SAR Journal of Psychiatry and Neuroscience

Abbreviated Key Title: *SAR J Psychiatry Neurosci*
Home page: https://sarpublication.com/journal/sarjpn/home
DOI: 10.36346/sarjpn.2024.v05i02.001

ISSN 2707-7764 (P)
ISSN 2709-6939 (O)

Original Research Article

Nutritional Approach to Ameliorate Demyelinating Changes Expressed in Spinal Olig2 Immunoreactivity in a Konzo Disease Rat Model

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Article History: | Received: 11.04.2024 | Accepted: 24.05.2024 | Published: 01.06.2024 |

Abstract: A nutritional approach to ameliorate demyelinating changes expressed in spinal Olig2 immunoreactivity in a Konzo disease rat model was investigated. 30 adult female Wistar rats weighing 200-250g were assigned to 4 groups. Group 1 (Control, n=5) was fed on animal pellets, whereas Group 2 (Protein control, n=5) was provided protein food. Bitter cassava flour was provided to Group 3 (Konzo induced, n=15). Protein and bitter cassava flour were supplied to Group 4 (protein treatment group, n=5). Body weight was taken weekly. The C3-C5 spinal regions were harvested through transcardiac perfusion for histological and immunohistochemical staining. Image J was used to quantify the cells in the spinal cord. Body weight showed a significant reduction in body weight in, Cassava at $p<0.01$, and Cassava + Protein group at $p<0.05$ when compared to the control and protein control groups. Examination of the neurons with cresyl violet showed a significant increase in the percentage of unhealthy neuron population in the Konzo-induced group [$p<0.001$] compared to the control and protein control groups. The rats induced with Konzo showed a significant decrease in Olig2 immunoreactive cells compared to control, protein control, and Cassava + protein groups, at $p<0.05$. Similarly, Olig2 immunoreactive was substantially increased in the Cassava + Protein group compared to the Cassava group [$p<0.05$]. This shows that an adequate diet, along with a protein supplement, can counteract the demyelinating and neurodegenerative effects of cyanogenic cassava consumption implicated in Konzo disease.

Keywords: Bitter cassava, Cyanogenic, Konzo, Spinal olig2, Demyelination, Remyelination.

INTRODUCTION

Konzo is a paralytic disease caused by the ingestion of improperly processed cassava root (*Manihot esculenta*) [1-5]. Although its incidence has declined, konzo still affects several populations in sub-Saharan Africa [6]. Konzo is characterized by demyelination of the spinal cord and motor neurons, which leads to a spastic gait, lower limb weakness, and upper motor neuron sign [7-10]. The onset of konzo usually occurs in children, teenagers, and women of childbearing age, and there is no effective treatment for this disease [11].

Demyelination is a hallmark of many neurological disorders, including multiple sclerosis (MS) and neuromyelitis optica (NMO) [12-16]. The oligodendrocyte transcription factor 2 (Olig2) plays a crucial role in the formation and maintenance of myelin in the central nervous system (CNS). Olig2 is expressed in oligodendrocytes, which are responsible for producing myelin sheaths that insulate axons and allow rapid and efficient transmission of nerve impulses [17]. Olig2 is also expressed in other cell types within the CNS, such as interneurons, astrocytes, and ependymal cells [18].

Olig2 is a critical transcription factor involved in the formation and maintenance of myelin in the CNS. It controls oligodendrocyte development, differentiation, survival, and myelin-related gene expression, thereby playing a vital role in proper myelination of CNS neurons [19-21].

Previous studies have shown that the nutritional quality of cassava may play a role in the development of Konzo [22-26]. Cassava is a starchy root crop that is a

dietary staple in many parts of Africa, Asia, and South America. Cassava contains cyanogenic glycosides that release hydrogen cyanide when ingested and are toxic to humans and animals. The processing of cassava involves soaking, grating, and fermenting the root to remove the cyanogenic compounds. However, the traditional cassava processing methods employed in many African countries are inadequate, resulting in residual cyanide in the final product [27].

