows – pyknotic cells; Arrowheads – Vacuoles.

The histology of the cerebellum, depicted in the control group, shows three distinct layers; granular, Purkinje, and molecular. The granular layer is composed of numerous granular neurons. Conversely, the molecular layer has very few cells. Lying in between the granular and molecular layer is the Purkinje layer, which is composed of large Purkinje neurons characterized by large round nuclei and prominent nucleoli. The cerebellum of the Mn-treated groups shows a loss of Purkinje cells and a consequent formation of vacuoles. In addition to the lost neurons, there are visible disintegrating neurons surrounded by perinuclear spaces. All other groups show intact cerebellar histology with no obvious tissue damage when compared to the control (Fig. 7).

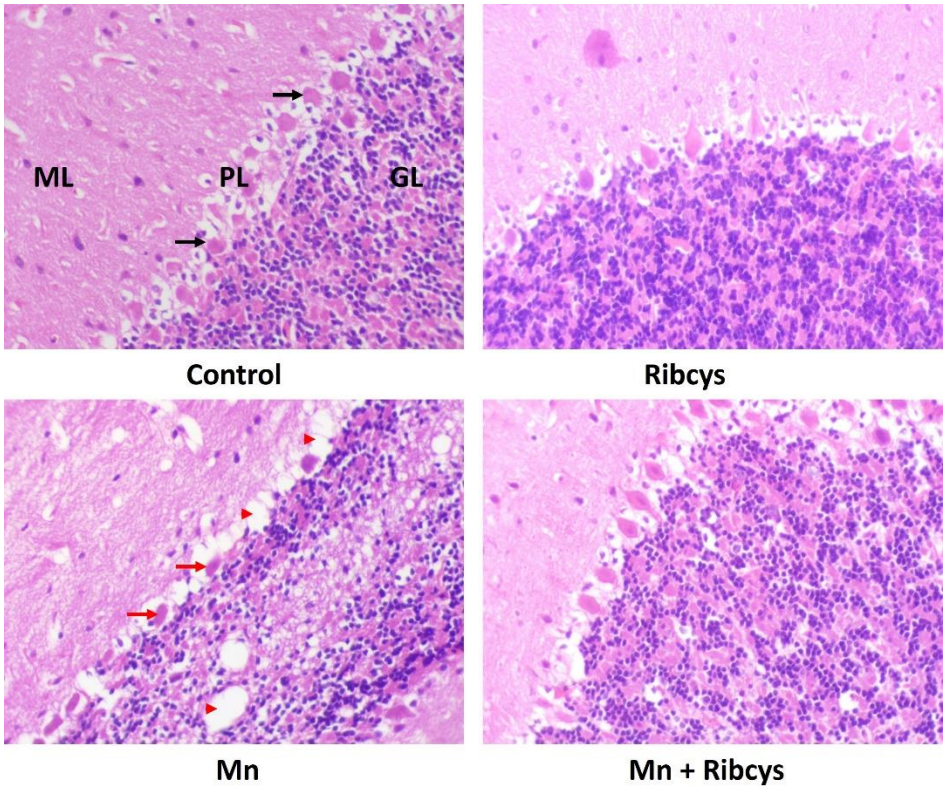

Figure 7: Histology of the cerebellum in control and experimental groups. H&E x400 magnification. ML – Molecular layer; PL – Purkinje layer; GL – Granular layer; Black arrows – intact Purkinje neurons; Red arrows – Degenerating Purkinje neurons; Arrow heads – Vacuoles.

