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Garcinia kola Biflavonoid Complex Mitigates Cerebellar Histomorphological Alterations and Oxidative Stress in Maternally Deprived Rats

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ABSTRACT

Background: Early maternal deprivation interferes with normal growth and development, with attendant morphological and biochemical alterations.

Objective: This study explored the protective action of kolaviron, a biflavonoid complex from Garcinia kola, on the cerebellum of maternally deprived Wistar rats.

Methods: Adult female Wistar rats were exposed to male rats for mating, and after delivery, the pups were randomly assigned into four groups. Group A was administered with normal saline, group B received kolaviron (200 mg/kg/bw) on postnatal day (PD) 21-35, groups C and D rats were maternally deprived on PD 9 for 24 hours, while group D rats received kolaviron (200 mg/kg/bw) on PD 21-35, in addition to earlier maternal deprivation. The pups were then anesthetized and sacrificed, followed by transcardial perfusion with normal saline and then 4% paraformaldehyde. The cerebellum was excised from the brain and processed for tissue histochemistry, immunohistochemistry and biochemical analysis.

Results: Findings revealed cytoarchitectural distortion, extreme chromatolysis in neuronal cells, astrogliosis and compromise in the oxidative state of the cerebellum. Treatment with kolaviron led to significant improvement in oxidative status and a comparatively normal cytoarchitectural profile.

Conclusion: In conclusion kolaviron could help ameliorate the oxidative stress and cerebellar histopathology associated with maternal deprivation.

Keywords: maternal deprivation, kolaviron, oxidative stress, chromatolysis, cerebellum, development

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INTRODUCTION

Maternal deprivation (MD) of pups during early life is one of the strongest stressors during neonatal development (1,2). In humans, stressors experienced during childhood are the main cause of subsequent emergence of mental disorders in adulthood (2). Studies using animal models revealed that MD causes behavioral changes such as increased anxiety, personality disorders like schizophrenia, depression and memory deficits (3,4,5). Maternal deprivation in rodents is an essential neurodevelopmental model for studying different behavioral changes which are similar to the symptoms of schizophrenia in humans (5).

The cerebellum plays an important role in higher cortical functions, including cognition and emotion and might have important implications in neurodevelopmental neuropsychiatric disorders such as autism and schizophrenia (1,6). At the cellular level, MD induces neuronal degeneration and astroglial changes in the cerebellar cortex of neonatal rats (7,8,9). Early maternal deprivation can lead to disruptions in the postnatal development of the brain, which eventually causes learning disabilities, behavioral anomalies and neurobehavioral disorders in later life (10). Such disruptions can be associated with the functions of various brain regions including the cerebellum, underlying some of the cognitive, neurological, and neuropsychiatric symptoms that are observed in schizophrenic patients (11). Reactive oxygen species play a role in the stress response in different tissues, and are known to be generated by NADPH oxidase in Maternally deprived rats (12). Studies have shown that early maternal deprivation leads to long-term redox alterations in the brain of rats (12).

Kolaviron is a natural biflavonoid complex isolated from the seeds of *Garcina kola* (G. kola) and is widely consumed in the West African subregion, including Nigeria. It is one of the several plant extracts and nutritional supplements that have been established to have a wide range of medicinal value including antioxidant and anti-inflammatory properties (13). As a result of the antioxidant characteristic of kolaviron, it has the ability to guard the neurons of the brain from toxic damage. Kolaviron is neuroprotective against gamma radiation-induced oxidative stress in rats (14), has hypoglycemic effects in normal and alloxan- and streptozotocin-induced diabetic animals (15,16), and also elicits strong antioxidant activity in both in vivo and in vitro experimental models (17).

The behavioral disorders (10) associated with MD do not have therapeutic cure; hence, the current study explored the role of kolaviron in ameliorating MD-induced cerebellar histomorphological changes and oxidative stress.

METHODOLOGY

Animal Care and Use

The study was approved by the University Ethical Review Committee, University of Ilorin, Nigeria, and the protocols and treatment procedures were carried out according to the guidelines. Thirty adult female Wistar rats aged 8 months and eight adult male Wistar rats aged 10 months were obtained from Veterinary Department of the University of Ilorin, and housed in the Animal Facility of the Faculty of Basic Medical Sciences, University of Ilorin. The rats were fed daily with standard rat pellets and had access to tap water ad libitum. They were allowed to acclimatize for two weeks before exposure of the female rats to the male rats for mating. This research was approved by the University of Ilorin Ethical Review Committee (UERC/ASN/2019/1742).

