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Lactational Kolaviron Mitigates Bilirubin-induced Neocortical Oxidative Damage and Neuroinflammation in Young Wistar Rats

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ABSTRACT

Oxidative stress and neuroinflammation play a central role in the pathogenesis of numerous neurological disorders by disrupting cellular redox balance and promoting neuronal degeneration. Phenylhydrazine (PHZ), a well-established inducer of oxidative stress and hemolytic anemia, generates reactive oxygen species capable of causing structural and functional damage to the brain. Kolaviron, a biflavonoid complex isolated from *Garcinia kola* seeds, possesses potent antioxidant and neuroprotective properties. However, its effects on PHZ-induced neocortical oxidative stress and neuroinflammation remain poorly understood. Twelve adult female Wistar rats were assigned into three groups (n = 4 per group) and allowed to breed. Hyperbilirubinemia was induced in neonatal pups using phenylhydrazine (75 mg/kg body weight, intraperitoneally), while 200 mg/kg of Kolaviron was administered orally to the mothers from postnatal day 1 to day 14. At the end of the experimental period, animals were euthanized, and neocortical tissues were harvested for biochemical assays of catalase, superoxide dismutase (SOD), and interleukin-1β (IL-1β), and histological evaluation. PHZ significantly altered antioxidant enzyme activities, evidenced by reduced catalase and SOD activities, while significantly elevating the concentration of pro-inflammatory cytokine IL-1β. However, KV regulated the concentrations of these biomarkers similar to the control. Histological examination of the cerebral neocortex also revealed varying degrees of alterations. The findings of this study demonstrate that phenylhydrazine induces oxidative stress, neuroinflammation, and mild neocortical neurodegeneration, while kolaviron effectively attenuates these alterations through its antioxidant and anti-inflammatory activities.

Keywords: Kolaviron, phenylhydrazinium chloride/phenylhydrazine, oxidative stress, neuroinflammation, neocortex

INTRODUCTION

Hyperbilirubinemia is a clinical condition characterized by elevated levels of bilirubin in the blood resulting from excessive bilirubin production, impaired hepatic uptake, defective conjugation, or reduced excretion. Bilirubin is a yellow pigment produced during the breakdown of hemoglobin from senescent red blood cells. Although bilirubin possesses antioxidant properties at physiological concentrations, excessive accumulation can exert toxic effects on various tissues, particularly the central nervous system[1].

The brain is highly susceptible to bilirubin-induced toxicity because of its high oxygen consumption, abundant lipid content, and relatively limited antioxidant defense mechanisms [2]. Excessive bilirubin accumulation may cross the blood-brain barrier and induce neuronal injury through oxidative stress, neuroinflammation, mitochondrial dysfunction, and apoptosis. These pathological processes can impair the structure and function of several brain regions, including the neocortex, which is responsible for higher cognitive functions such as sensory perception, learning, memory, language, decision-making, and motor planning [3].

The neocortex is the largest and most evolutionarily advanced region of the cerebral cortex. Structurally, it is characterized by numerous folds known as gyri and grooves called sulci, which increase the surface area of the brain. The neocortex is divided into four major lobes: the frontal, parietal, temporal, and occipital lobes, each performing specialized functions[4]. It contains complex neuronal circuits composed predominantly of pyramidal neurons and interneurons that facilitate communication and information processing within the brain[5].

Experimental models of hyperbilirubinemia are essential for investigating the mechanisms underlying bilirubin-induced neurotoxicity and for evaluating potential therapeutic interventions. Phenylhydrazinium chloride (Phenylhydrazine) (PHZ) is a well-established hemolytic agent commonly used to induce hyperbilirubinemia in laboratory animals[6]. PHZ causes oxidative damage to erythrocytes, leading to hemolysis and excessive hemoglobin degradation, which subsequently increases bilirubin production and mimics the pathological features of hyperbilirubinemia[7]. In addition to inducing hyperbilirubinemia, PHZ promotes the generation of reactive oxygen species and lipid peroxidation, resulting in oxidative stress and cellular damage in various tissues, including the brain[8].

