

Full Length Research Paper

Neurotoxicity of cassava: Mode of cell death in the visual relay centres of adult Wistar rats

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Cassava (*Manihot esculenta*) is an annual tuber root crop cultivated widely in the tropics and subtropics, it serves as a major food crop of low protein but high calorie content. At the cellular level, both free cyanide and hydrogen cyanide has been found to induce degeneration via increased lysosomal activity *in vivo* (Sotoblanco et al., 2002). In this study, we investigated the effects of cassava diet administered to adult Wistar rats at 2, 5, 10, 20 and 30 gms of cassava per animal per day for a period of 60 days using metachromasia of Cresyl fast violet for Nissl substance to demonstrate degenerating neurons. The mode of cell death observed in the V1 was found to be an apoptosis-necrosis continuum for moderate dose treatment and necrosis for extreme dose treatment while the SC and LGB was mainly necrosis irrespective of the dosage.

Key words: Primary cortex, superior colliculus, lateral geniculate body, apoptosis, necrosis, reactive oxygen species, cyanide, nitric oxide.

INTRODUCTION

Cyanide as earlier described is a potent neurotoxic substance that can initiate series of intracellular reactions leading to cell dysfunction and eventually cell death; it enhances NMDA (N-Methyl-D-Aspartate) receptor function (Fiske et al., 2001). In cultured neurons, cyanide neurotoxicity is linked with NMDA receptors, which mediated a rise in calcium that in turn activates series of biochemical reactions leading to generation of reactive oxygen species (ROS) and NO (Nitric Oxide), this anti-oxidants species then mediates peroxidation of lipids (Ha et al., 2010). It is concluded that oxidative stress plays an important role in cyanide induced neurodegeneration; this phenomena can cause cell death in two ways:

1. Apoptosis
2. Necrosis

Depending on the cell type or stimuli, a cell may die in

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Abbreviations: V1, Primary visual cortex; SC, superior colliculus; LGB, lateral geniculate body; CcOX, cytochrome C oxidase,

either of the two distinct ways. Apoptosis considers the physiological form of cell death; it is an acute process with distinct morphological and biochemical features (Bathacharya et al., 2001; Batharcharya and Tulwasami, 2008). Apoptotic cells are characterized by condensed and fragmented nuclei, whereas necrotic cells show less plasma membrane integrity without apparent nuclei damage. This two different forms of cell death can be elicited by the same stimuli depending on the intensity of the effects (Isom et al., 1999; Jerome et al., 2003), suggesting that an initial common event can be shared by both forms of cell death. It has been shown previously that cyanide induces cell death in a varying mode for different brain areas (Lee et al., 2010). Cell death occurs predominantly via apoptosis in the cortical region while necrosis was observed in the substansia nigra after the same dose of cyanide was administered in the mice. Selective vulnerability of different aspect of the brain to cyanide may be explained by the triggering of region specific toxic pathways in which oxidative stress may be a common activator (Isom et al., 1999; Lee et al., 2010; Ming et al., 2003). In the two modes of cell death, oxidative stress was a factor but the level of ROS generated varies with the cell types, other reports that oxidative stress can be involved in apoptosis or necrosis

(Way et al., 1984), it is possible that when high ROS accumulated in the cell, direct and irreversible damage to cell components can lead to necrosis. Moderate levels of ROS may function as cellular messengers and regulatory molecule which mediates apoptotic cell death (Li et al., 2000), in mesencephalic cells, cyanide induced a progressive but rather small increase in necrosis at the concentrations of 100 – 300 μm and at 400 μm , the data recorded shows a steep increase in necrotic index.

MATERIALS AND METHODS

Fifty adult Wistar rats, cassava tuber, cage, Cresyl fast violet, acetic acid, spring balance, microtome, staining jar and Olympus Research Microscope with JVC image processor were used in this study.