DISCUSSION

The role of D-Ribose-L-Cysteine (RibCys) in mitigating neurotoxicity and promoting neuroprotection has been of interest to researchers over the years. This study investigated the effects of RibCys on Mn-induced changes in behavior and brain histomorphology. The study assessed the impact of RibCys treatment on rat's, motor, cognitive and anxiety-related behavior following Mn exposure. Motor skill and anxiety-related behavior were assessed using the open field test (OFT) and elevated plus maze test (EPM), and cognitive behavior was assessed using the Y-maze test for spontaneous alternation. The open field test is commonly used to access locomotor and exploratory activities in experimental rats and mice and is also a common tool to assess anxiety behaviors in rodents [33]. Several studies have investigated the impact of Mn exposure on cognitive abilities. A study by Zhang *et al.*, [34] investigated the effect of Mn exposure on cognitive function with a focus on the elderly population. Their result revealed that exposure to Mn in elderly individuals correlated with a notable decline in cognitive function, thus, providing additional evidence that Mn exposure can lead to cognitive impairment. Similarly, Ruiz-Azcona *et al.* [35] investigated whether environmental Mn exposure in adults is associated with poorer results in motor and cognitive function. They observed a significant negative correlation (the higher the Mn levels, the poorer the scores) between Mn exposure and cognitive and motor functions. Also, Akingbade *et al.* [36] reported an impairment in motor and cognitive behavior following Mn exposure, an effect that was attenuated by RibCys administration. Studies by Kalueff *et al.*, [37] and Batschauer *et al.*, [38] revealed that an increase in grooming time reflects anxiety in Mn-exposed animals and may be related to cognitive and behavioral problems. In our study, motor and cognitive neurobehavioral assessments showed no significant impact of Mn and RibCys co-exposure on rat brains. However, increased grooming behavior was observed only on the OFT and not the EPM suggesting only mild anxiety-related behavioral change following Mn treatment, and this was documented by alterations in grooming frequency. Despite these tests revealing only mild behavioral changes following Mn exposure, the histopathological analysis demonstrated significant neurotoxic effects.

The structural integrity of neurons is known to be vital for transmitting and processing information, and damage to neuron structure in various brain regions can lead to impaired behavior [32,39]. Hence, this study investigated the effects of Mn and RibCys exposure on the histomorphology of the hippocampus, cortex, striatum, and cerebellum. The cytoarchitectural

alterations seen following Mn exposure presented as nuclear shrinkage (pyknosis), disintegration (vacuolations), and loss of cerebellar Purkinje cells. These structural alterations are indicative of neuronal damage and degeneration [40,41]. Furthermore, the lack of noticeable behavioral deficits, despite evident histopathological damage, suggests that the behavioral tests might not fully capture the extent of neurotoxic effects or the subtle changes caused by Mn exposure. This difference in results could be due to the sensitivity and specificity of the tests used. Additionally, the observed histopathological changes, such as the loss of Purkinje cells, could contribute to the mild behavioral alterations documented, suggesting a more nuanced relationship between neurotoxicity and behavioral outcomes. Purkinje cells play a critical role in motor control and coordination, and their loss might impact motor function and anxiety levels subtly, potentially explaining the mild changes seen in grooming frequency.

This study further revealed that RibCys mitigated the observed Mn-induced changes in grooming behavior, as well as distortions in neuronal architecture, effectively reversing the potential physiological impairment caused by Mn exposure. This corroborates previous work that RibCys could mitigate behavioral deficits in Mn-induced neurotoxicities, and attenuate histomorphological distortions in the hippocampus and striatum caused by Mn exposure [36]. A recent study by Adekomi *et al*., [42] investigated the neuroprotective effects of RibCys on alcohol-induced cerebellar dysfunctions in juvenile BALB/c mice. Their results showed that RibCys exposure mitigated the detrimental impact of ethanol in the cerebellum of young BALB/c mice through the modulation of monoamine neurotransmitters, lipid peroxidation, total antioxidant status, as well as the actions of superoxide dismutase and glutathione peroxidase pathways. Another study by Emokpae *et al*., [16] evaluated the effects of RibCys on memory deficits induced by Lipopolysaccharide (LPS) in mice. They found that RibCys alleviated the memory impairment caused by LPS in mice by means associated with the suppression of oxidative stress, the reduction of proinflammatory cytokine release, and the modulation of NF-κB expression in mice. This study acknowledges certain limitations that may affect result interpretation, including the potential insensitivity of the neurobehavioral tests to subtle changes from Mn exposure, which may not capture all neurotoxic effects or the full efficacy of RibCys. Therefore, while RibCys shows promise in mitigating Mn-induced neurotoxicity, further research is needed to understand its impact on behavior and optimize treatment. Future studies should explore different dosages, and additional tests, and involve various animal models or humans to better assess the effectiveness of RibCys and its clinical relevance. Understanding the link between behavior and neuronal damage will be key to developing effective interventions.

Conclusion

RibCys is effective in mitigating Mn-induced behavioral change and in maintaining neuronal architecture by preventing Mn-induced distortions in neuronal structures thereby preventing potential physiological impairments.

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Author's contribution

HOI and OMI conceptualized and designed the experiment. HOI and SOO performed the experiment and collected data. HOI, UKE, and SOO performed data analysis and interpretation. UKE and OMI supervised the study. HOI drafted the manuscript. OMI revised the manuscript. All authors read and approved the final version of the manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

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