Recent studies have shown that the consumption of foods rich in certain nutrients, such as vitamin D, vitamin B12, and omega-3 fatty acids, may have a positive impact on Olig2 expression and myelination in the CNS [28, 29]. Since konzo is characterized by demyelination and Olig2 immunoreactivity, it is possible that a nutritional approach may be effective in ameliorating the symptoms of konzo by improving myelin formation and Olig2 expression.

The present study aims to investigate the efficacy of a nutritional approach in ameliorating demyelination and Olig2 expression associated with Konzo disease in a rat model. The study examined the effects of a protein-rich diet on Olig2 expression and spinal cord demyelination in a rat model of konzo.

MATERIALS AND METHOD

Procurement of Animals

Thirty female Wistar rats weighing between 200 and 250 grams were obtained from the Department of Pharmacology's animal home University of Port Harcourt. All of the animals were kept in their own regular metal cages. The animals were acclimatized for three (3) weeks; kept in a conventional laboratory environment with 12-hour light/dark cycles and given unrestricted access to distilled water and a commercial pellet diet. The temperature in the room was kept under control for the animals' care. The cage's floor was cushioned by a layer of coarse sawdust that had been sprayed on top of carpet remnants. Daily changes were made to the coarse sawdust to remove waste droppings and maintain hygienic standards.

Plant Collection and Identification

The bitter cassava roots were collected from the Ministry of Agriculture, Agricultural Development Programme, Rivers State, while Soybeans and brown beans were purchased from the local market within the University environment and were identified in the Department of Plant Science and Biotechnology in the Faculty of Agriculture, University of Port Harcourt.

Bitter Cassava Root Processing

The farm's fresh cassava roots were uprooted. The cortex was scraped using a cutter shortly after harvesting to reveal the whitish interior layer. The cassava roots were then chopped into tiny pieces, similar to pommes frites, and sun-dried for three days as described by Enefa *et al.*, [30] and David *et al.*, [31].

Processing of Protein Food Supplement

For this study, a protein dietary supplement consisting of a mixture of Soybeans and brown beans was served. Brown beans and Soybeans were grinded together in a grinding machine into a powdered form that was offered as a protein diet nutrient to the "protein group" and "cassava + protein treatment group" animals, respectively.

Induction of Konzo

The method of Konzo induction is as described by David *et al*., [31] and David *et al.*, [32] with slight modification. Bitter cassava chow was made from pommes frites cassava that was grinded into a powdered form using a grinding machine and fed to the animals used for the experiment for the period of 5 weeks.

Experimental Design

The experimental design as described by Enefa *et al*., [30] and David *et al.*, [31] with slight modification was used in this study. The experimental animals were randomly divided into four groups: group 1 served as positive control and were identified as Normal control (NC) and they were fed with rat feed and water *ad libitum* only for 5 weeks. Group 2 served also as positive control and were identified as Protein control (PC) and they were fed Soybeans and brown beans and water only for 5 weeks. Group 3 served as the Konzo-induced control and was induced with Konzo disease by allowing them to feed from inappropriately processed cassava for 5 weeks, while group 4 was induced with Konzo disease by allowing them to feed from inappropriately processed cassava for 3 weeks and then rehabilitated with Soybeans and brown beans for 2 weeks. This experimental protocol is presented in the table below;

Table 1: Showing grouped experimental animal

Group	Description	No. of Rats	Treatment Protocol
Group 1	Normal control (NC)	5	Were fed on pellet animal feed and water for 5 weeks
Group 2	Protein control (PC)	5	Were fed with a protein food (Soybeans and brown beans) and water for 5 weeks
Group 3	Konzo induced	15	Were fed with bitter cassava flour for 5 weeks
Group 4	Rehabilitation/protein treated	5	Were fed with bitter cassava flour for 3 weeks (Konzo illness induction period) and then with Soybeans and brown beans for two weeks.

Tissue Collection and Histological Examination

In a desiccator, the animals were sedated with 10% chloroform inhalation before transcardiac perfusion was done [31]. The abdominal and thoracic regions were exposed through incisions. After the rat was completely exposed, 10 percent of normal saline solutions were used to do transcardiac perfusion. A 10% solution of formaldehyde was injected into the heart's ventricle. The spinal cord was taken out and put in a 10% formal saline solution to fix them. Levels C3 to C5 of the spinal cord were cut into four microns. The tissue was preserved in formalin for 48 hours.