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Extraction and Isolation of Kolaviron

The seeds of *Garcinia kola* were bought from a market and authenticated at the Department of Plant Biology, Faculty of Life Sciences, University of Ilorin. The seeds were then dried at room temperature (28–30°C) for three weeks. Isolation of kolaviron from Garcinia kola was according to the method of Iwu (18) as modified by Farombi et al. (19). The dried seeds were pulverized with an electric blender into fine powdery form and then extracted with light petroleum ether (boiling point (b.p.) 40–60°C) in soxhlet extractor which was placed in an electric water bath for 24 hours. After this, the defatted, dried marc was repacked and extracted with acetone (b.p. 56-60°C) in the water bath for 14 hours. Subsequently, the resulting extract was concentrated and diluted twice its volume with distilled water and then extracted with ethyl acetate (7.250 ml). The concentrated ethyl acetate fraction gave a yellow solid referred to as kolaviron, whose purity and identity can be determined by subjecting it to thin layer chromatography using silica gel GF 254 coated plates and solvent mixture of methanol and chloroform in a ratio 1:4 v/v.

Experimental Design

Kolaviron was dissolved in corn oil (Carlini, ALDI Inc. Batavia) (17,20) for ease of administration orally. Maternal deprivation was achieved by separating the pups from the mother on postnatal day (PD) 9 for a period of 24 hours (5), while kolaviron was administered via an oral cannula from PD 21 to PD 35. After the pregnant female rats were delivered, the pups were randomly assigned into a control and three treatment groups with each group containing 4 rats from different mothers. The rats in the control group A received normal saline (0.5 ml), group B received kolaviron (200 mg/kg/bw), group C were maternally deprived, while group D were maternally deprived followed by administration of kolaviron (200 mg/kg/bw) (21).

Tissue processing

After the termination of treatment, the rats were anesthesized with ketamine (5 ml/kg) and transcardially perfused using normal saline and 4% paraformaldehyde. The cerebellum was excised from the fixed brain and dehydrated in ascending grades of alcohol (50%, 70%, 90% and 100%). Tissues were then cleared in xylene for 2 times 15 minutes each. Infiltration and embedding were done with paraffin wax in Leica hot air oven at 56°C. Tissue sections were obtained serially using a rotary microtome (Leica RM2245) and then mounted on glass slides. Sections were taken at 30 µ for histology and 15 µ for immunohistochemistry (22). The cerebellum was processed for Hematoxylin and Eosin (H&E) staining according to Pearse (1975) (23) and Fischer et al. (2005) (22) to demonstrate the general cytoarchitecture of the cerebellar cortex, while the cresyl fast violet staining technique was adopted to demonstrate the integrity of the Nissl bodies of the cerebellar cortex.

Immunohistochemical technique using anti-glial fibrillary acidic protein (GFAP) antibody

Endogenous peroxidase blocking was carried out using hydrogen peroxide, while 5% bovine serum albumin (BSA) was used to reduce non-specific protein reactions. Diluted primary antibody (10% calf serum with 1% BSA and 0.1% Triton X-100 in 0.1 M PBS; Anti-GFAP –dilution 1:1000) was added to each slide and incubated overnight at 4°C. Slides were later treated with biotinylated secondary antibody (goat anti-rabbit 1:400 in block) and incubated for 2 hours at room temperature. Colour intensification was done using the chromogen 3.3'-Diaminobenzidine (DAB 1:1000 dilution) and counterstaining was done in hematoxylin and 1% acid alcohol.

Biochemical studies

Assay for superoxide dismutase, catalase and glutathione peroxidase levels were carried out in cerebellar homogenates using spectrophotometric techniques. The tissue was placed in 0.25 M sucrose solution and homogenized. Tissue homogenate was collected in a 5 ml serum bottle and centrifuged at 3000 rpm for 15 minutes using a centrifuge (Model 90-1; Jiangsu Jinyi Instrument Tech, Jiangsu, China). The supernatant was collected with Pasteur pipettes and placed in a freezer at -4 °C, and thereafter assayed. Biochemical assay was carried out as described by Sun and Zigma (24) for superoxide dismutase, Sinha et al. (25) for catalase, Reddy et al. (26) for glutathione peroxidase.

