

Model of Konzo Disease: Reviewing the Effect of Bitter Cassava Neurotoxicity on the Motor Neurons of Cassava-Induced Konzo Disease on Wistar Rats

Stella Enefa, Chikwuogwo W. Paul, Lekpa K. David*

Department of Anatomy, Faculty of Basic Medical Sciences, College of Health Sciences, University of Port Harcourt, Rivers State, Nigeria

DOI: [10.36348/sjm.2020.v05i11.005](https://doi.org/10.36348/sjm.2020.v05i11.005)

| Received: 05.10.2020 | Accepted: 21.10.2020 | Published: 28.11.2020

*Corresponding Author: Lekpa K. David

Abstract

Introduction: Cassava (*Manihot Esculenta*) is a staple food in tropical and subtropical regions in Africa, and is the main source of carbohydrate in these regions. Nevertheless, it contains cyanogenic glycosides metabolised to hydrogen cyanide, which has been shown by studies to affect the motor neurons of the central nervous system and causes neurodegenerative disease as konzo. However, the cassava-induced konzo disease and its neurotoxicity in rat model is yet to be explored. **Method:** 30 Adult female Wistar rats were assigned to 4 experimental groups (i) negative control n=5 (ii) positive control n=5, (iii) konzo-induced group n=15, (iv) protein-treated group n=5. The bitter cassava foods were taken by oral ingestion for a period of 5 weeks. Motor activity was evaluated using forelimb grip strength testing done weekly. **RESULTS:** There was significant difference in weight and forelimb grip strength between the negative control group and the konzo-induced group $p < 0.05$. Also, there was distortion of the pyramidal cells in the motor cortex and reduction of alpha cells in the ventral horn of the spinal cord which was not present in the protein treated group. **CONCLUSION:** Prolonged intake of insufficiently processed bitter cassava roots is associated with impaired motor coordination. Motor neurons are affected by neurological changes and distortion. These neurotoxicity effects of cassava can be ameliorated with consumption of cassava along with sulphur amino acid proteins and vitamins.

Keywords: Cassava; hydrogen cyanide; motor neurons; konzo, neurotoxicity, sulphur amino acid proteins.

Copyright © 2020 The Author(s): This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International License (CC BY-NC 4.0) which permits unrestricted use, distribution, and reproduction in any medium for non-commercial use provided the original author and source are credited.

INTRODUCTION

Konzo is a neurological disorder of selective upper motor neurons. It is characterized by a clinical spastic paresis (para/tetraparesis), which is non-progressive and permanent [1]. It is an irreversible paralytic disease associated with prolonged consumption of a monothrophic diet of mostly inappropriately processed bitter cassava roots [2]. The name "Konzo" was derived from the local Congolese dialect meaning 'tied legs'. This is a physical description of the resulting spastic gait. Konzo disease was firstly described by Giovanni Trolli, an Italian doctor in the 1930's in the former Belgian Congo now Democratic Republic of Congo. Nine decades ago, with outbreaks in Cameroon, Mozambique, Tanzania, and The Central Africa Republic amongst other, Konzo disease still remains a health problem in Africa. The disease is associated with poverty and agricultural crises provoked by drought or war. The Populations of people affected are mostly those living in remote rural areas, the reason why there is global silence about the disease and its dangers in the world. The disease is

believed to be caused by the consumption of inappropriately processed cassava root especially, the bitter cassava species, which are known to contain high dietary cyanogen. A prolonged consumption of this food will lead to cyanogenic poisoning which is the main cause of Konzo disease. The traditional methods of processing cassava practiced in some parts of Africa so as to remove cyanogenic toxins, such as sun drying and heap fermentation especially in East Africa, are inadequate in the removal of cyanogen from the cassava roots [2]. Cassava is a major staple food in some parts of the world including Africa, providing a basic diet (carbohydrate) for over half a billion of people in the world. After rice and maize, cassava is the third largest source of carbohydrate food in tropical Africa [3].

Proteins containing sulphur amino-acid helps the body metabolize and excrete cyanide. Protein insufficiency may increase the potential to cassava toxicity on the population. Communities may not be able to maintain this basic dietary defense due to a lack of access to meat, fish, beans and other protein rich foods.

The first outbreak of konzo disease was recorded in Mozambique, in 1981, during severe drought. More than 1000 cases of konzo disease broke out and were restricted to remote areas of the isolated northern province of Nampula. The disease outbreak was associated with an exclusively monothrophic diet of bitter cassava. The extent of the epidemic was only revealed after an extensive two months active detection

exercise [4]. Lessons from previous outbreaks indicate that the number of people affected by konzo is underestimated [5]. The possibility of Konzo being an infectious disease was considered in the early reported epidemics and cerebrospinal fluid studies were carried out. The results however, was normal serology for the retroviruses (HIV or HTLV-1) implicated in progressive spastic myelopathy were negative [6].

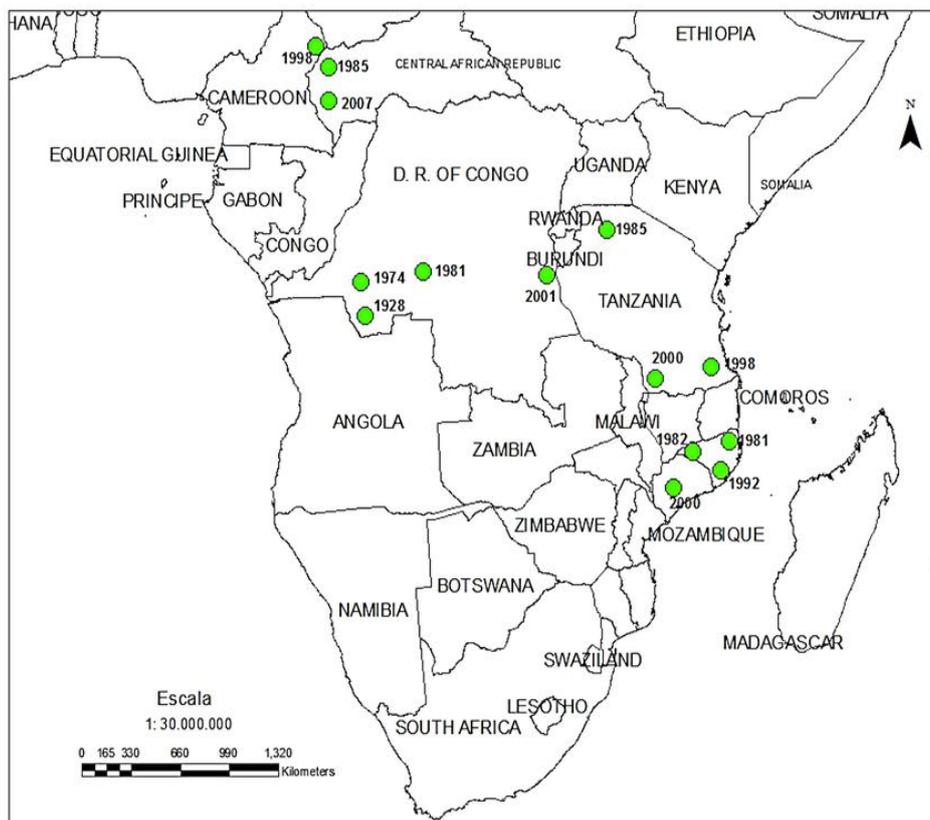


Fig-1.1: Map of countries in Africa where Konzo have been reported in green dots.