Oxidative stress and consequent inflammation remain a major contributor to neuronal injury and neurodegenerative disorders[9]. Persistent oxidative stress contributes significantly to neuronal degeneration by damaging cellular lipids, proteins, and nucleic acids, thereby impairing normal brain function[1]. Experimental induction of oxidative stress using phenylhydrazine has been widely employed to investigate mechanisms of tissue damage and evaluate the therapeutic potential of antioxidant agents[10, 11]. Although phenylhydrazine-induced hemolysis and oxidative stress have been extensively studied in hematological and hepatic systems, its effects on neocortical integrity remain relatively underexplored[12].

Kolaviron (KV) is a natural biflavonoid complex isolated from the defatted ethanolic extract of *Garcinia kola* Heckel seeds, a medicinal plant widely distributed in West Africa, particularly in Nigeria, Ghana, and Cameroon[13, 14]. Chemically, kolaviron consists predominantly of the biflavanones GB1, GB2, and kolaflavanone. Numerous studies have demonstrated that kolaviron possesses antioxidant, anti-inflammatory, anti-cancer, anti-diabetic, hepatoprotective, neuroprotective, anti-amnesic, anti-malarial, anti-asthmatic, anti-spasmodic, and anti-atherogenic properties[15-17]. These biological activities have generated considerable scientific interest in the potential use of kolaviron as a therapeutic agent against disorders associated with oxidative stress, neurotoxicity, and tissue injury.

Therefore, investigating the protective effects of kolaviron against PHZ-induced hyperbilirubinemia and its associated oxidative damage in the neocortex may provide valuable insight into the development of novel therapeutic strategies for the management of bilirubin-induced neurotoxicity.

MATERIALS AND METHODS

Experimental animals

Twelve (12) female Wistar rats weighing between 150–200 g were obtained from Ilorin, Kwara State, Nigeria. The animals were housed in well-ventilated wire-gauzed cages in the Animal House of the Department of Anatomy, Faculty of Basic Medical Sciences, College of Health Sciences, University of Ilorin, Nigeria. The animals were maintained under standard laboratory conditions and fed daily with standard rat feed and water provided *ad libitum*. They were acclimatized for fourteen (14) days before commencement of the experiment. Wood shavings were used as bedding material and were changed regularly to maintain hygiene. The animals were marked individually for identification purposes. The animals were mated and allowed to undergo a gestation period of approximately 21 days, after which rats littered, marking day 1 of the experiment. The pups were allowed to remain with their mothers.

Animal experiments were approved by the University Ethical Review Committee, University of Ilorin (UIL/ASN/2018/2025/3216), and all procedures complied with the Institutional Animal Care and Use Committee (IACUC) guidelines.

Drugs

Phenylhydrazinium chloride (or phenylhydrazine; PHZ) was obtained from Bristol Scientific Company Limited, Bristol Road, Apapa, Lagos State, and used at a dose of 75 mg/kg. For treatment, the standard substance Kolaviron (KV) was isolated from fresh seeds of *Garcinia kola* Heckel, which were purchased from a local market. The seeds were authenticated at the University herbarium, followed by extraction using a Soxhlet apparatus. KV was dissolved in corn oil and administered at a dose of 200 mg/kg[18].

Establishment and treatment of the PHZ-induced oxidative stress rat model

Twelve (12) Wistar rat pups were randomly assigned into three experimental groups (A–C) (n=4/group) (Table 1). The pups remained with their respective mothers throughout the experimental period.

Group A served as the control group; the mothers received corn oil (2 ml/kg) via oral cannula from postnatal Day 1-21; Group B: the pups received a single intraperitoneal dose of PHZ (75 mg/kg body weight) on postnatal day 15. PHZ was dissolved in normal saline; Group C: the mothers received kolaviron (200 mg/kg body weight, dissolved in corn oil, via oral cannula) from postnatal day 1 through 14,

while the pups received PHZ (75 mg/kg body weight intraperitoneally) on day 15.