Data analysis

All quantitative data were analyzed using GraphPad® (version 6) and SPSS (version 20) software. The outcomes were plotted in ANOVA followed with Tukey's multiple comparisons test. Significance was set- at $p<0.05$ (95% confidence interval). The results were represented in bar charts with error bars to show the mean and standard error of mean respectively.

RESULTS

Kolaviron mitigates cerebellar oxidative stress induced by maternal deprivation

Superoxide dismutase activity

The level of superoxide dismutase (SOD) enzyme in the cerebellum was highest in the control rats (Figure 1). Rats treated with kolaviron also had an elevated level of SOD but less than the control with no statistically significant difference. There was significant reduction in the level of SOD in maternally deprived rats when compared to both control ($p<0.01$) and kolaviron-treated group ($p<0.05$). Treatment of maternally deprived rats with kolaviron raised the SOD level higher than in maternally deprived rats that did not receive kolaviron treatment, though the level was lower than the control ($p<0.05$) and kolaviron-treated rats.

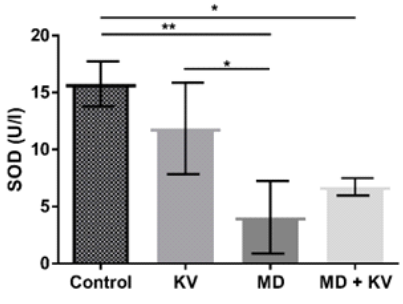


Figure 1: Activities of superoxide dismutase (SOD) in the cerebellum of rats. There was significant decrease in SOD activities in maternally deprived rats (MD) compared to control ($p < 0.01$) and kolaviron treated (KV) ($p < 0.05$), with elevated level in maternally deprived rats treated with kolaviron (MD+KV). * and ** were $p < 0.05$ and $p < 0.01$ respectively.

Catalase activity

Catalase activity was also significantly low in maternally deprived rats compared with the control ($p<0.0001$) and kolaviron-treated ($p<0.005$) rats, while the level was increased in maternally deprived rats that received kolaviron treatment (Figure 2). The control had the highest catalase activity.

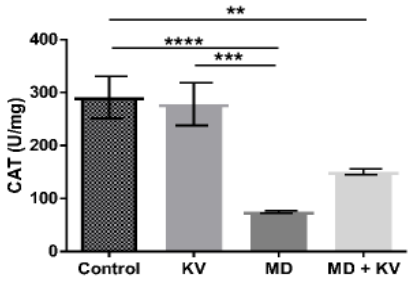


Figure 2: Catalase (CAT) level in the cerebellum across all groups, showing a significant decrease in enzyme activity in maternally deprived (MD) group compared to control group ($p < 0.0001$) and kolaviron (KV) group ($p< 0.005$). Enzyme activity increased in maternally deprived rats that received kolaviron intervention (MD + KV). **, *** and **** are $p<.01$ and $p<0.005$, $p<0.0001$ respectively.

Glutathione peroxidase activity

Expression of glutathione peroxidase (GPx) for stress-related metabolism in the cerebellar cortex showed a slight deviation from the preceding two enzymatic oxidative markers. GPx level was highest in kolaviron treated rats, though the difference from the control was not statistically significant, while the difference was significant when compared with the maternally deprived rats ($p<0.05$). GPx activity however increased in maternally deprived rats that received kolaviron intervention (Figure 3).

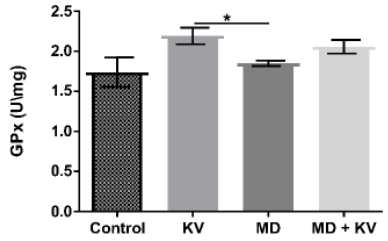


Figure 3: Expression of glutathione peroxidase (GPx) as a measure of oxidative redox states in the cerebellar cortices of Wistar rats, showing a significant decrease in GPx activity in the maternally deprived (MD) group compared to kolaviron (KV) group ($p < 0.05$). * was $p<0.05$ and MD + KV = maternally deprived + Kolaviron group.