Twenty F1 generation adult Wistar rats were divided at random into five groups. Group 1 - 5 are the major experimental groups. The duration of treatment was 60 days, unprocessed cassava uprooted immediately before administration was peeled to remove the external root cortex; the inner white part was chopped into pieces according to the method of Sotoblanco et al. (2002) (Table 1). The animals were sacrificed by cervical dislocation and the V1, SC and LGB were excised by dissection and fixed in Formolcalcium. The tissues were processed using the method of Pearse (1960) and stained with CFV (Cresyl fast violet) (Yin et al., 2007).

RESULTS

Group 5

On examination under the microscope, group 5 (Control group) – the six layered cortex appeared intact (Figure 1), (5A). The orientation of the horizontal cells in the molecular layer (Martinotti and Cajal) remains normal and a clear demarcation can be observed between the adjacent layers about 0.1 μm apart. The outer granular layer down to the multiform layer gives a normal orientation and cellular arrangement. The outer pyramidal layer is the most prominent (5A arrow head) illustrating the giant Betz cells. The superior colliculus displayed all the cellular and fibrous layers as they interlace from the stratum Zonale down to the stratum album Profundum, few pyramidal cells are observed in the stratum Griseum Intermedium (Figure 3), (5C).

The parvocellular and magnocellular layers of the lateral geniculate body and the intermediate layer separating them are prominent (Figure 2), (5B). In this low dose group, there are some degree of histological disarray in some regions of the molecular layers as compared with those of the control, the cells appeared slightly reduced in size about 2.5 μm and vacuolar spaces are observed around the cells in the outer granular layer (0.5 μm) (Figure 1 (3A), Figure 2 (3B) and Figure 3 (3C)).

However, the very few pyramidal cells did not show such vacuolar spaces and the outer pyramidal layer showed average sized pyramidal cells (3A). A few of

Table 1. Animal preparation and treatment.

Group	Cassava (gm)/rat	Feed (gm)/rat
1	40	0
2	20	20
3	10	30
4	2.5	37.5
5	0	40

HISTOLOGY I

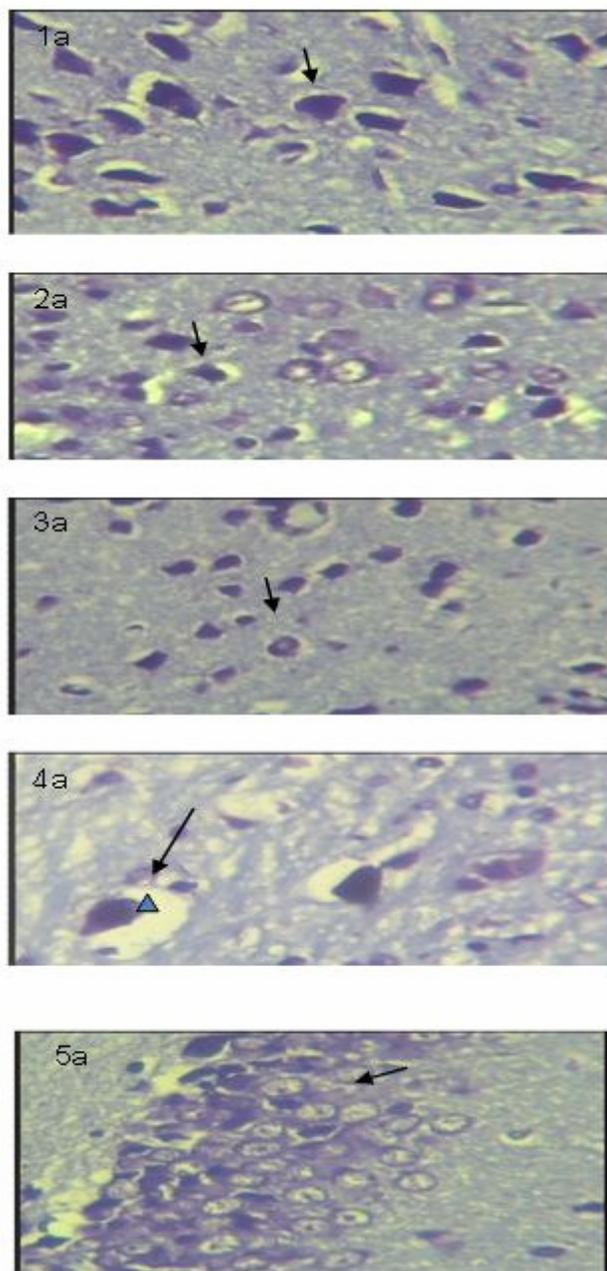


Figure 1. Comparative study of V1 for treatment groups. Magnification: X 1000 (Arrow heads indicates orientation and appearance of pyramidal cells).

