Cyanide cytotoxicity and the follicular cells of thyroid gland in male Wistar rats

***Adeniyi TD[1], Bejide RA[2], Musa AA[3], Adewole OS[4]**

Original Article

Abstract

Aim: This study was aimed at understanding the cytotoxic effect of cyanide as a result of ingesting inadequately processed cassava products linked with goitre formation as seen in cassava endemic region of Nigeria through cyanide-induced cytotoxicity in rats thyroid gland.

Materials and Methods: Twenty-one F_1 Male Wistar rats were randomly grouped into three of seven rats each. The treatment groups (1 & 2), were administered with different concentration of potassium cyanide, while group 3, the control group of the experiment was administered 0.25M sucrose for 30 days. On sacrifice, the rats were bled from which serum FT3, FT4 and TSH concentration were analysed. The excised thyroid gland was processed for light microscopic investigation while the activities of G6PDH, LDH, ALP, MDA and SOD were assayed from the thyroid tissue homogenates.

Results: Histological observation of thyroid gland of rats from the experimental treated groups revealed markedly distended follicles and diffusely hyperplastic thyroid follicles lined with tall columnar epithelial cells. Their colloids are vacuolated with scalloped edges. An increase in serum FT3 and FT4 with decrease serum TSH was obtained in the treated group. Increased levels of G6PDH, LDH, ALP, MDA and decreased SOD were also observed. Activities of G6PDH, LDH, ALP, MDA, SOD, FT3, FT4 and TSH were highly significant when likened to the control group using one-way ANOVA statistical analytical method.

Conclusion: Results of this study showed the effects of cyanide cytotoxicity on the follicular cells of thyroid gland.

Keywords: Cyanide, cytotoxicity, cassava, thyroid gland.

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La cytotoxicité cyanure et les cellules folliculaires de la thyroïde chez des hommes rats Wistar

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L'article d'origine

Résumé

Objectif : Cette étude visait à comprendre l' effet cytotoxique de cyanure à la suite de l'ingestion insuffisamment traitées le manioc produits liés avec le goitre formation comme on l'a vu dans le manioc région endémique du Nigéria avec cyanide- cytotoxicité induite chez le rat glande thyroïde.

Matériel et méthodes: Vingt-et-un F1 mâle Wistar rats ont été aléatoirement regroupées dans trois des sept rats chacun. Les deux groupes de traitement (1 & 2), ont été administrés avec différentes concentrations de cyanure de potassium, tandis que le groupe 3, le groupe de contrôle de l'expérience a été administré 0,25 M saccharose pendant 30 jours. Le sacrifice, les rats ont été purgés de qui serum FT3 FT4 et TSH concentration ont été analysées. L' excisée glande thyroïde a été traitée pour la lumière microscopiques enquête tandis que les activités de G6PDH, LDH, ALP, MDA et SOD ont été dosés dans la glande thyroïde homogénats tissulaires.

Résultats : observation histologique de la glande thyroïde de rats de l'expérimental groupes traités a révélé nettement distendu follicules et diffuse la thyroïde hyperplasiques follicules bordée de hautes colonnes cellules épithéliales. Les colloïdes sont vacuolated avec festonné. Une augmentation dans le sérum FT3 et FT4 avec diminution TSH sérique a été obtenu dans le groupe traité. Une augmentation des niveaux de G6PDH, LDH, ALP, MDA et diminué la SOD ont aussi été observés. Activités de G6PDH, LDH, ALP, la MDA, SOD, FT3 FT4 et TSH étaient hautement significatif lorsque comparé au groupe de contrôle utilisant un moyen statistique ANOVA méthode analytique.

Conclusion : Les résultats de cette étude ont montré les effets du cyanure sur la cytotoxicité des cellules folliculaires de la thyroïde.

Mots-clés : cyanure, cytotoxicité, le manioc, la glande thyroïde.

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Introduction

Cassava has high carbohydrate content which informs why it forms the main component in food of more than 800 million people (1). It is widely cultivated mostly in the tropics and sub-tropic for consumption as staple food (2). A major safety concern comes to bear since cassava roots contain cyanide in considerable quantities in the form of *Cyanogenic glycosides* (3). *Cyanogenic glycosides* found in cassava root is primarily *linamarin* and a small quantity of *lotaustralin* (ratio 93: 7) formed from Valine and Isoleucine amino acids respectively (4, 5).

These *Cyanogenic glycosides* when ingested are broken down during digestion relinquishing hydrogen cyanide gas which is cytotoxic (6). Cassava root is consumed in many rural households in Nigeria in forms of food products (7). These cassava products are usually processed to reduce the cyanide composition; they are mostly poorly processed containing certain level of cyanide. Due to the inadequacy of traditionally developed methods in removing cynogens from processed cassava products irrespective of whether the roots are low or high in cyanide content (8, 9), the result is cyanogenic cytotoxicity when consumed over time. These Cyanogens inhibits cellular enzymes of mitochondrial oxidative phosphorylation chain in cellular respiration and energy metabolism resulting in histotoxic hypoxia (10). More than required reactive oxygen species (ROS) are produced giving rise to oxidative stress which leads to cytotoxicity as a result of this inhibition (11).