Epidemics of spastic paraparesis that is associated with a combination of prolonged consumption of high dietary cyanide and low sulphur amino acid protein intake from inappropriately processed bitter cassava roots in tropical Africa countries. Thus is the need to establish a laboratory rat model of Konzo disease and the role of nutrition in the amelioration of its pathology. Hence, this study was done to establish a laboratory rat model of Konzo disease by reviewing the effect of bitter cassava neurotoxicity on the motor neurons of cassava-induced Konzo disease on Wistar rats [6].

Konzo

Konzo cases reported ranges from only hyperreflexia in the lower limbs to a severely disabled, bedridden patient with spastic paraparesis, associated weakness of the trunk and arms. Spasticity is present from the first day, without any initial phase of flaccidity. After the initial weeks of functional improvement, the spastic paraparesis remains stable for the rest of life [7]. Some patients may suffer an abrupt

aggravating episode, e.g. a sudden and permanent worsening of the spastic paraparesis. Such episodes are identical to the initial onset and can therefore be interpreted as a second onset. Occasional blurred vision and/or speech difficulties typically clear during the first month, except in severely affected patients. Although the severity varies from patient to patient, according to world health organization, the degree of severity of konzo are mild: in this form, subject is able to walk without support; moderate: subject uses one or two sticks to walk; and severe form: subject is unable to walk. The hallmark of konzo is difficulty in walking, and often the affected person walks with the help of a stick. The brain is the key organ involved in cyanide poisoning, and studies have shown that cyanide significantly increases brain lactate and decreases brain Adenosine triphosphate concentrations [8]. Highest levels of cyanide are typically found in the liver, lungs and brain [8]. However, people are not uniformly affected by konzo. Children above the age of three years and women in the fertile age group are more

affected than adult males [9]. This is why this research will be based on female wistar rats.

Symptoms of acute cyanide intoxication could appear at least four hours after injection of poorly processed cassava roots. Some symptoms include tachypnea, tachycardia, goiter, vertigo, vomiting, diarrhea, dizziness, collapse or even death [10]. These symptoms are sometimes reported during konzo epidemics and in acute poisoning cases are attributed to cassava consumption. Usually, in less than a week, an acute and non-progressive symmetric spastic paraparesis becomes installed. Individual susceptibility factors remains unknown

Characteristics of Konzo Disease

The case definition of konzo recommended by the World Health Organization (WHO) is based on the combination of visible sudden onset of spastic paraparesis, abnormalities in walking on the balls of the feet with rigid legs and often with ankle clonus in a formerly healthy person and bilaterally exaggerated knee or ankle jerks without signs of disease of the spine with no signs of sensory or genitourinary impairments. By using these criteria, people with clonus and mild spasticity, but without visible difficulty in walking, are not considered to have konzo. However, because these minor signs are present in apparently healthy people, and they are only recognizable if neurological examination is performed, case detection and comparison between case series would be difficult to carry out. Up to date, there is no treatment for konzo that has been documented. Signs of upper motor damage, in the form of spasticity resulting in exaggerated reflexes, ankle clonus, and extensor plantar reflexes are clinical markers in the absence of paraparesis [7-10].

A laboratory model of konzo disease can be created and used for further research work in unraveling the pathology of konzo disease in human. Rehabilitation and possible recovery of motor functions through nutritional neuromodulatory approaches in a laboratory model can also be applied/ achieved in the treatment and management of people with Konzo disease.

This study was carried out at the Department of Human Anatomy, Faculty of Basic Medical Sciences, University of Port Harcourt, and Rivers State, Nigeria.

MATERIALS AND METHODS

Research Design

Thirty female Wistar rats weighing between 200g to 250g used for this research work were acquired from the animal house of the Department of Anatomy. All animals were housed in their individual standard metal cages. Animals were allowed to acclimatize for one week in their cages, with pellet animal feed and water. The experimental animals were then divided into

four groups; Group 1 (positive control group) n=5, were fed with protein food and water. Group 2 (negative control group) n=5, were fed with pelleted animal feed and water. Group 3 (cassava induced Konzo group) n=15, were fed with bitter cassava flour. Group 4 (protein treated group) n=5, were fed with protein and bitter cassava flour and water. Animal feeding was by oral ingestion. Animals were weighed weekly with an electric weighing scale and the weights recorded. Animals were closely observed for physical manifestations and clinical signs. The experiment lasted for duration of five weeks.

Ethical Clearance

The experimental animals were obtained from the animal house of the department of Anatomy in the Faculty of Basic Medical Sciences. All procedures carried out during this research were done in accordance with the guiding principles of research involving animals as recommended by the Research Ethics Committee of the University of Port Harcourt. Animals were kept in standard metal cages and at normal room temperature.

Sample Size

The sample size of the study was thirty (30) female Wistar rats. The sample size for this experimental animal study was determined using Power Method.

Plant collection and Identification

The bitter cassava roots were collected from the agricultural farm of the Department of Agriculture in the University of Port Harcourt, and were identified by the Department of Plant Science and Biotechnology, in the same university.

Processing of Bitter Cassava Root

Fresh cassava roots were uprooted from the farm. Immediately after harvesting, the brownish peel (skin or cortex) was removed with knife to expose the white inner layer. Then the roots was sliced into smaller sizes like chips and allowed to sundry for 3 consecutive days. The dry cassava pieces were manually grinded into powdery form using a grinding machine and served to the experimental animals as cassava chow.

Processing of Protein food supplement

A combination of egg shells and brown beans served as the protein food supplement for this research work. Egg shells were washed, broken into small pieces and allowed to sundry for 24 hours. Brown beans and the egg shells were grinded together using a grinding machine, into powdery form and served as sulphur amino acid protein food supplement and it was given exclusively to the "positive control group" and to the "protein treated group" rats.

Process of Inducing Konzo disease in Rats

After one week of acclimatizing in their cages, fifteen Wistar rats were allowed to freely feed on the inappropriately processed bitter cassava flour exclusively and constantly for a duration of five weeks to induce Konzo disease and its manifestations. The oral-consumption method used in this study better mimics a real-world consumption scenario wherein the food enters the mouth, then passes through the esophagus and into the stomach following the entire alimentary canal, contacting the relevant visceral organs along with their associated fluids, such as saliva in the mouth and gastric juices, including any contribution provided via sublingual or buccal absorption during digestion.

Forelimb Grip Strength test

Grip strength test is a neurobehavioural test used to evaluate motor function and deficit in laboratory

models of central nervous system disorders. The grip strength tests are more convenient and give less stress to animals. Thus, grip strength test has been widely used alone or in combination with other test to assess neuromuscular disorder and to evaluate the effect of chemicals on motor performance. The conventional forelimb grip strength was modified in this study. The inspector horizontally pulled the tail of the rat, and the rat was placed on the horizontal wire grid, immediately the animal grips the wire grid, the horizontal wire bar is inverted downwards and was suspended from the ground. The rat grips the grid of the wire and the maximum time the rat stayed before releasing its forepaws from the wire grid was recorded with a stop watch that was monitored by another inspector. This procedure was carried out three times at 10 minutes intervals to allow animals rest and the average time is recorded.