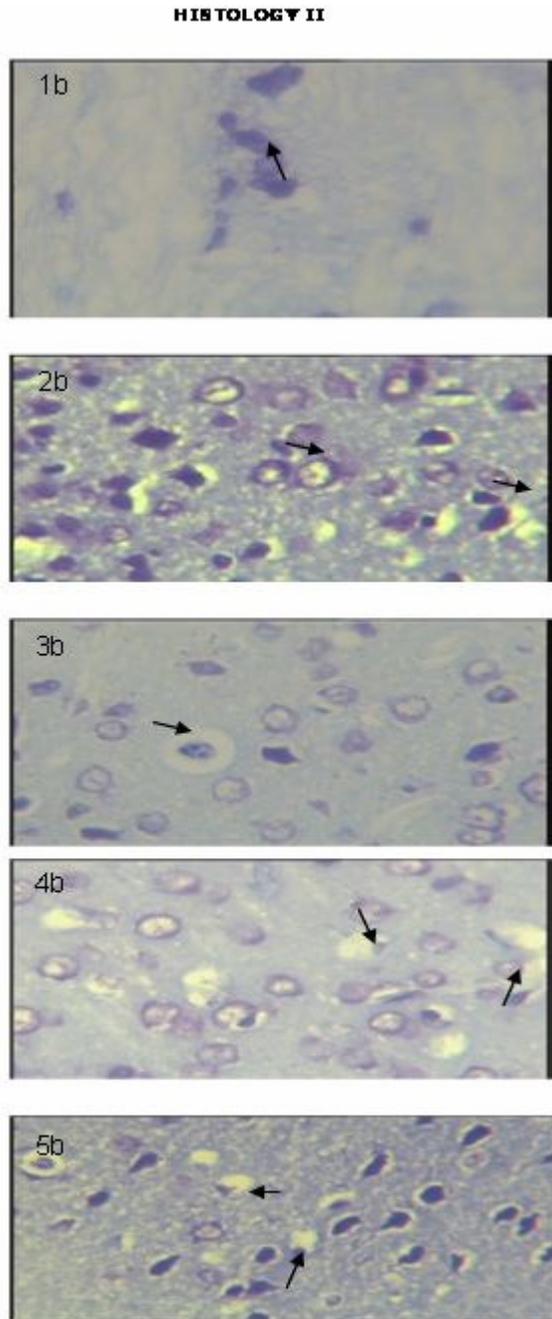


Figure 2. Comparative study of LGB (1b - 5b) for treatment and control groups. Magnification: X1000.

them are observed to be smaller than others and are surrounded by vacuolar spaces of 0.5 μm in diameter; 3A. The proportion varied with every position viewed and the degenerating cells appeared prominent in the upper portion of the outer pyramidal layer where cells are smaller and relatively more separated. They were less intensely stained relatively due to cellular degeneration and reduction in Nissl substance. In the superior colliculus, the deep layers showed more degenerative