Table 1: Experimental animals grouping

Groups	Treatment	Duration
A	Control - Corn oil (2 ml/kg)	Days 1–21
B	PHZ only - 75 mg/kg body weight, i.p. (single dose, pups)	Day 15
C	Kolaviron - 200 mg/kg body weight (mothers) & PHZ-75 mg/kg body weight (pups)	Days 1–14, Day 15

Animal sacrifice and tissue processing

The pups were euthanized about 24 hours after termination of treatment (postnatal day 16 for groups B and C, and postnatal day 22 for group C), following anaesthetization with ketamine hydrochloride (100 mg/kg body weight, intraperitoneally). The rats were euthanized, and the skull was exposed to excise and weigh the brain. The cerebrum was homogenized in phosphate buffer and centrifuged at 3000 rpm for 15 minutes. The supernatant was collected for biochemical assays. Neocortical homogenates were prepared and analyzed using biomarkers such as superoxide dismutase (SOD), catalase, and interleukin-1β. Transcardial perfusion fixation was performed for tissues for histological evaluation using 10% neutral-buffered formalin. The fixed cerebrum was processed using Hematoxylin and Eosin (H&E) staining[19] as modified by Fischer *et al*. [20] and cresyl fast violet staining[21].

Photomicrography

All histological sections, including both H&E- and CFV-stained preparations, were examined and photomicrographed using a binocular compound light microscope (AmScope Microscopy, USA) equipped with a calibrated digital camera attachment.

Statistical analysis

All quantitative data were expressed as the mean ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism software, version 7.0 (GraphPad Software Inc., San Diego, California, USA). Group differences were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc multiple comparison test to determine the statistical significance of pairwise differences between all experimental groups. The threshold for statistical significance was set at $p < 0.05$ for all comparisons. Results were presented graphically as bar charts with error bars representing the SEM, generated using GraphPad Prism version 7.0. All analyses were performed by an investigator blinded to the group assignments of the animals.

RESULTS

Whole brain weight observation

There was a statistically significant overall difference in whole brain weights among the experimental groups. PHZ administration alone resulted in a significant reduction in whole brain weight compared to the Control group ($p < 0.05$; Figure 1). However, treatment with PHZ + KV resulted in increased whole brain weights compared with the PHZ-induced pups, though not statistically significant (Control: $p > 0.05$; PHZ-treated group: $p > 0.05$).

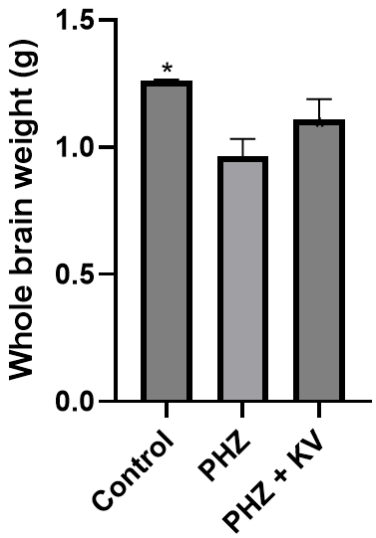

Figure 1. Whole brain weights of pups across experimental groups: PHZ: phenylhydrazine; KV: kolaviron; PHZ+KV: phenylhydrazine and lactational kolaviron. *$p < 0.05$ Control vs. PHZ

Histological observation of the neocortex

The cerebral neocortex of the control group revealed well-preserved tissue architecture with uniform eosinophilic neuropil background (Figure 2a). Neurons were evenly distributed with distinct cell bodies, visible nuclei showing clear nuclear membranes, and prominent nucleoli. The cortical layers maintained normal laminar organization. No evidence of pyknosis, vacuolation, or inflammatory