Cresyl Staining

Sectioned spinal cord tissues (5um) were stained with cresyl violet to detect the physiological state of the nerve cells as described by Yilmazer-Hanke *et al*., [33]. These were then viewed with a Bright Field Microscope.

Olig 2 Staining

ImmPRESS® HRP Horse Anti-Rabbit IgG Polymer Detection Kit, (Peroxidase), a Polymer Reagent, manufactured by Vector Laboratories headquartered in Newark, California, United States, was purchased and was incubated on sections for 30 minutes. The colour was created using a DAB Peroxidase (HRP) Substrate Kit from Vector® Labs in the USA. Olig2 immunoreactivity was measured by counting positive immunoreactive cells, as previously described by Ijomone and Nwoha [34] and Akingbade *et al*., [35]. Using the ImmunoRatio plugin for Image J, which divides and calculates the percentage of DAB (positive immunoreactivity),

Method of Data Collection

After the histological (H&E and Cresyl fast violet) and immunohistochemical (Olig2) staining process, a quantitative assessment of the percentage number of surviving neurons was conducted. The assessment was based on the selection of four sections from each group viewed using a Light Microscope (Leica® DM5000B) under 400x magnification. The cells were counted serologically for surviving neurons using Image J software. Healthy neurons were statistically analyzed.

Statistical Analysis

The data was examined using Graph Pad Prism (version 8.0) and Microsoft Excel (2016 edition). Values were presented in descriptive statistics as Mean ± Standard Error of Mean (SEM). A Tukey post-hoc multiple comparison tests was used after a one-way analysis of variance (ANOVA) to assess for significant differences between the groups. A result of *p<0.05, **p<0.01, and ***p<0.001 was considered significant.

Ethical Consideration

The ethical use of animals in research was approved by the Research Ethics Committee of the University of Port Harcourt and issued the ethical approval number UPH/CEREMAD/REC/MM87/037. The rats had free access to water and feed and the weights of the animals were recorded on a weekly basis using a digital electronic weighing scale (Digital Electronic Laboratory Scale -500G x 0.01G - SF-400C - White). The study took place over a five-week period.

RESULT

General Observation

The ameliorated demyelination in spinal C3-C5 corresponds to the improved motor functions observed in rats co-fed with a protein diet. Overall, this study has shown that the protein food supplements used in this study have neuroprotective effects on the motor cortex and C3-C5 spinal regions. Also, the presence of Flavonoids, Isoflavones, and Tannin in the Soybeans and brown beans was able to ameliorate the neurotoxic effects of bitter cassava in the spinal C3-C5 and restore perturbed motor functions.

Effect of Cassava and Protein Diets on Body Weight

One-way ANOVA showed a significant reduction in mean body weight (kg) in, Cassava [178.6±10.43], and Cassava + Protein [187.0±7.437] groups compared to the Control [244.0±3.895; p<0.001] and Protein [220.9±6.585; p<0.01] groups.

Further, there was a steady rise in body weight in the control animals and protein-fed across the five weeks of administration. In contrast, there was an observed decline in the average body weight of animals in the Cassava and Cassava + Protein groups. Interestingly, mean body weight rose in the Cassava + Protein group in the last week of administration (see Figures 1 and 2).

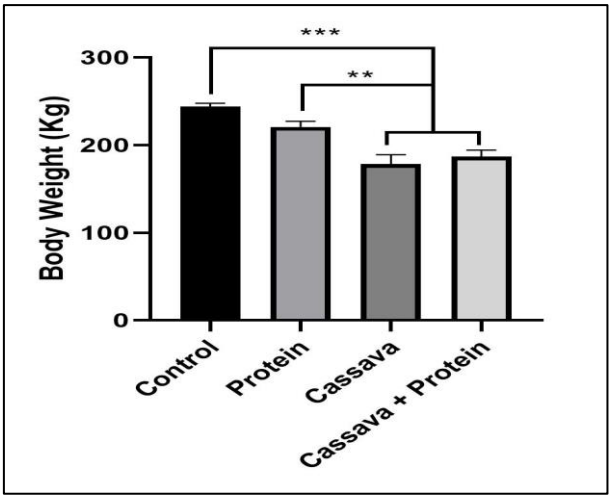

Figure 1: Shows mean weight of control and treated groups. Bars are Mean ± SEM of N=5/group. Each column represents mean ± S.E.M. Data was examined. Using one-way analysis variance followed by Tukey's posthoc-test. **p<0.01, ***p<0.001