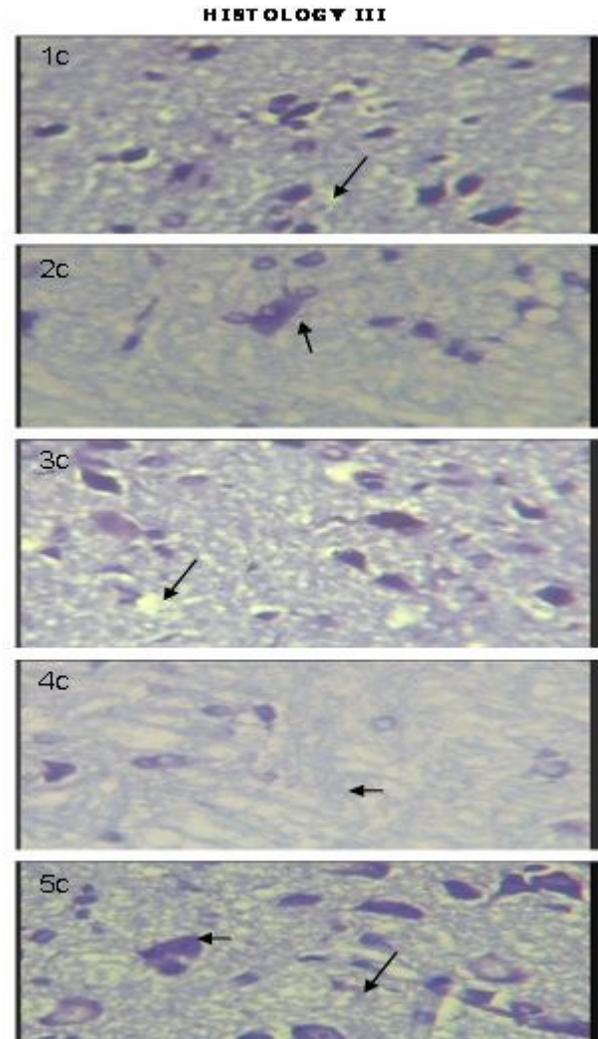


Figure 3. Comparative study of SC (1c-5c) for treatment groups. Magnification: X 1000 (Arrow heads indicates cell architecture and interlacing fibrous layers). Observe the Metachromasia in 5C the prominence of the axons radiating from the basket cells and the connections of the fibrous layers in between the cell rich layers.

changes, wherein the quantity of fibrous tissue had surpassed those of other layers (3C). Fibrous layers: The lateral geniculate body showed minimal degenerative changes in the parvocellular part and the magnocellular part remained relatively unchanged as one could distinguish between the spiny and non spiny neurons (3C).

The tufted ones are more distinct than the few bitufted and Chandler cells in the parvocellular layers. The neurons are dilated.

Group 2

There is an overall distortion of the general cellular arrangements as compared with those of the two

previous groups. The border between the molecular and the outer granular layer is obscured; arrow head in (Figure 1), 2A. Large vacuolar spaces of 0.8 μm in diameter are seen in the pyramidal and stellate cells of the outer granular and pyramidal layer and cells seemed to be undergoing degenerative changes. The inner granular and pyramidal layers show a decrease in the number of cells with enlarged cells interspersed among abnormally small cells. The fibrous layer is predominant throughout the superior colliculus in the stratum opticum just beneath the stratum Griseum Superficiale.

The cellular component is almost completely altered. Cells are small, with large prominent vacuoles while fibrous strands were observed in both directions rather than being interlaced as seen in the earlier groups. Presumably due to degenerating changes, the stratum Zonale however, remained relatively unchanged (Figures 3 and 2C). The parvocellular and magno-cellular layers (Figure 2), (3B) showed changes similar to those of the superior colliculus, the synapses appeared distorted and are seen as swollen knobs while the few cells observed are small and themselves surrounded by halos such that spaces are seen in between bundles, although certain areas appeared relatively unchanged (2C).

Group 1

The general, histological feature appeared conspicuously distorted and intensely stained, affecting cells in all layers of the visual cortex. This layer displayed large vacuolar spaces of about 1 μm (Figure 1), (1A) and obscured intercellular/interlayer bordering, in the superior colliculus and the lateral geniculate body. Identification is difficult even at $\times 1,000$ (light microscopy). In the superior colliculus, the fibrous strands predominates with small cells scattered in between the fibrous layers. The lateral geniculate body was observed as being more fibrous with fibres directed in one direction. Vacuoles are numerous and neurons are small and spiny (Figure 2 (1B) and Figure 3(1C)).