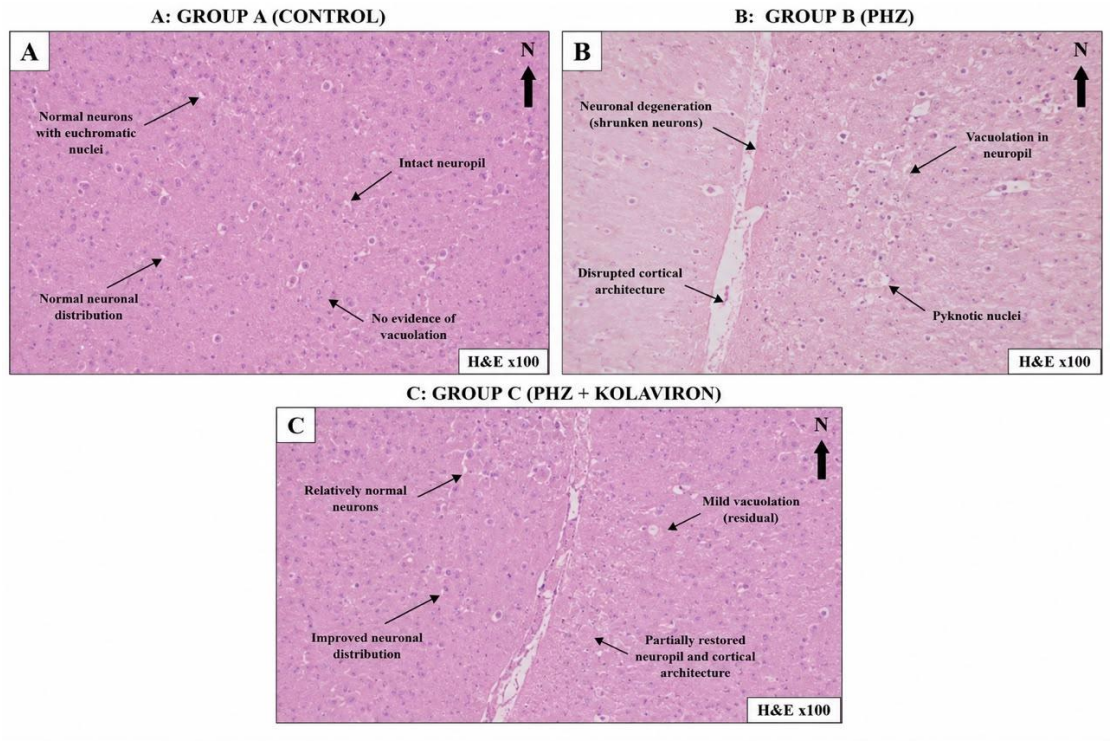

Figure 2: Photomicrographs of Hematoxylin and Eosin (H&E)-stained sections of the neocortex of pups showing the histological alterations in the Control, PHZ-treated, and PHZ + Kolaviron-treated groups.

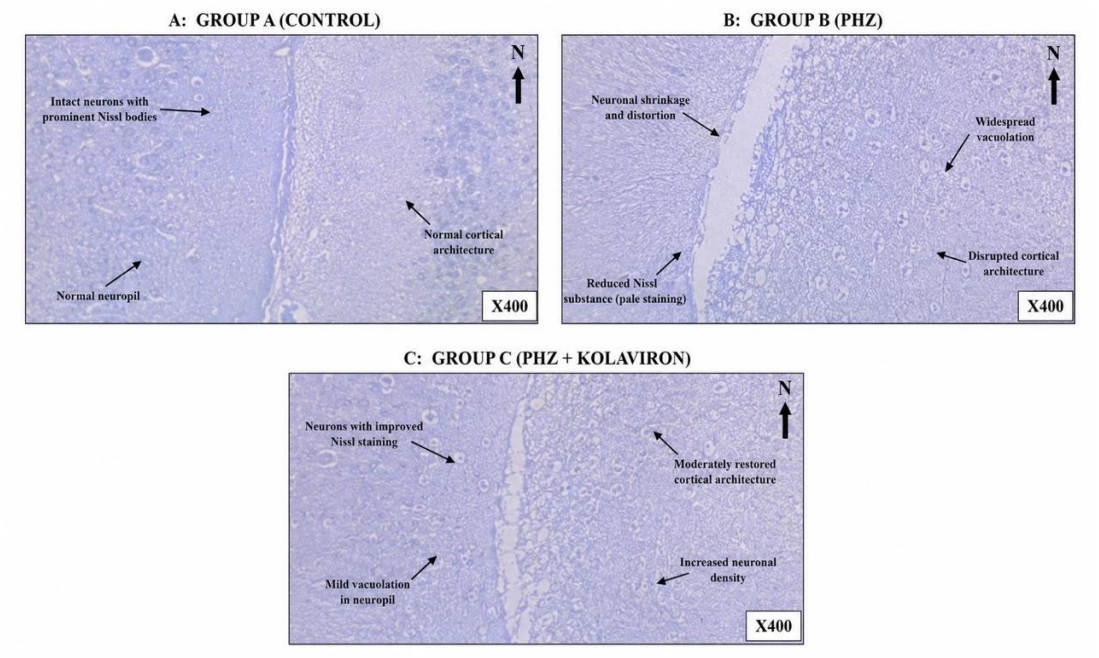

Figure 3: Photomicrographs of Cresyl Fast Violet (CFV)-stained sections of the neocortex showing the histoarchitectural changes and Nissl bodies distribution in the Control, PHZ-treated, and PHZ + Kolaviron-treated groups.