Fig-3.1: A picture showing a Wistar rat grip on the wire grid of the horizontal wired bar.

Histology

Anesthesia, Perfusion and Fixative

The animals were anaesthetized by inhalation using chloroform in a desiccator. Incisions were made to expose the thoracic region. Incisions were made to expose the thoracic and abdominal regions. When fully exposed, transcardiac perfusion was done using 10% formal saline solutions. The 10% formal saline was injected into the ventricle of the heart. The brain and spinal cord were harvested and fixed by immersion in 10% formal saline solution. Coronal sections were taken on the cerebrum of the brain and at spinal cord level C2 – C6. The tissue was fixed for 48 hours.

Tissue Processing

Dehydration is the first step in tissue processing followed by clearing, impregnation, embedding, trimming, sectioning and staining respectively. Dehydration is a process by which all water molecules are removed from aqueous fixed tissue. This is done by running tissues through ascending grades of alcohol, from 50%, 70%, and 95% to absolute

alcohol. The tissues were immersed in 50% alcohol for 6 hours, then in 70% alcohol for another 6 hours, then in 95% alcohol for 6 hours and in absolute alcohol for 3 hours. Clearing was done using xylene. The tissues were kept in xylene for 3 hours. Next, the tissues were impregnated with paraffin wax. Tissues were placed in cassettes with their labels and put into molten paraffin wax and allowed for some time. The tissues were embedded with paraffin wax in embedding moulds. Extra wax was trimmed with a microtome blade. Sections between 5-10 microns were made using the microtome. Sections were floated on a hot water bath to unfold the tissues and mounted on well labeled frosted slides.

Staining

Nissl Substance

The form, size and distribution of Nissl substance vary in different types of neurons. The Neurons are well stained by basic anilines dyes such as thionin, azure A and Cresyl echt violet because of their rich content of ribonucleic acid (RNA). The loss of

Nissl substance during neurological injury or disease is 'chromatolysis', and it is valuable in microscopically assessing the physiological status of the nerve cells. The appearance of the granules is indication of recovery of functions and conduction of the neuron. 0.5% aqueous solution of cresyl echt violet was prepared and allowed to ripen for 24-48 hours and was filtered before use. Balsam xylene solution was prepared with equal parts of Canada balsam and xylene. Brought section to distilled water before staining with cresyl echt violet solution for five minutes, then rinsed in two changes of distilled water, rinsed in 95% alcohol for 30 seconds, rinsed in absolute alcohol for 30 seconds, rinsed in xylene for one minute, rinsed in balsam xylene mixture for two minutes and also rinsed in two changes of absolute alcohol for 10-30 seconds. Then washed in 3-4 changes of xylene and mounted.

Hematoxylin and Eosin (H & E)

Hematoxylin and Eosin staining technique was performed to demonstrate the general structure of tissues. After taking section down to water, tissue was stained in hematoxylin solution for 5-10 minutes, and then rinsed in water for a few seconds. It was next differentiated in 1% acid alcohol with continuous agitation for 10-15 seconds, washed in running tap water for 5 minutes and stained again in 1% aqueous eosin solution for 5 minutes. Washed in running tap water for 30 seconds and dehydrated in alcohol, cleared in xylene and mounted.

Immunohistochemistry

The laboratory Wistar rats were deeply anaesthetized with chloroform in a desiccator by inhalation. After anesthetizing, the rats were placed in supine position. The rats were dissected to expose the heart and at the apex of the heart, and perfused transcardially with 0.1M phosphate buffered saline (PBS) followed by 250 ml of 4% paraformaldehyde fixative in phosphate buffer (pH 7.4) into the chambers of the heart until the rats were fixed and specimens of the brains with spinal cords intact were extracted and immediately fixed in 4% paraformaldehyde solution for about 48 hours and cryoprotected in 30% sucrose in 0.1 M phosphate buffer at 4°C. All brains were sectioned frozen at 40µm thickness on a sliding microtome and stored at 0°C in a cryoprotectant solution prior to processing.

Preparation of 4% paraformaldehyde

For the preparation of one litre of 4% paraformaldehyde, 1000ml of 1X PBS (phosphate

buffered solution) was prepared with 0.145M NaCl, 0.0027M KCl, 0.0081M Na₂HPO₄, 0.0015M KH₂PO₄ at pH 7.4 and stored. 800ml of 1X PBS was then added to a glass beaker on a stir plate in a ventilated hood. The solution was heated to 60°C while stirring. 40g of paraformaldehyde powder was added to the heated PBS. The powder did not immediately dissolve in the solution. 1 Normal sodium hydroxide (NaOH) was added slowly from a pipette to raise the pH until the solution was clear. The solution was allowed to cool and was filtered. The volume of the solution was adjusted with 1X PBS to one litre and the pH was adjusted with small amounts of dilute hydrochloric acid (HCl) to approximately 6.9.

Immunofluorescence Staining

The brain sections were left in PBS with PBS containing 0.3% Triton X-100 (Sigma-Aldrich) for twenty minutes for permeability, then blocked for unspecific binding site with 5% goat serum for 30 min [11]. Goat anti-choline acetyltransferase (ChAT) primary antibody (1: 100 diluted in PBS; Merck Millipore; AB144P) was then added and allow incubating for 48 h at 4 °C. Slides were washed in PBS (3 × 5 min) and Alexa Fluor 594 donkey anti-goat IgG (1: 500; Life Technologies, A-11058) secondary antibody applied for 2 h at room temperature. After PBS washes, blocking solution was added again followed by mouse anti-neuronal nuclei (NeuN) primary antibody, clone A60 (1: 100; Merck Millipore; MAB377) for 2 h at room temperature. After PBS washes, Alexa Fluor 488 donkey anti-mouse IgG (1: 500; Life Technologies, A21202) secondary antibody was applied for 2 h at room temperature. Following PBS washes, DAPI nuclei stain (1: 1000; Life Technologies, D1306) was applied for 10 min. After final PBS washes, coverslips were placed on slides using Mowiol mounting media. A high resolution Zeiss 880 Airyscan confocal microscope imaging was used to take micrographs.

STATISTICAL ANALYSIS

Data were analyzed using Statistical Package for the Social Sciences (SPSS IBM version 23.0) and Microsoft excel 2019 edition. Values were expressed as mean±SD in descriptive statistics. One-way analysis of variance (ANOVA) was used to analyze the significant difference between the groups followed by posthoc test multiple comparison test (LSD). Confidence interval was set at 95%, therefore p < 0.05 was considered significant.

