Research Paper: The Protective Effect of Kolaviron on Molecular, Cellular, and Behavioral Characterization of Cerebellum in the Rat Model of Demyelinating Diseases

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ABSTRACT

Introduction: This study aimed at assessing the protective mechanisms of Kolaviron (KV) on the cerebellum in a rat model of demyelination.

Methods: Twenty-eight male Wistar rats were used in the present study. They were randomly divided into 4 groups of 7 rats. Group A (control) received corn oil (0.5 mL/kg/d); group B received 0.2% Cuprizone (CPZ); group C was treated with 200 mg/kg/d of KV, and group D received 0.2% CPZ and 200 mg/kg/d KV for 6 weeks. CPZ powder was mixed with the regular diet while KV was dissolved in corn oil and administered orally. A behavioral test was conducted at the termination of the experiment. Thereafter, the animals were sacrificed and their brains were removed with the excision of the cerebellum. A part of the cerebelli underwent tissue processing with a series of 5 µm thick sections cut from paraffin blocks for histological and immunohistochemical assessment. Besides, the remaining cerebellar tissues were homogenized for the spectrophotometric assays of Oxidative Stress (OS) parameters.

Results: The current research findings revealed minimal weight gain following CPZ treatment, but significant weight increase in KV-treated rats. CPZ treatment was associated with a reduction in the number of the line crossed, rearing frequency, rearing duration, center square entry, and center square duration; however, it increased the freezing time, i.e. significantly reversed in the KV-treated animals. Oxidative markers, such as Superoxide Dismutase (SOD) and GPx were reduced in CPZ-treated rats with elevated MDA levels. However, these data were significantly reversed by the co-administration of CPZ and KV. At the tissue level, the cerebellar cortex was characterized by poorly defined layers, cryptic granules, as well as chromatolysis and pyknotic Purkinje cells with the evidence of hypertrophic astrogliosis.

Conclusion: CPZ treatment significantly depressed locomotor and exploratory activities. Furthermore, it increased OS and cerebellar toxicity. However, KV intervention significantly enhanced behavioral functions and ameliorated CPZ-induced cerebellar degeneration. Moreover, it considerably regulated OS markers in the cerebellum of the rat model of demyelinating diseases.

Keywords: Demyelinating disease, Cerebellum, Cuprizone, Kolaviron, Oxidative stress

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1. Introduction

Demyelination is a condition that causes the loss of myelin with the relative preservation of axons. It results from diseases that damage myelin sheaths or the myelinating cells of the Central Nervous System (CNS), oligodendrocytes. The loss of myelin is considered as the cause of numerous CNS conditions (Love, 2006). Demyelination of focal white matter injury can occur in any location of the CNS (Ahmad, Satriotomo, Fazal, Nadeau & Doré, 2015). The most frequent form of demyelination is observed in Multiple Sclerosis (MS). The volume of white matter lesions in the cerebellum is small, compared to other parts of the CNS; however, the lesions are detrimental to health (Ahmad et al., 2015). Recent insight into the function of the cerebellum indicates that the cerebellum not only controls movement but also impacts motor learning, cognitive behavior, as well as injury-induced sensation and adaptation due to changes in the strength of the connections among its neurons (Houk & Miller, 2001; Diedrichsen & Bastian, 2014). The defining clinical manifestation of MS is reported to be associated with cerebellar demyelination (Weinschenker, Issa & Baskerville, 1996; Rot, Ledinek & Jazbec, 2008). Despite these interesting findings, there is a paucity of information regarding the molecular, cellular, and behavioral characterization of the cerebellum in demyelinating diseases. Understanding such characterization could help identify the underlying mechanism involved in demyelination and potential therapeutic targets.

There is a rise in the prevalence of some demyelinating diseases. Accordingly, various animal models have been used to study and find preventive measures or potential cure for these conditions. The cuprizone model, toxin-induced demyelination, has been frequently implemented; it represents a reversible demyelination and remyelination system (Kipp, Clarner, Dang, Copray & Beyer, 2009; Faizi et al., 2016).