infiltration was observed. Blood vessels appeared normal with intact walls and no perivascular edema, consistent with normal neocortical histology. However, the cerebral neocortex of the PHZ-treated group showed significant histopathological alterations (Figure 2b). There was marked disruption of cortical architecture with evidence of tissue fragmentation, as demonstrated by the prominent tissue cleft observed across the section. Neuronal density was visibly reduced with widespread cell loss and neuropil rarefaction. Pyknotic nuclei and shrunken cell bodies were noted, indicative of ongoing neurodegeneration. Micro-vacuolation of the neuropil was observed, consistent with tissue edema and cellular swelling secondary to oxidative injury. The overall eosinophilic staining was pale and disorganized, reflecting loss of cellular integrity. The cerebral neocortex of the PHZ + Kolaviron group demonstrated partial restoration of neocortical architecture compared to Group B (Figure 2c). Neuronal density was notably higher, with clusters of neurons retaining recognizable cell morphology visible particularly in the upper cortical regions. Nuclear staining with hematoxylin was more pronounced compared to the PHZ group, suggesting improved nuclear integrity. However, some residual tissue disorganization and vascular irregularities persisted, including areas of mild neuropil disruption.

Demonstration of Nissl staining of the neocortex of pups

The neocortex of the control group displayed normal neuronal morphology with well-preserved cytoarchitecture (Figure 3a). The neurons exhibited regular arrangement with distinct cellular outlines and intact nuclei. Nissl staining was strong and well-defined, indicating preserved Nissl substance and normal neuronal metabolic activity. The neuronal morphology in the PHZ group was significantly altered, with evidence of cellular shrinkage, distortion, and structural degeneration (Figure 3b). Pyknosis and vacuolation were prominent, characterized by numerous dark condensed nuclei and widespread microcystic spaces consistent with tissue edema. Nissl staining was markedly reduced and pale, indicating loss of Nissl substance and impaired neuronal integrity. The neocortex of the PHZ-exposed rats that were pre-treated with kolaviron showed moderate to relatively high neuronal density compared to Group B (Figure 3c). Neocortical architecture showed partial restoration with improved laminar organization and reduced structural fragmentation. Pyknosis and vacuolation were mild to moderate, with residual but reduced microcystic changes within the neuropil, and Nissl staining intensity was moderate.

Lactational kolaviron improved neocortical antioxidant activity

There was an overall difference in catalase concentrations among the experimental groups. PHZ administration alone resulted in a marked decrease in the level of catalase enzyme compared with the other groups (Figure 4). However, treatment with PHZ + KV led to an increase in catalase levels when compared to the PHZ-treated group, though not up to the level of the Control baseline.

The activity of superoxide dismutase (SOD) enzyme was lowest in the neocortex of pups induced with PHZ compared with other groups. The pups in the Control group undoubtedly had the highest activity of SOD (Figure 5). However, the PHZ pups that received prior lactational kolaviron treatment had a slightly higher level of SOD compared with the PHZ-only group, though still lower than the control group.

Lactational kolaviron prevented neuroinflammation associated with PHZ neurotoxicity

Using IL-1β as an inflammatory marker to quantify the degree of neuroinflammation in the neocortex, the study observed a marked elevation of the marker in the brain of pups induced with PHZ, above the Control and the intervention group levels (Figure 6). However, retreatment with kolaviron significantly reduced IL-1β levels when compared to the PHZ-treated group and the control. The level of IL-1β in the kolaviron group was lower than the Control.

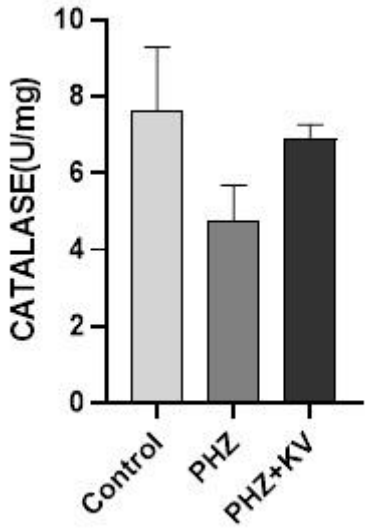

Figure. 4: Catalase concentrations across experimental groups. PHZ: phenylhydrazine; KV: kolaviron; PHZ+KV: phenylhydrazine and kolaviron.