RESULTS

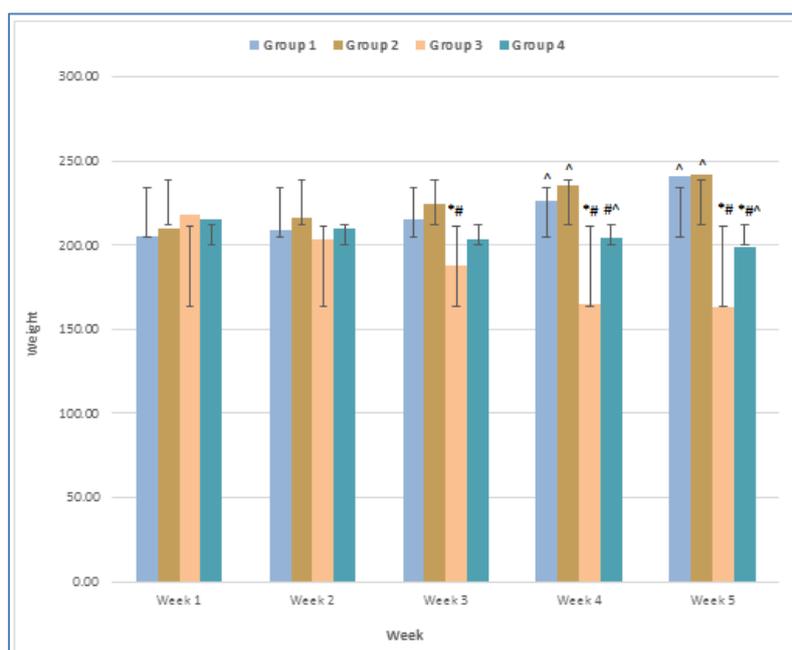


Fig-1: Bar chart showing the effect of bitter cassava on the weight of albino wistar rats.

Each value represents mean \pm SD, Values marked (*) differ significantly from positive control, values marked (#) differ significantly from the negative control while those marked with (^) differ significantly from cassava only group at ($p < 0.05$).

From this study, it was observed that there were significant changes in the body weight of the experimental animals during the 5 weeks duration of the experiment. The result shows weekly body weight differences in experimental animals.

There was significant weight reduction in the body weight of the cassava- induced Konzo group experimental Wistar rats compared to the significant

weight increase observed in the negative control group Wistar rats.

There was weight loss of approximately 7g to 13g observed weekly from the cassava-induced Konzo Wistar rats compared to approximately 6g to 11g weekly weight gain in negative control Wistar rats.

Also, significant increase in weight was observed in the protein treated group animals compared to the cassava- induced Konzo Wistar rats.

The positive control groups also recorded significant weight gain when compared to the cassava-induced Konzo Wistar rats.

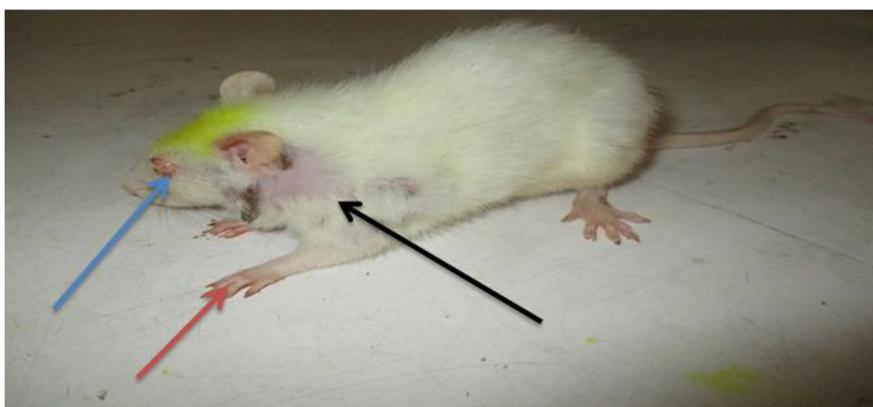


Fig-2: Picture showing a laboratory Wistar rat model of cassava-induced Konzo disease.

Black arrow: showing swollen neck and swollen forelimbs on the left side of the body.

Blue arrow: showing redness of the eyes in a cassava-induced Konzo rat model.

Red arrow: showing paralysis of the left forelimb.



Fig-3: Black arrow showing Alopecia (hair loss) at the base of the tail.

The picture above is showing alopecia (hair loss) initially observed at the base of the tail and over the lumbar region and progressed cranially and

ventrally to the head and face of cassava-induced Konzo Wistar rats after 5 weeks of oral ingestion of bitter cassava flour.

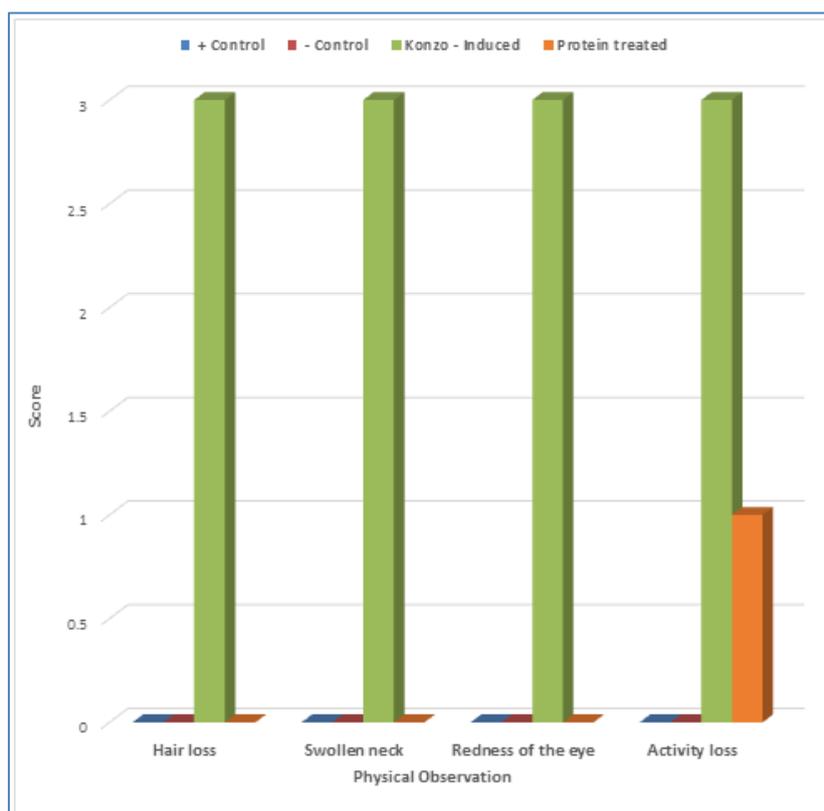


Fig-4: Physical observation on the effect of bitter cassava-induced konzo rat models
 Score interpretation: 0 = none, 1 = Mild, 2 = Moderate, 3 = High

From this study, it was observed that there was significantly higher level of hair loss in the cassava-induced Konzo group when compared with protein treated group. There was no observable hair loss in the positive and negative control groups.

The present study also show significant higher incidence of swollen neck observed in the cassava-

induced Konzo group in comparison to the protein treated group. There was no observable incidence of swollen neck in positive and negative control groups.

Redness of the eyes was significantly higher in the cassava-induced Konzo group when compared with the protein treated group. There was no observable

redness of the eye seen in the positive or negative control group neither the protein treated group.

It was also observed from this study that the level of physical activity in the cassava-induced Konzo

group was significantly low in comparison with the protein treated group which was moderate and the positive and negative control groups showed a high activity level as seen in normal healthy rats.