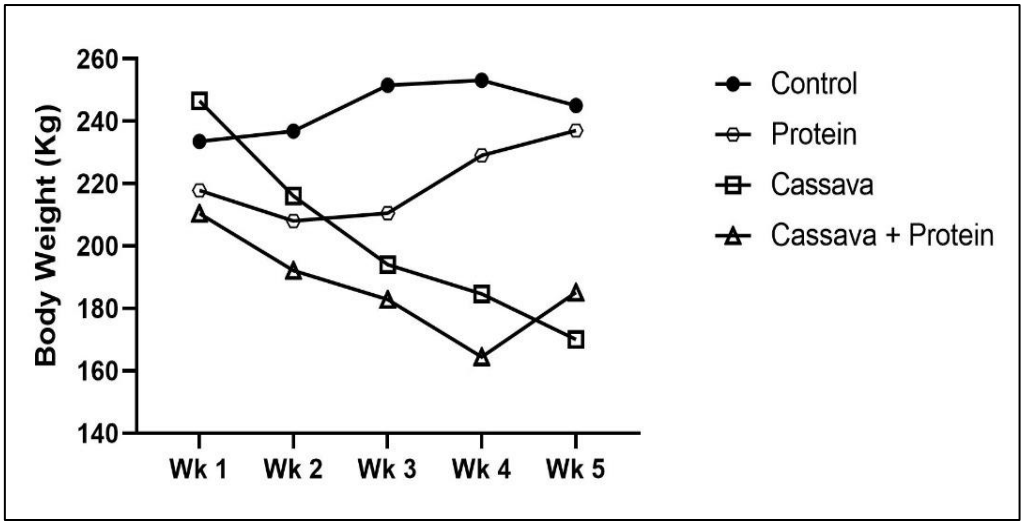

Figure 2: Shows the progression of average weight of control and treated groups during the five weeks of administration. Control groups a steady increase in body weight, in contrast, other groups exhibit gradual decrease of body weight. In particular, rats in the cassava + protein group gained significant weight in the last week of administration. N=5/group

Cresyl Violet Demonstration in the Spinal Cord Following Exposure to Cassava and/or Protein Diets

Cresyl Violet was used to demonstrate Nissl substance. It differentiates between neurons and non-neuronal cell populations. Unhealthy neurons are smaller with denser nuclei, less obvious nucleoli, and a cell body rich in rough endoplasmic reticulum. Healthy neurons are larger with a well-defined nucleolus and a cell body rich in rough endoplasmic reticulum.

Neuronal (healthy and unhealthy) count was performed on both horns of the gray mater. One way ANOVA showed significant increase in percentage of unhealthy neuron population in Cassava [57.73±2.221; $p<0.001$] and Cassava + Protein [46.75±3.684; $p<0.001$] groups compared to the Control [23.47±2.496] and Protein [29.38±2.755] groups. In addition, compared to the Cassava group [57.73±2.221], there is reduced percentage of unhealthy neurons in the Cassava + Protein [46.75±3.684; $p<0.01$] group (see Figures 3, 4 and 5).