Cuprizone [oxalic acid bis(cyclohexylidenehydrazide)] (CPZ), is a copper chelator that induces demyelination in regions where white matter are located in the rodents’ CNS (Sachs et al., 2014). Its underlying mechanism remains controversial; however, studies have reported that rodents fed with CPZ diet presented megamitochondria, elevated free radicals level due to Oxidative Stress (OS), uncoupling of the oxidative phosphorylation process, oligodendrocyte apoptosis, the disturbance of neurotransmitter homeostasis, synaptic dysfunction, and axonal degeneration (Kipp et al., 2009; Norkute et al., 2009; Hesse et al., 2010; Skripuletz, Gudi, Hackstette & Stangel., 2011; Tandler & Hoppel, 1973; Wakabayashi, Asano, & Kurono, 1975). Although CPZ toxicity targets oligodendrocytes, other macroglia, especially astrocytes could be affected in this respect.
Astrocytes are specialized glial cells that provide structural and functional support for neurons (Şovrea & Boşca, 2013). They are involved in synaptogenesis, as well as regulating the communication between already formed synaptic connections (Ota, Zanetti, & Hallock, 2013). Astrocytes participate in controlling brain homeostasis, and the intrinsic brain defense system (Kettenmann and Verkman, 1994); subsequently, they make them respond in case of an insult to the brain.

Contrarily, the treatment and management of complex demyelinating diseases could be achieved through applying the phytochemical constituents of certain plants with medicinal values (Omotoso, Gbadamosi, Afolabi, Abdulwahab & Akinlolu, 2018). Kolaviron (KV) is among such phytochemicals due to the properties attributed to it (Olal-eye and Farombi, 2006; Farombi, Abarikwu, Adedara & Oyeyemi, 2007). KV is a bioflavonoid complex isolated from the seed of Garcinia kola (bitter kola). The plant is used as an herbal remedy for treating several conditions. It provides antiviral, antibacterial, antifungal, and analgesic uses and has many other activities (Olajide et al., 2016). The present study aimed to assess the cellular, molecular, and behavioral changes associated with cuprizone toxicity in the cerebellum of Wistar rats, following KV intervention.

2. Materials and Methods

Twenty-eight male 9-week-old Wistar rats were obtained from a private animal holding (Tanke, Ilorin). The rats were housed in the Animal House of the Faculty of Basic Medical Sciences, University of Ilorin. They were allowed to acclimatize for 7 days before the onset of the experiment. They were fed on rat chow and water ad libitum. The relevant Ethical approval was obtained from the Ethics Committee of the College of Health Sciences, University of Ilorin, Nigeria. Animal handling and protocols were conducted according to the guidelines of the Ethics Committee. Garcia kola seeds were procured from a market in Ilorin, Nigeria. Subsequently, they were verified at the herbarium of Botany Department, Faculty of Life Sciences, University of Ilorin (verification code: UILH/001/1217). CPZ was procured from Sigmar-Aldrich (Germany), while Phosphate-Buffered Solution (PBS; pH 7.0) was freshly prepared. Superoxide Dismutase (SOD), Glutathione Peroxidase (GPx), and Malondialdehyde (MDA) assay kits were also obtained (Abcam®, USA). Rats anti-Glial Fibrillary Acidic Protein (GFAP) were acquired from Cell Signaling Technologies, Massachusetts, USA.

The rats used for enzyme studies were sacrificed by cervical dislocation. Their brains were immediately removed and post-fixed in 4% PFA for 24 hours. The cerebelli were routinely excised and processed to obtain paraffin wax-embedded blocks. The tissues were stained using Haematoxylin and Eosin and Cresyl fast violet, as described by Fischer, Jacobson, Rose & Zeller (2008) and Bancroft and Stevens (1982), respectively. The rats used for enzyme studies were sacrificed by cervical dislocation. Their brains were immediately removed and rinsed in 0.25 M sucrose solution thrice for 5 min each; accordingly, they were placed in 30% sucrose at 4°C.

Sequel to last administration and behavioral study, the rats classified for histological and immunohistochemical evaluation were anesthetized using 20 mg/kg bw ketamine intraperitoneally; subsequently, they received perfused transcardially with 0.4 M Phosphate-Buffered Saline (PBS), followed by 4% Paraformaldehyde (PFA). The brain tissues were thereafter excised, each rinsed in 0.25 M sucrose solution thrice for 5 min; then, post-fixed in 4% PFA for 24 hours. The cerebella were routinely excised and processed to obtain paraffin wax-embedded blocks. The methods employed in the isolation and identification of KV were as earlier described (Farombi, Shrotiya & Surh, 2009; Olajide et al., 2017). These involved drying Garcia kola seeds at room temperature, pulverization, extraction, as well as the assessment and confirmation of the purity and identity of the extract (KV) obtained from these processes.