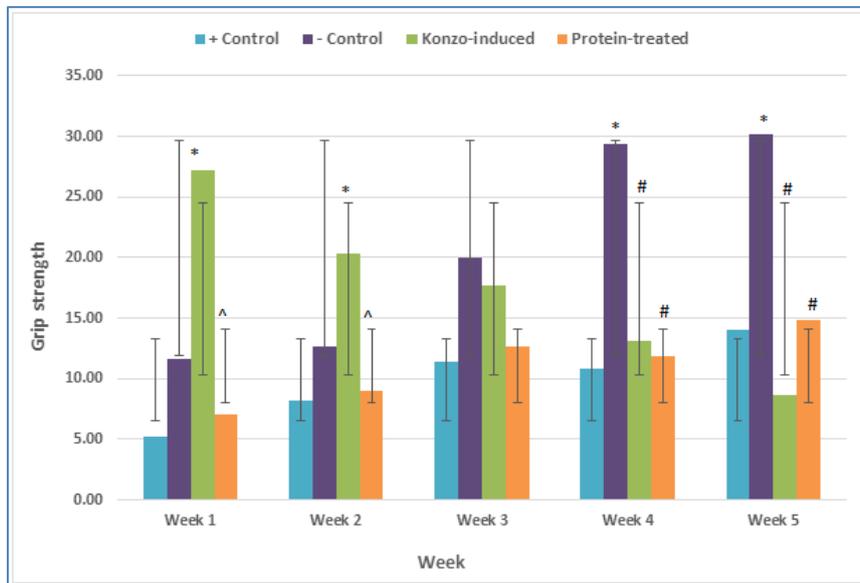


Fig-5: Bar chart showing the effect of bitter cassava on the grip strength of Wistar rats.

Each value represents mean±SD, Values marked (*) differ significantly from positive control, values marked (#) differ significantly from the negative control while those marked with (^) differ significantly from cassava only group at (p < 0.05).

From this study, it was observed that there were significant difference/changes in muscular activity of the forelimbs assessed weekly using the grip strength test results.

The cassava-induced Konzo Wistar rats recorded significant decrease in gripping time as the experiment progressed compared to the negative control Wistar rats that had significant increase in gripping time recorded in seconds as the weeks went by.

Also, the protein treated Wistar rats also differed significantly from the negative control. Although there was increase in gripping time, but it was not as much compared to the negative control group or compared to the cassava-induced Wistar rats that had reduced gripping time.

The negative control Wistar rats had recorded significant increase in gripping time as the experiment progressed. The positive control groups also had recorded significant increase in gripping time as the week progressed.

Histology of the Cerebral Cortex of the Brain Using H/E Stain

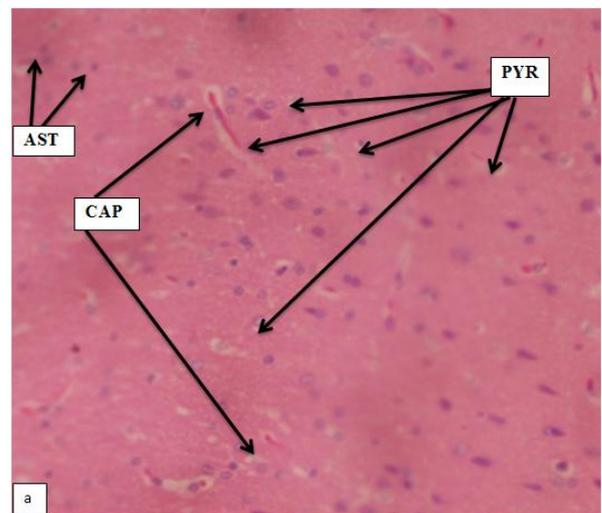


Fig-6a: Figure 7: Photomicrograph of H and E stain of the cerebral cortex of the brain of negative control group Wistar rat. Magnification:x400. Pyr- Pyramidal Cells, Cap-Capillaries, Ast-Astrocytes.

This micrograph shows a motor neuron from an animal that consumed animal food and water for 5 weeks. Within this micrograph we also can see that the negative control group Wistar rats shows numerous pyramidal cells and there is no cytoplasmic vacuolation within the cells. Also, the capillaries are seen and the neurons are closely packed.

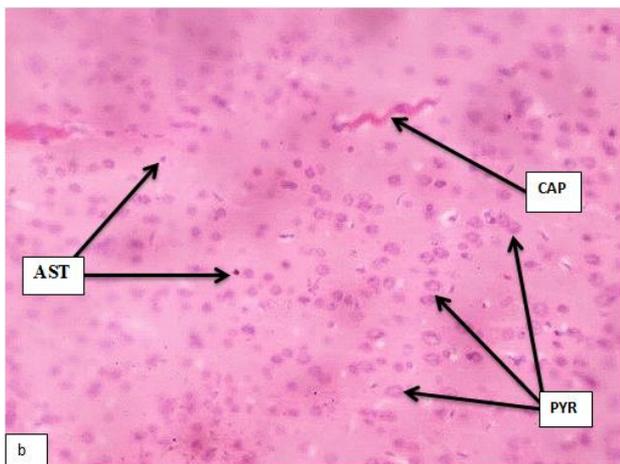


Fig-6b: Figure 5: Figure 7: Photomicrograph of H and E stain of the cerebral cortex of the brain of positive control Wistar rat fed with protein and water. Magnification: x400. Pyr- Pyramidal Cells, Cap-Capillaries, Ast-Astrocytes

This micrograph shows a motor neuron from an animal that consumed protein food and water for 5 weeks. Within this micrograph we also can see that the protein treated group Wistar rats shows numerous pyramidal cells and there is no cytoplasmic vacuolation within the cells. Also, the capillaries are seen and the neurons are closely as seen in negative control group.

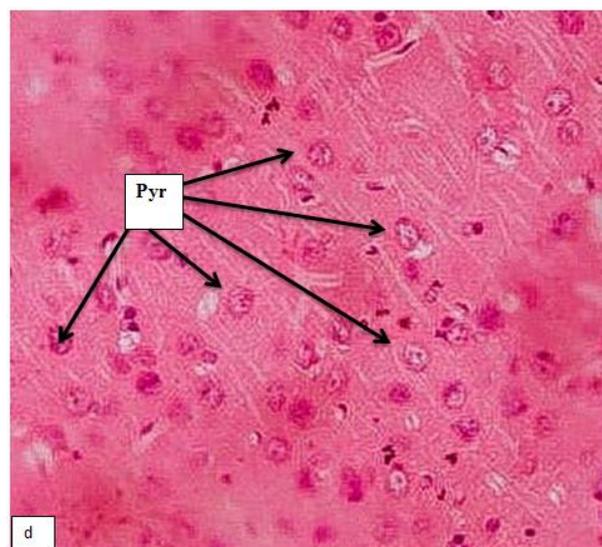


Fig-6d: Photomicrograph of H and E stain of the cerebral cortex of the brain of protein treated group Wistar rat. Magnification: x400. Pyr- Pyramidal Cells

This micrograph shows a motor neuron from an animal that consumed bitter cassava flour for 5 weeks and treated with protein. Within this micrograph we also can see that the protein treated group Wistar rats shows numerous pyramidal cells and there is no cytoplasmic vacuolation within the cells. Also, the neurons are closely packed compared to the control group.



Fig-6c: Photomicrograph of H and E stain of the cerebral cortex of the brain of Wistar rat fed with bitter cassava. Magnification: x400. Pyr- Pyramidal Cells

This micrograph shows a motor neuron from an animal that consumed bitter cassava flour for 5 weeks. Within this micrograph we also can see that the control group shows reduced number of pyramidal cells and there is cytoplasmic vacuolation within the cells. Also, the neurons are dispersed compared to the control group.