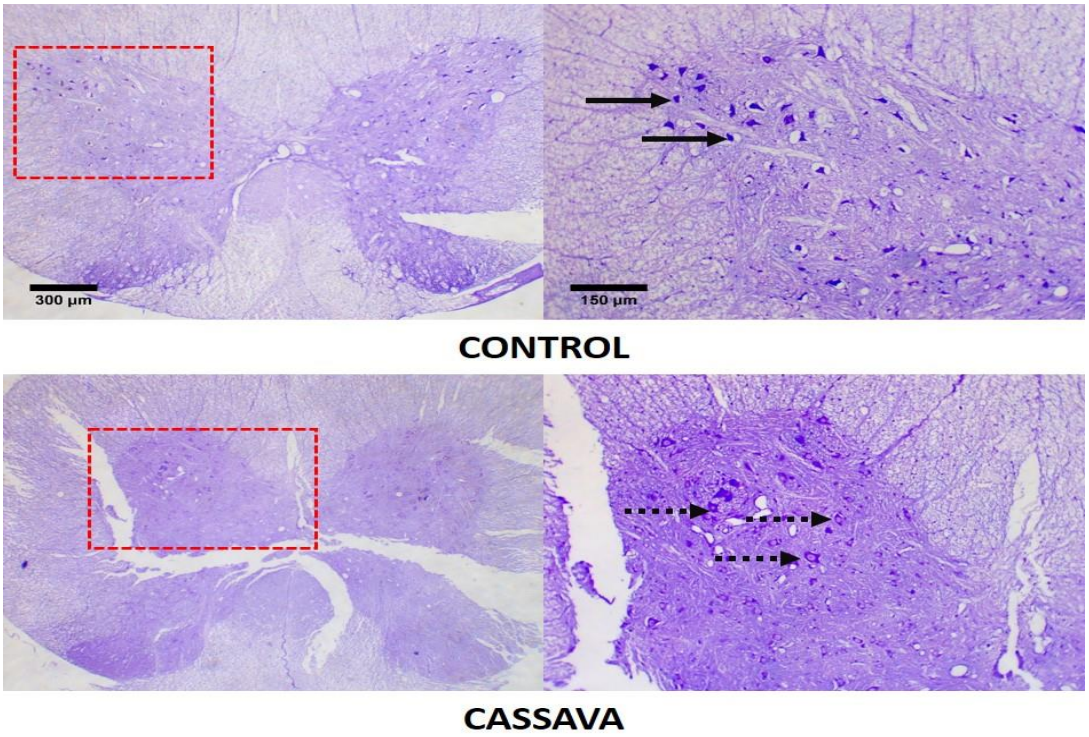

Figure 3: Photomicrograph of Nissl stain in the cervical spinal cord region of experimental groups. Magnification = x40 and x100. Black arrows – intact neurons; dashed arrows – unhealthy neurons