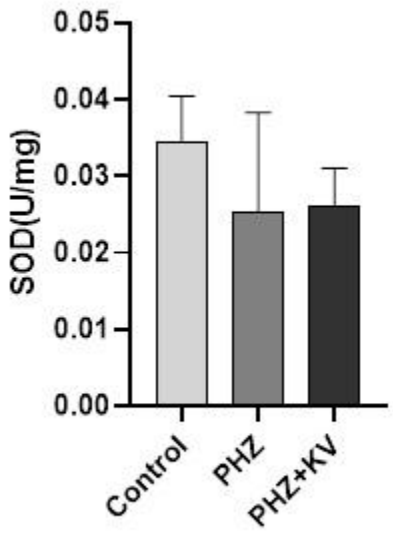

Figure 5: Superoxide dismutase (SOD) concentrations across experimental groups: PHZ: phenylhydrazine; KV: kolaviron; PHZ+KV: co-exposure to phenylhydrazine and kolaviron.

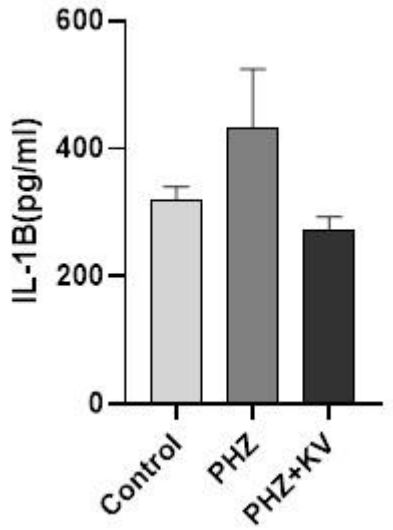

Figure 6: Interleukin 1-beta (IL-1β) concentrations across experimental groups: PHZ: phenylhydrazine; KV: kolaviron; PHZ+KV: phenylhydrazine and kolaviron

DISCUSSION

The present study employed phenylhydrazine (PHZ) as a model of hemolytic-induced neocortex oxidative damage. This model bears direct and clinically significant relevance to neonatal hyperbilirubinemia, one of the most common and potentially devastating conditions of the neonatal period, particularly within sub-Saharan Africa[22, 23]. We investigated the effects of kolaviron on phenylhydrazine (PHZ)-induced oxidative stress and neuroinflammation in the neocortex of Wistar rats using biochemical analyses and histological evaluation. PHZ administration produced biochemical alterations consistent with oxidative stress and mild cerebral histopathological changes, whereas kolaviron treatment ameliorated many of these changes.

The histological findings demonstrated partial restoration of neocortical architecture, increased neuronal density, and improved nuclear integrity in the treatment group compared to the PHZ-only group. Although some residual disorganization and vascular irregularities persisted in the treatment group, the overall improvement suggests that Kolaviron exerts a neuroprotective effect, likely through its antioxidant properties. Kolaviron, a biflavonoid complex, has been extensively characterized for its potent antioxidant, anti-inflammatory, and anti-apoptotic properties[17]. The histological improvements observed in the KV+PHZ group in this study are consistent with these established pharmacological actions[24]. Neocortical sections from the control group demonstrated intense cresyl fast violet staining with well-defined layers, characteristic of healthy cerebral tissue, and indicate normal neuronal metabolism and protein synthesis. Conversely, the PHZ-treated group demonstrated reduced Nissl staining intensity, mild chromatolysis, and decreased cellular density within the granular layer. The reduction in Nissl substance suggests impairment of neuronal protein synthesis secondary to oxidative injury. Chromatolysis is a well-established response of neurons to cellular stress and is associated with dispersion or loss of rough endoplasmic reticulum following oxidative damage[25].

The neuronal alterations observed following PHZ administration are consistent with the known mechanism of PHZ toxicity. During its metabolism, PHZ generates reactive oxygen species that promote lipid peroxidation, mitochondrial dysfunction, oxidation of intracellular proteins, and DNA damage[10, 12]. These oxidative processes compromise neuronal homeostasis and interfere with protein synthesis, resulting in depletion of Nissl substance and subsequent neuronal degeneration. The mild chromatolysis observed in the present study therefore provides additional histological evidence that PHZ successfully induced oxidative stress within neocortical tissue.