The rats were randomly grouped into 4 classes, labeled as A-D (n=7/group). Group A received 0.5 mL of Corn Oil (CO) and served as the Control; group B received 0.2% CPZ diet (Praet et al., 2015); group C received 200 mg/kg bw of KV (Farombi et al., 2009; Omotoso, Olajide, Gbadamosi, Rasheed & Izuogu, 2018), and, group D received KV (200 mg/kg bw) and CPZ (0.2%) diet. CO served as the vehicle in which KV was dissolved for the ease of oral administration. CPZ diet was constituted to 0.2% by mixing 0.2 g CPZ with a 100 g standard rat diet. The presented treatment lasted for 42 days.

The open-field test was performed to determine the locomotor activity and exploratory behaviors of rats. Each rat was placed at the center of an open-field box (Yan et al., 2015) and tested for 10 min; in this location, the rats usually have the freedom of movement. The activities of each rat were recorded by a video-camera situated above the area. Following the completion of the exercise, the following data were obtained: the Number of Lines Crossed (NLC), Center Squared Duration (CSD), Center Square Entry (CSE), Freezing Duration (FD), Rearing Frequency (RF), and Rearing Duration (RD).
Figure 1. The weights of animals

Bodyweight changes across the treated groups: A: Control; B: CPZ; C: KV; and D: CPZ plus KV;

A: Shows the initial and final weights, with all the groups having increased weight differences, though the CPZ group presented very minimal weight differences;

B: The control group (A) had the highest weight gain, while the CPZ-treated group (B) encountered the least weight gain with statistically significant differences, compared to the controls (P<0.001). Weight gain in the KV-treated group (C) was also high with a statistically significant difference, compared with the controls (P<0.01); the differences between the KV-treated group and CPZ-treated group was statistically significant (P<0.001); weight gain in the CPZ+KV group (D) was significantly higher than the CPZ-treated group (B) (P<0.05), but lower than the control group (A) (P<0.001) and KV-treated group (C) (P<0.01). Key: * P<0.05, **P<0.01, and *** P<0.001.

Figure 2. Behavioral tests for locomotion and exploratory activities

The locomotor and exploratory activities of rats across the research groups. A: Control; B: CPZ; C: KV; and D: CPZ and KV. In comparison with CPZ and CPZ+KV treatment groups, animals given KV treatment experienced an increase in the number of the line crossed (A) (P<0.001 & P<0.01, respectively), rearing frequency (B) (P<0.001: KV vs. CPZ groups), rearing duration (C) (P<0.001 & P<0.05, respectively), center square entry (D) (P<0.01: KV vs. CPZ groups) and center square duration (E) (P<0.001: KV vs. CPZ groups). However, there was a decrease in freezing time (F), compared with the groups solely treated with CPZ and KV (P<0.001 & P<0.05, respectively). Animals simultaneously treated with CPZ and KV generally demonstrated significantly improved number of the line crossed (A) (P<0.05), rearing frequency (B) (P<0.05), rearing duration (C) (P<0.01), center square entry (D) (P<0.05), and center square duration (E) (P<0.01), compared with the CPZ group.
Moreover, the tissues were stained with anti-Glial Fibrillary Acidic Protein (GFAP) antibody (as a primary antibody). Next, they were treated with biotinylated secondary anti-body (goat anti-rabbit) to detect the GFAP-positive cells (astrocytes) in the cerebellum. The mounted slides were viewed with the aid of an Olympus binocular research mi-croscope (Olympus, New Jersey, USA) connected to an Amscope Camera (5.0 MP).

Cerebellar tissue homogenate was prepared with a cold 0.25 M sucrose solution using an automated homog-enizer at 4°C. The tissue homogenate was centrifuged for 10 min in a microcentrifuge with a centrifugal force of 16099 ×g. The supernatants obtained were thereafter aspirated into plain bottles and analyzed for the activi-ties of Superoxide Dismutase (SOD), Glutathione Peroxidase (GPx), and Malondialdehyde (MDA) according to the manufacturer’s instruction in the biochemical kits.

All quantitative data were analyzed using GraphPad Prism. The obtained data were presented as the mean and standard error of the mean, using Analysis of Variance (ANOVA) by Tukey’s multiple comparisons test. Statistical significance was also considered as P<0.05.