Histology of the Spinal Cord using Nissl Stain

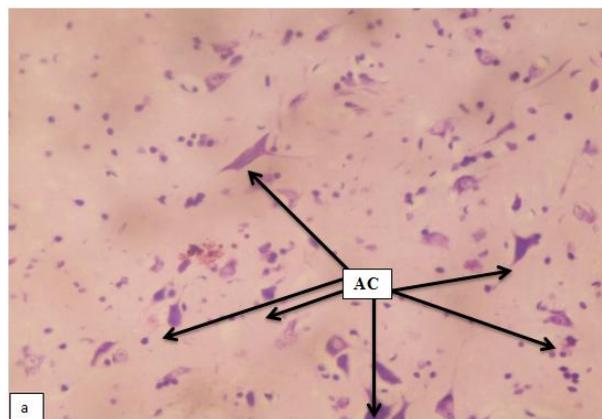


Fig-7a: Photomicrograph of Nissl stain of the ventral horn of the spinal cord of negative control group. Magnification: x400. AC-Alpha Cells

This micrograph shows a motor neuron from an animal that consumed protein and water for 5 weeks. Within this micrograph we also can see that the control group shows a higher number of alpha neurons and there is no cytoplasmic vacuolation. Also, the neurons are closely packed with supporting cells.

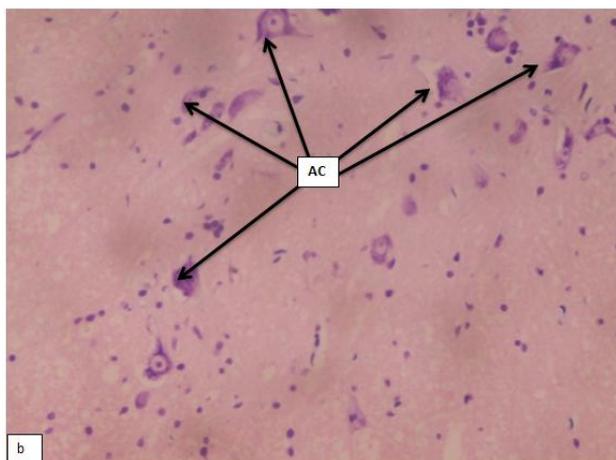


Fig-7b: Photomicrograph of Nissl stain of the ventral horn of the spinal cord of Positive control group. Magnification: x400. AC-Alpha Cells

This micrograph shows a motor neuron from an animal that consumed normal rat food and water for 5 weeks. Within this micrograph we also can see that the control group shows a higher number of alpha neurons and there is no cytoplasmic vacuolation. Also, the neurons are closely packed with supporting cells.

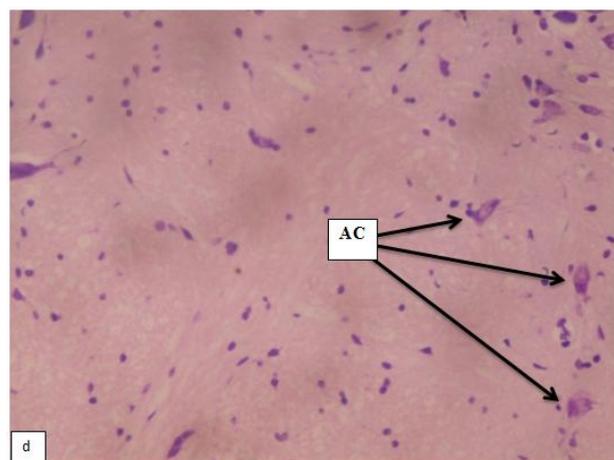


Fig-7d: Photomicrograph of Nissl stain of the ventral horn of the spinal cord of Protein treated group. Magnification: x400. AC-Alpha Cells

This micrograph shows a motor neuron from an animal that consumed sulphur amino acid protein supplement (beans and egg shells) with bitter cassava flour. Within this micrograph we can see reduced number of alpha cells compared to the negative control group.

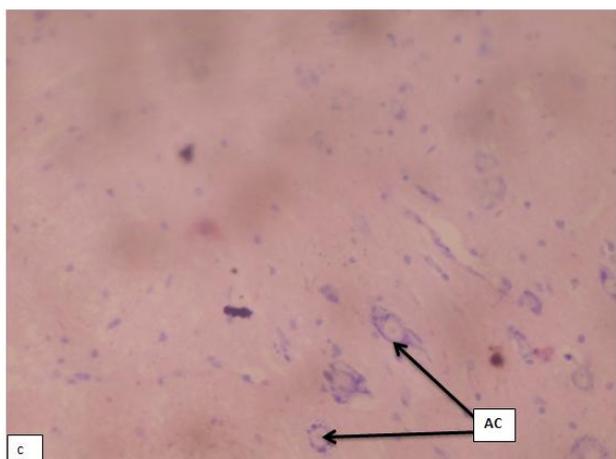


Fig-7c: Photomicrograph of Nissl stain of the ventral horn of the spinal cord of Cassava-induced Konzo group. Magnification: x400. AC-Alpha Cells

This micrograph shows a motor neuron from an animal that consumed bitter cassava flour for 5 weeks. Within this micrograph we also can see dispersed and reduced alpha cells of the motor neurons at the ventral horn of the spinal cord compared to the control group that showed a higher number of neurons.

Also, the micrograph shows cytoplasmic vacuolations, whereas the control group's alpha neurons are closely packed and not separated by vacuolations.

The immunohistochemistry of the ventral horn of the spinal cord using ChAT Stain

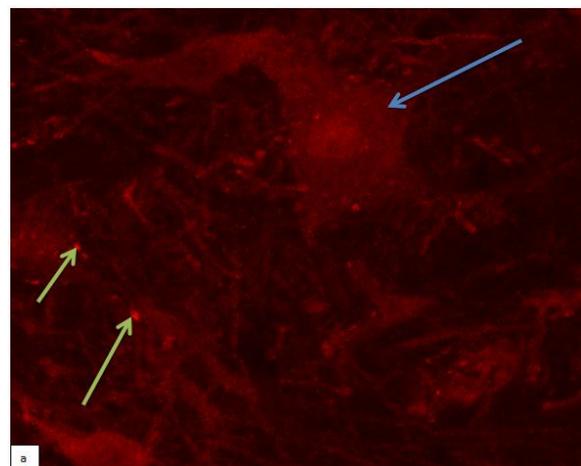


Fig-8a: is a Zeiss 880 Airyscan confocal micrograph stained for the motoneuron marker Choline acetyltransferase (ChAT). Blue arrowing showing ChAT+ on the alpha motor neurons on the spinal cord, and green arrow showing the C- boutons

This micrograph shows a motor neuron from an animal that consumed bitter cassava flour for 5 weeks. Within this micrograph we also can see numerous ChAT+ processes and some C-boutons (bright puncta of label). The C-boutons play a role in cholinergic modulation of motor neurons for the locomotor compensation for severe motor neuron loss.