However, treatment with kolaviron markedly improved the Nissl staining characteristics of the neocortex. The treated animals exhibited increased staining intensity, better preservation of neurons, restoration of the normal laminar organization of the neocortex, and improved cellular density. The histochemical analysis from this study provided more specific evidence of neuronal survival by demonstrating maintenance of Nissl substance within

neuronal cells. The concordance between the histological/histochemical findings together with the biochemical evidence of improved antioxidant status strongly supports the conclusion that pre-treatment with kolaviron effectively attenuated PHZ-induced oxidative damage in the neocortex.

Effect of kolaviron on antioxidant parameters

We have previously demonstrated the anti-oxidative and cytoprotective activities of kolaviron in the brain regions[26]. Phenylhydrazine is known to generate reactive oxygen species through oxidative metabolism, resulting in erythrocyte membrane oxidation, lipid peroxidation, and increased production of hydrogen peroxide[10, 12]. Reduction in the activity of antioxidant enzymes under these conditions reflects depletion of endogenous antioxidant defense mechanisms in response to oxidative damage.

The antioxidant ability of Kolaviron is well-researched and documented in the literature. There are several pharmacological effects of KV documented in the literature which are associated with its antioxidant capacity and ability to reduce oxidative stress[13]. Our findings in this study are consistent with the literature in which KV elevated antioxidant parameters (catalase and SOD) in the neocortex of experimental animals.

In the present study, PHZ administration significantly reduced catalase and SOD activities compared with the control group, whereas kolaviron treatment restored them toward normal values. Oxidative stress is frequently associated with depletion of antioxidant enzymes during prolonged injury; a decrease in catalase and SOD activities, as observed in our study, further corroborates this. These findings are also comparable to those reported by Ajibade *et al.*[27], who demonstrated that kolaviron normalized antioxidant enzyme-SOD activities in potassium dichromate-induced oxidative injury in rats. Similarly, Ishola *et al.*[28] reported restoration of antioxidant enzyme balance following kolaviron treatment in scopolamine-induced neurotoxicity in rats. Similar observations were made by Adedara *et al.*[29], who attributed enhanced antioxidant enzyme activity to the redox-modulating effects of kolaviron.

Effect of kolaviron on neuroinflammation

In the present study, Kolaviron treatment abrogated neuroinflammation, demonstrating its potential as a neuroprotective agent. Kolaviron has been shown to possess potent anti-inflammatory properties through various mechanisms. Abarikwu[30] demonstrated that kolaviron inhibited the production of pro-inflammatory cytokines such as interleukin-1 beta (IL-1β) in lipopolysaccharide (LPS)-stimulated macrophages. Similarly, Farombi *et al.* [31] reported that KV significantly reduced the concentration of IL-1β following an increase in a striatal redo-inflammation associated with the rotenone model of Parkinson's disease. These findings strengthen the results in our study where co-treatment with KV reduced the high concentration of IL-1β observed in the PHZ-treated group.

The neuroprotective effects of kolaviron observed in the present study (antioxidative and anti-inflammatory) are likely attributable to its potent antioxidant properties. Kolaviron is a biflavonoid complex capable of scavenging reactive oxygen species, inhibiting lipid peroxidation, preserving mitochondrial integrity, and modulating endogenous antioxidant enzyme systems[17]. Through reduction in oxidative stress, kolaviron preserves the structural integrity of the rough endoplasmic reticulum and ribosomes, thereby preventing chromatolysis and maintaining normal neuronal protein synthesis. This mechanism is consistent with the observed increase in catalase and SOD activities in the kolaviron-treated animals.

CONCLUSION

This study provides substantial histological and biochemical evidence that kolaviron attenuates PHZ-induced neocortical oxidative stress and neuroinflammation in a neonatal rodent model that closely recapitulates the pathophysiology of neonatal hyperbilirubinemia and kernicterus. The preservation of neocortical cytoarchitecture and neuronal integrity in kolaviron-pretreated rats suggests that this locally available bioflavonoid may represent a promising candidate for further investigation as a neuroprotective strategy in the management of neonatal jaundice-associated brain injury.

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