Group 4

This group shows histological features similar to those of the control. The major changes observed were in the outer pyramidal layer where certain pyramidal cells become enlarged compared to the surrounding cells. Vacuoles are more prominent and pale staining (Figure 1), (4A). Vacuoles are greatly decreased and the cells are smaller in size. Physical assessment of the laboratory animals; lethargy, sluggishness, and a progressive general weakness was observed with increasing dosage. Weight loss at the end of the period of administration was almost in high dose treatment group, which was abruptly compared to the low dose treatment and control groups. It is important to note the general decrease in the size of

the vacuolar spaces with reduction in the quantity of cassava in the diet.

DISCUSSION

In correlation with the histological appearance, the fourth group (0.12 mg of cyanide in 10 gms of cassava); showed an increased intercellular distance and implies high membrane transport activity indicated by a rise in alkaline phosphatase (ALP) activity. For the major experimental groups, there is a decreasing trend in (ALP) activity with increasing cyanide concentration. In comparing each major experiment against its corresponding withdrawal group, it was shown that the first two pairs displayed a decreased alkaline phosphatase activity, while the third category showed an increase. Thus, it could be inferred that high doses caused an increased alkaline phosphatase activities. Low doses also increased alkaline phosphatase activities but did not attain levels observed in the high dose treatment groups. In another experiment, withdrawal of group 3 coupled with banana extract administration (Methionine) caused neutralization of the cyanogenic glycoside leading to an increase in the rate of cellular regeneration in the withdrawal groups treated with the aqueous banana extract. As generally observed, increase in vacuolar spaces coupled with an increase in the distribution of such affected areas correlates with an increase in acid phosphatase activity per minute similar to the result of Di-Filipo et al. (2008).

Although, they went further to examine these parameters at higher dosage of cyanide extract but found out that the effects of cyanide at these doses were deleterious and almost irreversible on long term treatment. However, Osuntokun et al. (1981) reported an increase in the level of β -glucuronidase and sulphur excretion. All these structural changes and shift in enzyme activity was observed as a case of induced oxidative stress. However, statistically, the values are deviant from the median such that no regular pattern of change was observed (Oke, 1979; Nishimura et al., 2010), which implies that a multiple mode exist and the smallest value observed was zero. The mean activity at 180 s is 4.500 ± 0.49 . The changes in the level of activities of these enzymes were observed to be characteristic of cells undergoing oxidative stress. The distension observed in the cells of the molecular layer in the (30 gms of cassava) treatment group coupled with the increase in the size of the vacuolar space at first and a fluctuation afterwards will imply a phasic mechanism of neuronal damage, while the initial increase in acid and alkaline phosphatase activity will imply an early response to oxidative stress. Recall that in the (2.5 gms of cassava) treatment group, minimal changes were observed structurally, whereas in the histochemical quantification, they showed the highest fluctuation in enzyme activity. This was similar to the report of Okafor et al. (2002). Lower doses however, are capable of

Table 2. Result summary.

Group	V1	LGB	SC
1	Necrosis	Necrosis	Necrosis
2	Necrosis	Necrosis	Necro/Apoptosis
3	Apoptosis	Necrosis	Necro/Apoptosis
4	Necro/Apoptosis	Apoptosis	Necro/Apoptosis
5	Apoptosis	Necrosis	Necro/Apoptosis

inhibiting mitochondria activity thus causing the cell to heat up and expand as it is in a shift from aerobic to anaerobic metabolism. This is due to the effects of cyanide being capable of binding to the Fe^{2+} and Fe^{3+} present in Cytochrome oxidase thus impairing the electron transport chain. The integrity of the membrane is altered and its ability to mediate proper transport becomes jeopardized thus alkaline phosphatase activity tends to increase at first since it is the enzyme that mainly responsible for membrane transport.