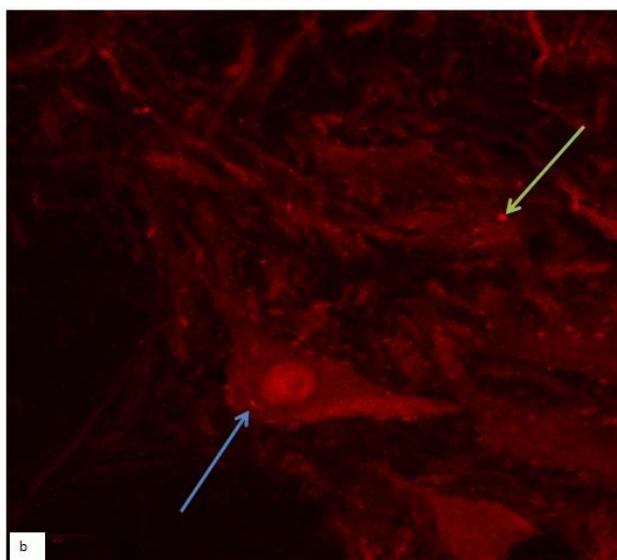


Fig-8b: is a Zeiss 880 Airyscan confocal micrograph stained for the motoneuron marker Choline acetyltransferase (ChAT). Blue arrow showing ChAT+ on the alpha motor neuron and the green arrow showing C-boutons

This micrograph shows a motor neuron from an animal that consumed cassava for 5 weeks. Within this micrograph we also can see numerous ChAT+ processes and some C-boutons (bright puncta of label). The C-boutons play a role in cholinergic modulation of motor neurons for the locomotor compensation for severe motor neuron loss.

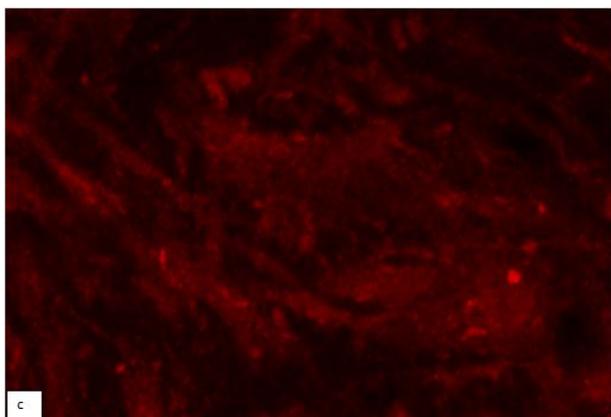


Fig-8c: is a Zeiss 880 Airyscan confocal micrograph stained for the motor neuron marker Choline acetyltransferase (ChAT)

This micrograph shows a motoneuron from an animal that consumed cassava and protein for 5 weeks. Within this micrograph we also can see numerous ChAT+ processes and some the C-boutons were not present. The C-boutons play a role in cholinergic modulation of motor neurons for the locomotor compensation for severe motor neuron loss.

DISCUSSIONS

Exposure to cyanide from prolonged consumption of insufficiently processed cassava roots

containing cyanogenic glycosides may lead to growth retardation, weight loss, outright death and neurological disorders resulting from tissue damage in the central nervous system (CNS). This study aimed to replicate Konzo disease and its manifestations in a laboratory animal model to evaluate the neurotoxic effect on the motor neurons and the skeletal muscle functions and how it can be ameliorated using nutrition.

This study demonstrated that oral ingestion of inappropriately processed bitter cassava flour leads to a significant reduction in the body weight. The experimental animals were weighed weekly throughout the duration of this research. It was observed that the negative control group animals had weight gain weekly. Also, positive control group animals that were fed with protein food supplement also had weight gain per week. The cassava induced Konzo group animals fed exclusively with cassava flour, had recorded severe weight loss. The protein treated group animals fed with equal amounts of cassava and protein food, also had weight loss per week as in a slower rate compared to the cassava induced group animals. This observations agrees with previous studies from other works that have also shown significant weight loss in animals injected with cyanogenic glycoside: linamarin [12] and in laboratory Wistar rats fed with cassava root chips (tapioca) and cassava flour (gari) [13].

Alopecia (hair loss) was observed in cassava induced Konzo Wistar rats

Hair losses were observed at the base of the tail, head and face of the cassava-induced Konzo Wistar rats that were exclusively fed with bitter cassava flour. This group of animals was the only experimental group animals that recorded hair loss. They had drastic body hair loss that got worse by the week. But, the other animals in the other groups had no loss of body hairs. Alopecia could be an indication of toxicity that is causing hormonal changes in the animal by the cyanogenic toxins that are present in the body and increased with prolonged consumption of the bitter cassava root. Alopecia have been reported in rats fed with cassava product "purupuru" for 18 months, and the result was reported to be due to nutritional imbalances of intake of 100% cassava diet as done in this study, particularly due to lack of the sulphur amino acids in diet [14].

Other physical manifestations observed where clinical signs such as bloody eyes, swollen neck, swollen limbs, motor weakness and experimental animals becoming less active and also loss of movement of the neck, forelimb and hind limb and even death. Bloody eyes were developed at week 2 and by week 3, cassava induced animals became weak and less active. Swollen neck seen as goiter and swelling of the hind limb was developed at week 3. First animal death was recorded at week 3. These clinical signs indicate severe discomfort in the animals, and also indicate that

the animal has been exposed to cyanogenic toxins in cassava roots which cause konzo disease [15].

One of the rats in the cassava induced group had paralysis of the left forelimb observed at 4th week. Paralysis is one of the pronounced effects of damage to motor neurons. Loss of alpha motor neurons at the ventral horn of the spinal cord severs the connection between the brain and the spinal cord and the muscles they innervate. Muscle weakness and reduced physical activities observed may be due to loss of upper motor neuron [16].

Voluntary muscles controls were lost at the forelimb of some animals and at the hind limb of other animals. This was due to loss of the alpha motor neurons that relay voluntary signals from the upper motor neurons to skeletal muscle fibers, while involuntary control loss was as a result from interruption of the reflex circuit which could lead to reduced muscle tone and flaccid paresis.

Toxicity studies in experimental animals aim to detect unusual behaviours prior to the occurrence of irreversible neurological damage, as observed with excessive consumption of inappropriately processed bitter cassava that has been shown to cause Konzo. In this study, the evaluation of muscle strength is essential for researching neuromuscular disorder in cassava-induced group konzo rat models. The forelimb grip strength test carried out was a method used to assess skeletal muscle functions in the experimental animals. The reduced muscular activities observed in the cassava induced Konzo animals were related to muscle weakness reflected in reduced time duration in gripping the wire grid. The muscle weakness is associated with the ingestion of the inappropriately processed bitter cassava as reported in previous studies [17].

The study indicates that there were distortions of the large pyramidal cells on the motor cortex. The pyramidal shape of the cells were distorted and appeared like a half moon or crystal shape with cytoplasmic vacuolation. There was increase in microglial and other glial cells which may be due to inflammation reaction and increased vacuolization in the brain vessels, nerve cell death and astroglial reaction following neurotoxicity in the central nervous system. In spite of several studies regarding the effect of bitter cassava on the central nervous system, this study demonstrated that raw or inappropriately processed bitter cassava has a neurotoxic effect on the corticospinal axonal outgrowth in the upper limb musculature [17].

Moreover, this study has shown that the proteins food supplements used in this study has neuroprotective effects on the motor neurons. Also the presence of sulphur amino acid in the diet can ameliorate the neurotoxic effect/s of bitter cassava on

the motor neurons in the central nervous system by detoxifying the hydrogen cyanide as shown in other studies.