3. Results

The feeding pattern among rats treated with CPZ reduced in the first two weeks of treatment; however, their feeding pattern gradually returned to normal. Additionally, that given KV experienced an increase in eating habits. However, after the fourth week of KV administration, their feeding habit dropped slightly. The animals treated with both CPZ and KV indicated increased feeding patterns during the early stage of administration; however, there was an observable gradual decline in their feeding pattern. Animals treated with CPZ demonstrated no appreciable weight gain (Figure 1A & B). The group treated with KV and those that concomitantly received KV and CPZ revealed a significant weight gain, compared with the group that was treated with CPZ. However, compared with the control group, there was a reduction in their weight (Figure 1A & B).

The open-field test data suggested that demyelinating and degenerative changes observed in CPZ decreased locomotor and exploratory activities (P<0.001). KV treatment was found to be protective against cuprizone-induced behavioral deficits; it recorded an alternation in locomotor and exploratory activities, compared to the CPZ group (Figure 2).
The activities of endogenous oxidative enzymes were assessed to illustrate their involvement in CPZ-induced demyelination and understand the mechanistic inhibitory roles of KV. The spectrophotometric assay data of the SOD profile from cerebellar homogenates presented a normal SOD level in the cerebellum of the control and KV groups; these data were associated with a significant increase in group C. The SOD level significantly reduced in rats intoxicated with CPZ, compared with other groups. However, rats that concomitantly received CPZ and KV revealed a significant increase in SOD level, compared to the group that received CPZ (Figure 3A).

The GPx level of cerebellar lysates was examined (Figure 3B). The related findings revealed that the control and KV-treated rats presented elevated GPx levels, compared to those treated with CPZ and adjunct CPZ and KV. Furthermore, animals co-treated with CPZ and KV recorded a higher GPx level, compared with the CPZ-treated rats. MDA level was also assessed to determine the degree of lipid peroxidation in cerebellar lysates in the research groups. The relevant results (Figure 3C) suggested that KV-treated rats experienced decreased expression of MDA in the cerebellar homogenate. The CPZ-treated had increased MDA level, compared to the other groups. The rats treated concomitantly with KV and CPZ demonstrated a significant downregulation of MDA expression, compared to the CPZ-treated group.

The histochemistry of the cerebellum was demonstrated using Haematoxylin and Eosin (H & E) and Cresyl Fast Violet (CFV) staining methods. Figure 4 shows the panoramic view and high power magnification of the microarchitecture of cerebellar cortices with distinct cell types and cell layers. The Control and KV-treated rats presented well-arranged cerebellar layers with obvious soma and dendrites; they project deep into the molecular layers, having a fan-like shape nucleus with well-stained white matter regions. Furthermore, the granular cell layers in the groups were comprised of well-arranged small granule cells. Neuronal morphology of rats intoxicated with CPZ demonstrated fragmented cerebellar layers with cryptic granules, degenerating Purkinje cells with pyknotic cell bodies, and short dendritic processes. However, the rats treated with both CPZ and KV indicated cerebellar layers and neuronal morphology, i.e. similar to that of the controls.

GFAP immunohistochemistry method was used to demonstrate astrocytic morphology and distribution in the cerebellar cortex of the explored Wistar rats. GFAP immunopositive cells within the cerebellar cortex of...
the control and KV-treated rats appeared scanty around neurons; they also were observable in between layers, with regular processes, distribution, and sizes within the neuropil. However, increased astrocytic densities with reactive astroglia within the granule cell layer and hypertrophic cells appeared within the cerebellar layers in CPZ-treated rats. Expressing astrocytes within the cerebellar cortex of rats treated with Kv and CPZ had close similarities with the control and KV-treated groups. Astrocytic processes, cellular distribution, and size were normal in the control and KV-treated rats.

4. Discussion

Demyelination of focal white matter injury can occur in any location of the CNS (Ahmad, Satriotomo, Fazal, Nadeau & Doré, 2015). The most frequent form of demyelination is observed in Multiple Sclerosis (MS). Eating and diets are essential factors in controlling body weight (Drapeau et al., 2004). Studies reported reduced feeding habits during the first 2 weeks of CPZ consumption and gradual improvement in feeding habits post CPZ diet withdrawal (Sachs et al., 2014; Praet et al., 2015; Steel-
Therefore, it is suggested that the observed bodyweight reduction in this study was caused by reduced feeding pattern and the disruption of healthy energy flow. KV treatment significantly prevented CPZ-associated weight loss; i.e. probably caused by its ability to prevent normal energy disruption owing to its potential to mop up excess free radical. This might be the reason for the detected disruption.