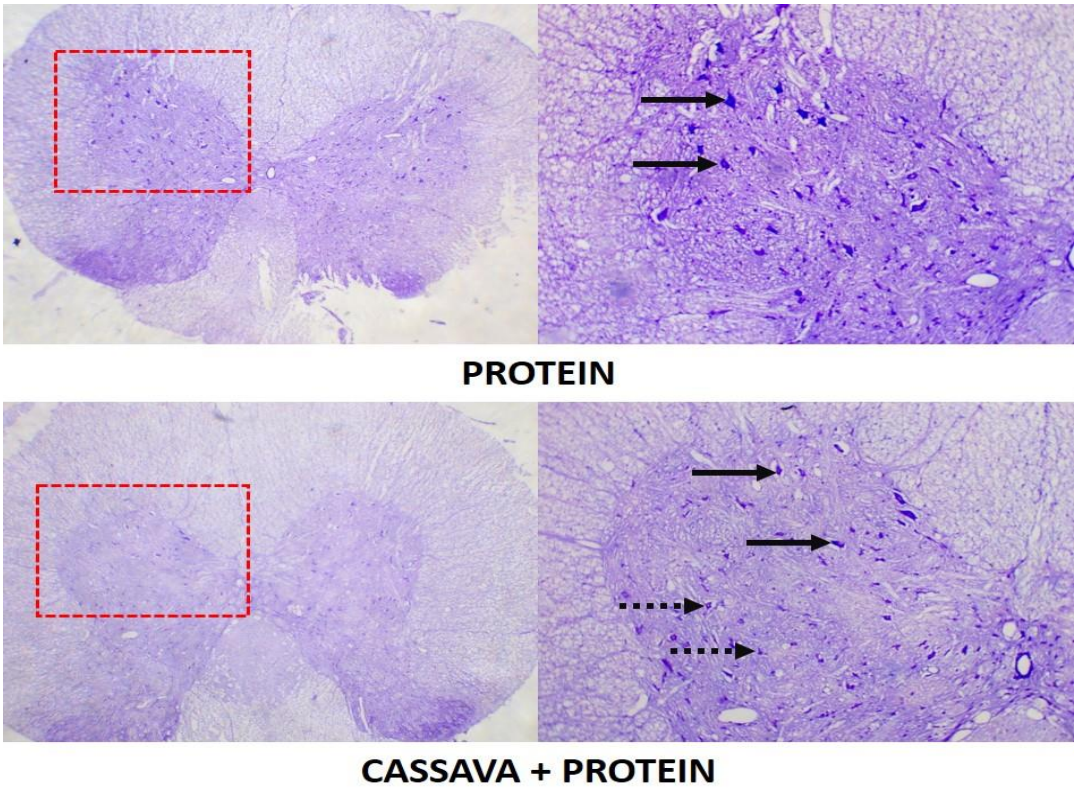

Figure 4: Photomicrograph of Nissl stain in the cervical spinal cord region of experimental groups. Magnification = x40 and x100. Black arrows – intact neurons; dashed arrows – unhealthy neurons

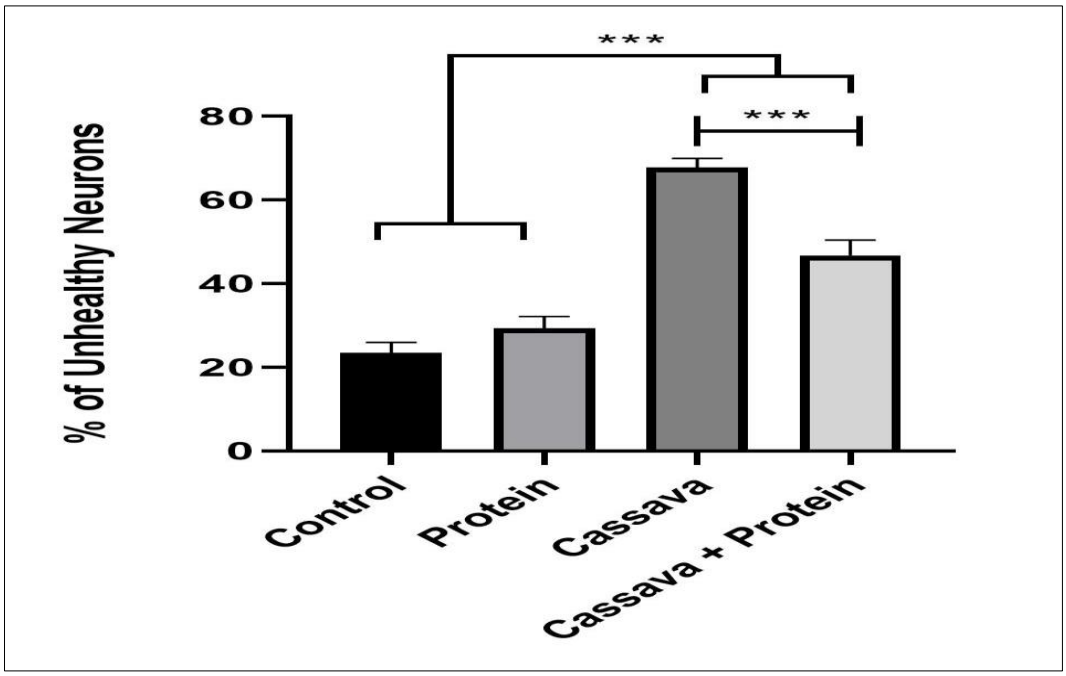

Figure 5: Shows the percentage of unhealthy neurons in the spinal cord of control and treated groups. Cassava-only treated rats showed significant increase in the number of unhealthy neurons compared to control and protein groups. Co-administration of protein with cassava significantly reduced the number of unhealthy neurons in the grey matter of the spinal cord

Each column represents mean ± S.E.M. N=5/group. Data were analyzed using one-way analysis of variance followed by Tukey's post-test. ***p <0.001.