Bitter cassava has been shown to induce Konzo disease in a laboratory rat model. There was visible paralysis of the left forearm of a Wistar rat. This indicates that Konzo is a paralytic disease [2, 16].

CONCLUSION

Cassava is a staple food in West Africa and some parts of East Africa. There are several ways and manners in which cassava is served as food. Cassava is eaten as whole root, grated root (garri) or root chips (tapioca). It has been realized that the various methods of processing and serving cassava may yield different results with debilitating consequences. For instance, when cassava is processed through the proper processing of fermentation and heating either by frying (as seen in garri) at very high temperatures of over 35°C, or by boiling sliced pieces of cassava (as we have in tapioca) over very high temperatures, it is eaten without any consequences, physiological or pharmacological consequences. However, some people because of poverty, hunger and agricultural crises may not have the resources needed to process cassava properly and may use the quickest but not safe means of processing cassava root as food which is almost in its raw state. For such people, especially in the remote rural areas, the consequences of eating these insufficiently processed cassava roots are grave. Processing methods can detoxify cyanogenic glycosides and reduce the risk of cyanide poisoning which affects those individuals who have regular long term consumption of cassava with poor nutrition status. The boiling or frying of cassava changes the metabolism of the cyanide content of the cassava from the toxic acetone cyanohydrin to hydrogen cyanide which is then evaporates. However, not processing the cassava properly in this form may leave acetone cyanohydrin in a grand state where the cyanide molecules are left freely as radicals and they combine with other agents in the body to build up the compound that leads to acute cyanogenic intoxication. Sustained high cyanogens intake due to prolonged consumption of inappropriately processed bitter cassava food in combination with a low intake of sulphur amino acid proteins has been reported to cause Konzo, women and children are mostly affected because they are always involved in the processing (WHO 1984). Appropriate processing of bitter cassava roots will aid in reducing cyanogenic toxins, and in eliminating the problems of unintentional cyanide toxicity and further neurological disorders such as Konzo disease affecting the population will be eradicated.

This study has shown that insufficiently processed bitter cassava is toxic and has neurotoxicity effects on the central nervous system (CNS) especially on the upper motor neurons. Balanced diet with

sufficient nutrients also, can ameliorate the effect/s of konzo disease as there was no effect on motor neurons of the brain and spinal cord of the animals that had taken protein in the protein treated group. Nutritional insufficiency may likely increase the potential toxicity of bitter cassava roots.

RECOMMENDATION

Cyanogenic plants such as cassava should be cut into smaller pieces, soaked in water for at least 5 days, washed and cooked thoroughly under very high temperatures and pH greater than 6.0, to liberate hydrogen cyanide before consumption in order to reduce the level of toxins.

Laboratory experimental studies are required to evaluate the biochemical role of sulphur amino acid proteins in neuroprotective action/s by tried protein sources in this research work, in the amelioration of bitter cassava neurotoxicity.

ACKNOWLEDGEMENTS

We appreciate the participants and the research assistants who took part in the study.

Conflict of Interest

We write to state that there is no conflict of interest.

Author's Contribution

We write to state that all authors contributed significantly and are in agreement with the contents of the manuscript. 'Author A' (Stella Enefa) designed the study and protocol, literature search and wrote the first draft of the manuscript; Authors B' (Chikwuogwo W. Paul) reviewed the design, protocol, and 'Authors C' (Lekpa K. David) designed the study protocol, examined the intellectual content of the manuscript, and did the analysis of the study. All authors read and approved the final manuscript.

REFERENCES

1. Cliff, J., Lundquist, P., Ma^ortensson, J., Rosling, H., So^rbo, B. (1985). Association of high cyanide and low sulphur intake in cassava-induced spastic paraparesis. *Lancet*, 326; 1211–1213.
2. Tylleska^r, T., Banea, M., Bikangi, N., Cooke, R. D., Poulter, N. H., Rosling, H. (1992). Cassava cyanogens and konzo, an upper motoneuron disease found in Africa. *Lancet*, 339; 208–211.
3. FAO. (2001). FAO Production yearbook for 2000. Rome: Food and Agriculture Organization of the United Nations.
4. Ministry of Health of Mozambique Mantakassa. (1984). An epidemic of spastic paraparesis associated with chronic cyanide intoxication in a cassava staple area of Mozambique. *Epidemiology and clinical and laboratory findings in patients. Bull World Health*, 62; 477–484.
5. Bradley, W. G., Daroff, R. B., Fenichel, G. M., Jankovic, J. (2004). Neurology in clinical practice. 4th edition. *New York: Elsevier*, 1693–1707.
6. Howlett, W. P., Brubaker, G. R., Mlingi, N., Rosling, H. (1990). Konzo, an epidemic upper motor neuron disease studied in Tanzania. *Brain*, 113: 223–235.
7. WHO. (1996). Konzo, a distinct type of upper motor neuron disease. *Weekly Epidemiology Record*, 71: 225–232.
8. Dufour, D. L. (2011). Assessing diet in populations at risk for konzo and neuropathy. *Food Chemical Toxicology*, 49: 655–661.
9. Cliff, J., Nicala, D., Saute, F., Givragy, R., Gani, A. (1999). Ankle clonus and thiocyanate, linamarin, and inorganic sulphate excretion in school children in communities with konzo, Mozambique. *Journal Tropical Pediatrics*, 3: 139–142.
10. Rosling, H. (1994). Measuring effects in humans of dietary cyanide exposure from cassava. *Acta Hort*, 375: 271–283.
11. Wang, Z.Y., Lian, H., Zhou, L., Zhang, Y.M., Cai, Q. Q., Zheng, L. F., et al. (2016) Altered expression of D1 and D2 dopamine receptors in vagal neurons innervating the gastric muscularis externa in a parkinson's disease rat model. *Journal of Parkinsons Disease*. 6, 317–323. doi: 10.3233/JPD- 160817
12. Rivadeneyra et al, 2016) Rivadeneyra D. E, Rodriguez-Landa J. F (2016). Motor impairments induced by injection of linamarin in the dorsal hippocampus of Wistar rats. *Journal of neurology*. 31(8): 516-522.
13. Ebeye O. A (2018). The effect of processed cassava products ("Tapioca and Gari") on weight and haematological indices of Wistar rats. *International Journal of Basic, Applied and Innovative Research*. 7(1): 35-40.
14. Osuntokun B. O (1970). Cassava diet and cyanide metabolism in Wistar rats. *British Journal of Nutrition*. 24 : 377.
15. Rivadeneyra D. E, Rodriguez-Landa J. F (2019). Preclinical and clinical research on the toxic and neurological effects of cassava (*Manihot esculenta* Crantz) consumption. *Journal of Metabolic Brain Disease*. Vol 35(1) pp 65-74.
16. T. Banea M, Bikangi N, Fresco L, Tylleska^r Persson L. A, Rosling H (1991). Epidemiological evidence from Zaire for a dietary etiology of konzo, an upper motor neuron disease. *Bull World Health Organ* 69: pp 581–589.
17. Tshala-Katumbay, D., Eeg-Olofsson, K., Kazadi-Kayembe, T., Tylleska^r, T., Fa^llmar, P. (2002). Analysis of motor pathway involvement in konzo using transcranial electrical and magnetic stimulation. *Muscle Nerve*, 25: 230–235.