As a correlative test for cellular and neuropathological changes in the cerebellar cortex in this study, the exploratory activities of rats were assessed using the open field test (Gould, Dao, & Kovacsics, 2010).

Our results revealed a significant reduction in exploratory activities in CPZ-treated rats, corroborating a previous study by Faizi et al. (2016). However, KV significantly countered these effects. Thus, locomotor and exploratory activities depend on the intact integrity of the myelin sheath. Prior research suggested increased exploratory activities of rats following KV treatment after NaN3-induced neurodegeneration (Olajide et al., 2016).

Decreased SOD and GPx levels, as observed in the current study, has been implicated in the mechanism through which CPZ induces demyelination (Biancotti, Kumar & de Vellis, 2008; Witherick, Wilkins, Scolding, & Kemp, 2010; Praet et al., 2014). Faizi et al. (2016) reported a decrease in the SOD level following the administration of CPZ due to the reduced level of Cu++, i.e. required for the proper functioning of SOD. KV treatment improved these antioxidants’ levels within the cerebellar cortex of rats. SOD catalyzes the dismutation of superoxide (O2−) radical (Sun and Trumpower, 2003; Hayyan et al., 2016); if upregulated by KV, it could prevent the cytotoxic effects of O2− molecules. Thus, it prevents oxidative-related damage. GPx is an enzyme capable of detoxifying Reactive Oxygen Species (ROS) and nucleophilic compounds. They could initiate lipid hydroperoxides to their corresponding alcohols and water, respectively (Muller et al., 2007). Lipid peroxidation and OS following CPZ treatment have been previously documented (Xuan et al., 2015). However, treating rats with KV significantly reduced the MDA level. Such data suggest that KV could inhibit lipid peroxidation due to its antioxidant properties.

CPZ-induced cerebellar injury revealed various degrees of structural damage to the cellular components and the normal architectural pattern of the cerebellum. These alterations in the cellular morphology of the cerebellum following CPZ treatment adversely affect signal processing. It also impacts the functionality of the synaptic complex, as the common features in patients with demyelinating diseases (Kutzelnigg et al., 2007; Rot et al., 2008).

Furthermore, CPZ intoxication induces megamitochondria and OS, leading to energy flow disruption and shortage (Tandler and Hoppel, 1973). The energy flow disruption and depletion alter the proper functioning of the endoplasmic reticulum, i.e. important in neuronal protein synthesis. This often causes the cellular degeneration and disintegration of oligodendrocytes perikaryon and myelin sheath (Praet et al., 2014). The neurotoxic nature of CPZ may explain the mechanisms by which it induces cellular degeneration and white matter demyelination. Interestingly, rats were given KV concomitantly reflected cerebellar morphology, i.e. similar to the controls. The ability of KV to restore the chromogenic nature of the Nissl substance following CPZ-induced damage might be due to its ability to prevent ER stress; this process inhibits the pathways that lead to failure of protein production owing to its antioxidative property and ROS scavenging ability.

Studies have revealed an increase in astrocytic activity following one-week CPZ intoxication (Zatta et al., 2005). The activation of astrocytes could result in extensive astrogliosis which may persist through the period of remyelination (Zaaraoui et al., 2008; Gudi et al., 2009). In the current work, changes observed on the cerebellum were characteristic of reactive astrogliosis. Accordingly, these were however absent in the cerebellar cortex of rats treated with KV. This finding suggests a cytoprotective role of KV.

5. Conclusion

KV exhibited neuroprotection against the behavioral deficit, OS, astrogliosis, demyelination, and CPZ-induced cortical neuronal damage in adult male Wistar rats. These findings provide a lead on the potentials of KV, as a neuroprotective agent against demyelinating diseases.

Ethical Considerations

Compliance with ethical guidelines

The Study was approved by the Ethical Review Committee, University of Ilorin (UERC/ASN/2017/969).

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Funding

This research did not receive any grant from funding agencies in the public, commercial, or non-profit sectors.

Conflict of interest

The authors declared no conflicts of interest.

Acknowledgments

The authors acknowledge the assistance and guidance of Dr. O.J. Olajide, affiliated with the Department of Anatomy, University of Ilorin.

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