Olig2 in the Spinal Cord

The immunohistochemical localization of Olig2 shows distinct Olig2 expressing cells interspersed within the gray matter of the spinal cord. The Cassava group

show less number of Olig2 immunoreactive cells than Control, Protein, and Cassava + Protein groups.

One way ANOVA showed significant decrease in the Olig2 immunoreactivity in Cassava group [26.43±4.815] compared to the Control [44.00±1.238; p<0.05], Protein [61.33±4.507; p<0.001] and Cassava + Protein [42.71±4.529; p<0.05] groups. In addition, Protein group [61.33±4.507; p<0.05] showed increased Olig2 expressing cells compared to the Control group [44.00±1.238; p<0.05]. Similarly, Olig2 immunoreactive was substantially increased in the Cassava + Protein [42.71±4.529] group compared to the Cassava group [26.43±4.815; p<0.05] (see Figure 6 and 7).

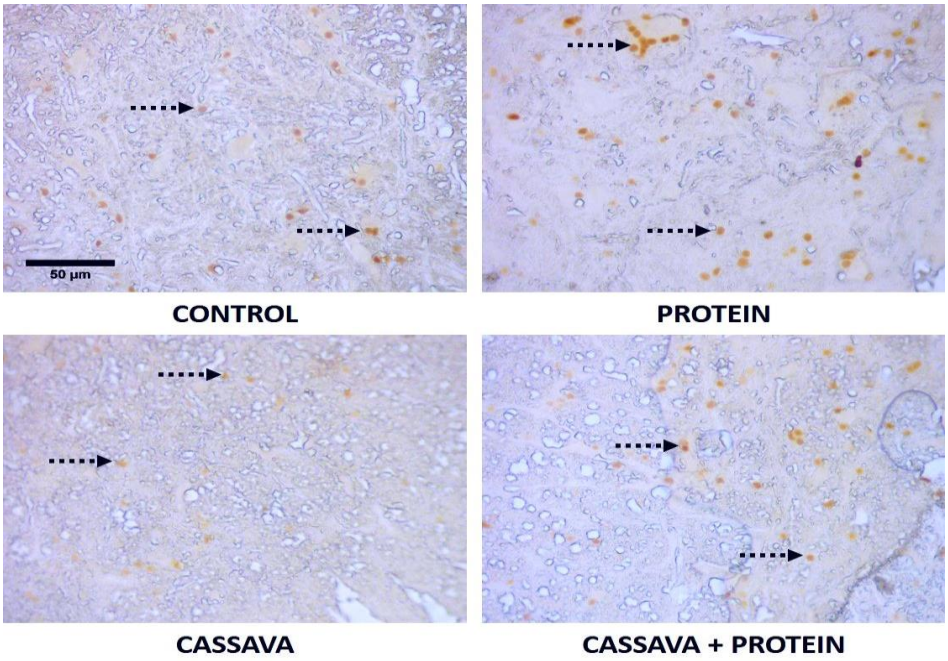

Figure 6: Immunohistochemical changes in the spinal cord of experimental groups. Oilg2; Magnification = x400. Dashed arrows – Olig2 expressing cells