Hubel et al. (1987) reported that a major association occurs in cyanide metabolism and calcium uptake which serves as a form of neutralizing mechanism in neurons. It is noteworthy to emphasize that calcium ions are capable of stimulating vacuole formation as observed in the normal electrophysiology of neurons, but may contain different membrane proteins that are not recognized as the usual cell inclusions, they are thus digested by lysosomal enzymes from the primary stage till the stage of formation of secondary autophagic bodies which were observed as vacuolar spaces in the treatment groups. This can be further elucidated by the results of the histochemical quantification which shows the initial high levels of acid phosphatase (a major lysosomal enzyme). In the moderate dose treatment group, some of the cells (Neurons) of the presumptive visual cortex appeared dead and shrunken and some other ones were found to be undergoing cellular degeneration, This may be attributed to a potential shift to anaerobic metabolism despite the presence of oxygen thus causing a type II Histotoxic anoxia, coupled with an increase and gradual build up of oxygen species (Isom et al., 1999; Nelson, 2006). The mode of cell degeneration of neurons found the high and low dose groups thus have the following features; distortion of membrane and axons, presence of large vacuolar spaces which are characteristic of necrosis while the moderate dose group showed decreased Nissl substance with larger vacuolar spaces and with little distortion in membrane activity compared to the high dose groups; the moderate dose have features of both apoptosis and necrosis which we describe as partial apoptosis and necrosis (Table 2), (Tor-Agbydye et al., 2003; Joshua et al., 2007).

In the superior colliculus and lateral geniculate body, as stated in the result, there was an increased fibrosis because the cells have regressed, and the axons are responding to the anoxic state of its corresponding

neuronal cytoplasm characteristic of necrosis, although vacuoles are present but are of negligible size, the prominent observation was obliteration of the cellular layer by fibrous layers. Thus, they showed a marked decrease in length and knobbed ends creating spaces in between cells. Osuntokun et al. (1981) and Oke (1979a) reported similar results but stated that it was non conclusive and that no positive correlation existed between the histological and statistical findings, no mechanism was described and the only enzyme assayed was β -gluco-ronidase in which an early increase was also reported to demonstrate the initial increase in lysosomal activity (Denison et al., 2009; Sotoblanco et al., 2002; de Haro, 2009), assayed for neurotransmitter hormones and they observed low activity and fluctuations in the level of cerebral calcium.

Osuntokun et al. (1981) treated the Wistar rats with amino acid supplements of tyrosine and methionine (Batharcharya and Tulwasami, 2008). Adopting the methods of De la Cruz et al. (2009), treatment with banana extract which contains sulphur and sulphur (containing amino acids) gave a faster regenerating effects in the withdrawal groups. Statistical analysis showed tendency towards normal in these withdrawal groups compared to the untreated withdrawal group. At this point, it will be emphasized that the cyanide in unprocessed *Manihot esculenta* even at low concentration are capable of inducing oxidative stress and state of histotoxic anoxia which may be restricted to certain regions of the visual relay centers depending on dosage and that this effects depends on the dietary pattern of the individual.

Conclusion

Cassava can be described as being neurotoxic, the gradual deteriorating vision in individuals in cassava endemic region can be attribute to the neurotoxic effects of the cyanogenic glycosides and other phytotoxins found in cassava, the mode of cell death and the morphological changes observed, is dose dependent and most important are protein diet dependent, therefore, if two persons are exposed to the same dose of cassava diet and one has protein supplements in addition, the neurotoxic effects of cassava will be less deleterious in the individual exposed to protein diets (Osuntokun, 1981). Sulphur containing amino acids like cysteine and tyrosine have been found to play an important role in the removal of cyanide as thiocyanate, the presence of this sulphur groups could be said to be the rate limiting factor in the excretion of cyanide as thiocyanate which is the major defense of the body against cyanide intoxication (Ernesto et al., 2002).

The toxicity of the phytotoxins follow a similar mechanism by inhibiting Cytochrome C oxidase, a terminal enzyme in the electron transport chain, thus generation NO(Nitric oxide) tension at complex I and III

(Isom and Way, 1984). NO are endogenous modulators of cell activity, but if present in excess concentration, it could trigger cell death, the concentration also determines the mode of cell death (Isom et al., 1999), other mechanism of toxicity involves cyanide binding to the binuclear centre by displacing molecular oxygen to create oxygen tension, this has been found to induce a state of histotoxic anoxia in cells (Li et al., 2000). The dose of the cassava in diet was found to have played a major role in the mode of cell death. The mode observed in the V1 was found to be an apoptosis-necrosis continuum for moderate dose treatment and necrosis for extreme dose treatment, while the SC and LGB was mainly necrosis irrespective of the dosage.

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