Histological and histochemical observations

The general histomorphology of the cerebellar tissue was demonstrated to reveal the molecular layer, Purkinje cell layer and the granular layer (Figure 4). In the control group, arrangement of cells within the cerebellar cortex was well organized and characterized by Purkinje cells within the Purkinje cell layer with cell bodies and dendrites projecting into the molecular layer, while the granule neurons were densely packed within the granular layer. The cerebellar cortex of maternally deprived rats showed some signs of degeneration as both cell bodies and dendrites were sparsely visible across the cortical layers when compared to control. The granular layer was not as densely populated as the Control. However, the cells in the Purkinje cell layer and granular layer of maternally deprived rats treated with kolaviron were somewhat more distinct morphologically compared to the maternal deprivation group.

A demonstration of Nissl proteins using cresyl fast violet staining methods across cerebellar sections (Figure 4) revealed normal Nissl profiles in the control and kolaviron groups compared to maternally deprived group, which was characterized by sparse Nissl distribution. However, kolaviron treatment markedly improved the distribution and population of Nissl bodies within the somata of neurons of rats treated with kolaviron after maternal deprivation. The Purkinje and granule cells were well stained in the control and kolaviron-treated group whereas in the maternally deprived, with or without kolaviron intervention, there were degeneration of Purkinje cells and chromatolysis as revealed by poorly stained Nissl profile (Figure 4).

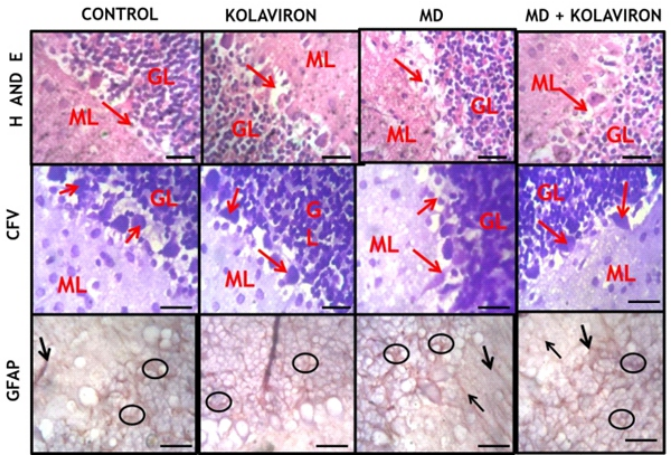


Figure 4: Representative photomicrographs of cerebellar cortices of rats stained with Hematoxylin and Eosin (H and E) for general histomorphology, cresyl fast violet (CFV) for Nissl profile and anti-glial fibrillary acidic protein antibody (GFAP) for astrocyte expression.

The H and E photomicrographs revealed a general cytoarchitecture showing the molecular layer (ML), granular layer (GL) and the Purkinje cell layer (between ML and GL). Deeply stained and characteristically normal cellular layering of the granule cell layers with well-outlined Purkinje cell layers can be seen in control, maternal deprivation + kolaviron (MD+KV) and kolaviron (KV) groups. These groups also showed large Purkinje cells (red arrows) with axons jetting into the molecular layer, whereas the Purkinje cells in the Purkinje layer in the maternally deprived group appeared degenerated as they were laconically expressed.

The CFV sections showed deeply stained and well expressed (red arrow) Nissl bodies in the control, MD+KV and KV groups. The Purkinje cells were deeply stained and well expressed in these groups. Maternally deprived group presented with chromatolytic Purkinje cell (red arrow). There was increased GFAP expression in the MD group, though the processes (black arrows) were mostly expressed in the molecular layer, the granular had more cell bodies (circles) expressing the GFAP compared with the control group. GFAP was expressed in the kolaviron group mostly in the granular layer (circles). In the molecular layer, the expression was only in the astrocytic processes (arrow heads). GFAP was expressed in the MD + kolaviron group mostly in the granular layer (circles). The molecular layer had expression of the astrocytic processes (arrows), with no difference with the control group (scale bar = 25μ)

Expression of astrocytes in the cerebellar cortex

Glial fibrillary acidic protein (GFAP) was expressed in the astrocytes of the cerebellar cortex. Astrocytic processes in the control were mostly expressed in the molecular layer, with the cell bodies expressed in the granular layer (Figure 4). There was increased GFAP expression in the maternal deprivation group; although the processes were mostly expressed in the molecular layer, the granular layer had more cell bodies expressing GFAP compared with the control group. Protein expression in the kolaviron group was more in the granular layer, similar to observation in rats that received kolaviron after maternal deprivation.