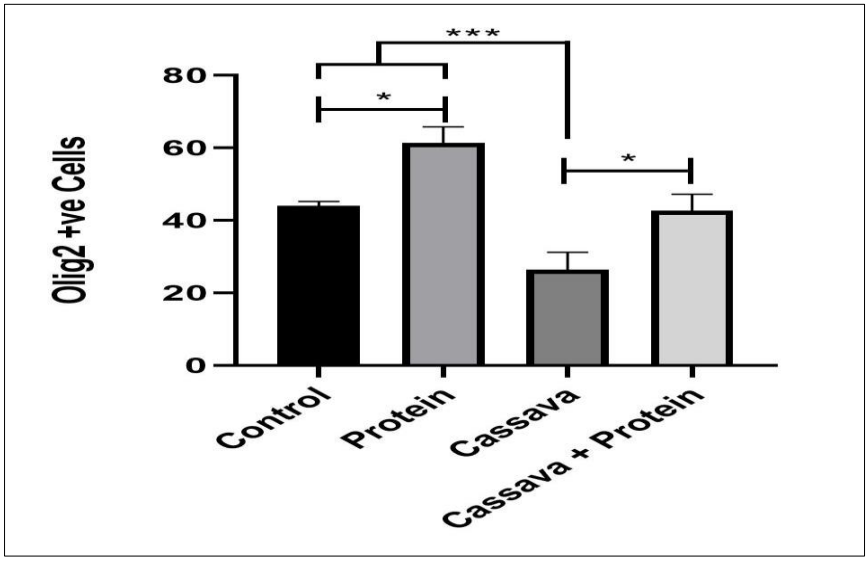

Figure 7: Shows the number of Olig2 expressing cells in control and treated groups. Each column represents mean ± S.E.M. N=5/group. Data was examined using one-way analysis of variance followed by Tukey's post-test. *p <0.05, ***p <0.001

DISCUSSION

Spastic paresis is a defining feature of the neurological condition termed Konzo, which affects just certain upper motor neurons [30, 36]. The typical warning signs of prolonged exposure to cyanide from improperly processed cassava roots include growth retardation, weight loss, and neurological diseases brought on by tissue damage in the central nervous system [31].

This study demonstrates that oral ingestion of inappropriately processed bitter cassava flour led to a significant reduction in body weight that can be rescued by a protein-based diet. It was observed that the control and protein group animals had steadily gained weight weekly. There was a drastic downward trend in the body weight of cassava-fed animals and cassava + protein-fed animals. However, the protein diet rescued body weight in the last week of the experiment for animals that were co-fed cassava with protein supplements. These observations agree with previous studies from other authors that have also shown significant weight loss in animals injected with cyanogenic glycoside: linamarin [37] and in laboratory Wistar rats feed with cassava root chips and cassava flour [30, 38, 39].

Damage to the spinal cord is hypothesized in Konzo [40], but no pathological data are available. Therefore, this study utilized cresyl violet stain to distinguish between healthy and unhealthy neurons, which were identified by the Nissl accumulation [41]. The cresyl violet stain revealed a greater number of unhealthy neurons, characterized by intense purple staining of the "unhealthy" nuclei. Prolonged cassava exposure resulted in an increased number of damaged motor neurons in the C3-C5 of the spinal cord.

In addition, the spinal cord is the site for several long-running myelinated nerve fibers. Proper myelination of these nerve fibers is required for the proper functioning of the nerve supplying the forelimbs [42, 43]. Therefore, this study utilized immunohistochemistry to demonstrate Olig2 expression. Olig2 protein is a marker for oligodendrocytes, which are the glial cells responsible for the proper myelination of CNS neurons, including the spinal cord [44, 45]. Upon data analysis, the study revealed a significant reduction of Olig2-expressing cells in cassava-exposed rats which is an indication of demyelination in the spinal neurons [46]. The nerve fibers in the C3-C5 region of the spinal cord are important for the innervation of forelimb muscles [43]. This supports the data reported for the forelimb grip test by Chijioke *et al*., [47] where cassava reduced both grip strength and latency fall. This suggests that the motor deficit is as a result of improper C3-C5 myelination. Nonetheless, there is increased Olig2 expressing cells in rats co-fed with protein diets compared to those with only cassava diet indicative of increased myelination.

CONCLUSION

Inadequately processed bitter cassava is toxic and has neurotoxic effects on the central nervous system (CNS), particularly on the motor cortex and spinal C3-C5 areas, as demonstrated in this study. The findings from this study suggest the potential of the nutritional approach in promoting remyelination and attenuating the negative effects of Konzo disease. This research contributes to the understanding of the pathophysiology of Konzo disease and offers a promising avenue for therapeutic interventions.

Acknowledgements: Appreciation goes to the research assistants who took part in the study.

Conflict of Interest: No conflict of interest exists.

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