DISCUSSION

In this study, the effects of maternal deprivation on the cerebellum of Wistar rats were investigated. We earlier demonstrated changes in body weights that followed maternal deprivation with or without kolaviron intervention, and noted that maternal deprivation was associated with reduced body weights (27). MD is a perinatal stressor that has long-term effects on the brain,

especially when exposed in early postnatal life. Early perinatal stress causes various short-term disturbances in cognitive, emotional, and other behavioural performances (12, 28, 29), and kolaviron has been demonstrated to improve behavioral outcomes associated with MD (27).

A balanced oxidative status is critical for normal brain development and function. To assess the redox system of the maternally deprived rats, we employed the use of enzymatic oxidative markers such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase. The use of kolaviron as an intervention is due to its proven antioxidant properties (21, 30). Antioxidants possess free radicals scavenging ability through which they mitigate the untoward effects of reactive oxygen species (ROS) in the system. Kolaviron, a biflavonoid from Garcinia kola seeds, has notably been demonstrated as one of such antioxidants (21, 30).

SOD enhances the separation of superoxide (O_2^-) radical into either regular molecular oxygen or hydrogen peroxide (31). The significant reduction in SOD activity in the cerebellum of maternally deprived rats observed in the current study indicated that separating pups from their mothers in early postnatal days predisposes infants to oxidative stress potent enough to cause neurochemical injury to the developing brain. Meanwhile, appreciable improvement in the SOD level occurred following the introduction of kolaviron, signifying the anti-oxidative property of this biflavonoid in maternal deprivation-induced oxidative stress. Similar effects were noticed with the use of other oxidative enzymes.

Catalase is an endogenous oxidative enzyme that rapidly catalyses the breakdown of hydrogen peroxide into oxygen and water molecules, so as to protect the cell from the harmful effect of hydrogen peroxide during cellular metabolism (32,33). Its reduced activity in maternally deprived rats could be a consequence of the overwhelming nature of the postnatal stress associated with maternal separation which the developing brain could not readily overcome. A reduction in catalase activity therefore could enhance further cellular damage arising from the detrimental activity of hydrogen peroxide. However, administration of kolaviron to maternally deprived rats raised the tissue level of catalase, thereby providing some level of cytoprotection for the cerebellum.

Glutathione peroxidase, under normal circumstance, prevents lipid peroxidation and maintains redox balance. Although the sensitivity of glutathione peroxidase to maternal deprivation-induced stress was not as remarkable as that seen with catalase and superoxide dismutase, the slight reduction in cerebellar GPx activity might still be able to negatively affect intracellular homeostasis and redox balance. Decrease in the GPx levels has been implicated in the mechanism through which maternal deprivation causes redox alterations (34).

MD-induced neurotoxicity affects different regions of the brain, and as demonstrated in this study, microarchitectural alterations in the structural integrity of the cerebellum and its cellular components were common occurrence. These changes in cellular morphology of the cerebellum can affect signal processing and the functionality of the synaptic complex, which are common features in patients with demyelinating diseases or Schizophrenia (4). In addition, maternal deprivation causes oxidative stress leading to energy flow disruption and shortage (34). Expectedly the neuronal morphology and cerebellar cortical layers of rats treated with kolaviron after maternal deprivation were generally characterized by neurons with apparently normal cell bodies, appreciable axons and numerous dendritic processes within neurophil, showing the neuroprotective effect of kolaviron. This is in consonance with Olajide et al. (35) that kolaviron protects the microarchitecture of the brain following neurotoxicity. These observations could improve neurological functions adversely affected by maternal deprivation.

As a consequence of the oxidative disruption that followed maternal deprivation, cellular integrity and functions became compromised. Maternally deprived rats showed loosely arranged and cryptic granule cells, with degenerating Purkinje cells that had pyknotic cell bodies and short dendritic processes.

The ability of kolaviron to return the chromatogenic nature of the Nissl bodies following MD-induced neuronal damage was as a result of its anti-oxidative property and ROS scavenging activity. Using the same anti-oxidative mechanism, kolaviron was reported to protect against astrogliosis in chemical-induced brain injury (36). In the same vein, MD-induced cerebellar astrogliosis was ameliorated in the current study.

CONCLUSION

Kolaviron showed remarkable ameliorative effect on cerebellar oxidative stress and neurohistochemical alterations associated with early maternal deprivation in rats.

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