Literatures established that goitre formation and cretinism is aggravated by continuing ingestion of inadequately processed cassava products due to iodine deficiency (12, 13, 14, 15, 16, 17). The possibility of cassava root inducing goitre formation does not solely depend on the proportional concentrations of cyanogenic component present in this fresh plant but likewise on its processing as food (12). Few publications exist regarding safe level of cyanide content in cassava products for humans. In 1993 the Joint committee of FAO/WHO on Food Additives and contaminant (JECFA) estimated the safe level of cyanogenic glucosides intake (18). The Committee concluded that in the absence of valued toxicological and epidemiological data, a common safe level could not be determined, but up to 10mg HCN equivalent/Kg dry weight, as defined by the codex Alimentarius (19, 20). Therefore, presence of cyanide above the safe level of 10mg HCN/Kg dry weight by FAO/WHO (20) of any cassava products, may pose health risk to the consumers (17, 21, 22).

This study aimed at understanding the cytotoxic effect involving cyanide exposure from ingesting inadequately processed cassava products linked with the goitre formation as seen in cassava endemic region of Nigeria (23) through cyanide-induced cytotoxicity in the rats thyroid gland.

Materials and methods

Experimental Animals

Twenty one 21 first filial (F1) generation in bred adult male Wistar rats (*Rattus novergicus*) with mean weight of 200 gm were procured from the animal facility of Department of Biomedical Sciences, College of Health Sciences, Ladoke Akintola University of Technology, Osogbo, Osun State, Nigeria. All animals were at random separated to three groups comprising seven rats in each group. Groups 1 and 2 represented the treatment groups while group 3 was the control of the experiment. The cyanide treatment dose were; group1 (6 mg/KgBW) and group 2 (12 mg/KgBW) while the control group (Group 3) received 0.25M Sucrose (solvent for cyanide solution). This research was conducted in consonance with the stipulations in "Guide for the Care and Use of Laboratory Animals." (24)

Treatment solution and mode of administration

A standard isotonic solution of 0.25 M sucrose was prepared to dissolve the potassium hexacyanoferrate III, $K_3Fe(CN)_6$; Mol Wgt = 329.25: in order to obtain a final working solution of concentration 5 mg/ml of potassium hexacyanoferrate in 0.25 M sucrose solution. 5 gm of the CN salt was dissolved in 1000 ml of 0.25M sucrose solution (β-D-Fructofuranosyl-α-D-Glycopyranoside; $C_{12}H_{22}O_{11}$; Mol. Wgt = 342.30) (25).

Method of administration
The animals were fed orally using oral cannula with a ball point at the tip. The animals were held with a glove with the left hand such that the neck region was held by the fingers to stabilize the neck while being fed with the cannula. Treatment was done at 07.00 hour every day before the animals were fed.

Treatment of Animals
The treatment duration was 30 days. The animals were kept under standard laboratory condition of good lighting (12 hours light and 12 hours dark), moderate temperature with average prevailing temperature of 27°C, and adequate ventilation in a hygienic environment. They were fed on standard rat chow containing proteins, carbohydrate, fats, vitamins, minerals and water ad libitum.

Methods
The animals were sacrificed by cervical dislocation in a humane way. Plain universal bottles were used to collect blood under aseptic conditions. The thyroid gland was excised following midline-abdominal incision to the neck region. The thyroid gland is a dark solid organ on the ventral aspect of the trachea. Specimens for histological investigations were fixed in formol saline and processed for paraffin wax embedding. Sections of 3µm thickness were sectioned on Leica rotary microtome (Leica RM2125RT) and stained with Haematoxylin and Eosin (26) and Periodic Acid Schiff (PAS) (27). Those for quantitative histochemical were preserved separately in cold 0.25M sucrose (Isotonic solution) and were homogenized with Polter-Elvhjem homogenizer.
The homogenates were centrifuged at 5000rpm for 10 minutes. The supernatants were immediately stored in the freezer (-20°C) and assayed within 48 hours. The activities of G6PDH, LDH, ALP, MDA and SOD were determined spectrophotometrically in the homogenate by the methods of Kletzien *et al.* (1994) (28), Wei Bhaar *et al.* (1974) (29), Babson *et al.* (1966) (30), Pasha and Sadasivadu (1984) (31) and Marklund and Marklund (1974) (32) respectively.
Serum blood was assayed through immunoenzymometry method for the concentration of FT3, FT4 and TSH by the methods of *Wild (1994) (33), Lee et al. (2009) (34) and Fisher (1996) (35) respectively.*

Statistical Analysis
Values were expressed as mean ± SEM (standard error of the mean). Statistical software SPSS version 17 was used to determine differences between means and regarded statistically significant at $P<0.05$ by application of one-way analysis of variance (ANOVA).

Results
Histological observations
The control group (group 3): the thyroid gland of the control rats administered with 0.25M sucrose was composed of follicles lined with a single layer of columnar follicular cells (Figure 1a and 1b). The follicular cells had vesicular nuclei and prominent nucleoli (Figure 1b).
The experimental groups (Group 1 - 2): on examination of thyroid gland of rats of the experimental groups administered with different concentration of potassium hexacyanoferrate revealed markedly distended follicles and diffusely hyperplastic thyroid follicles lined with follicular cells ranging between squamous and tall columnar epithelial cells. These thyroid epithelial cells are crowded and enlarged (*i.e.* showing cell increase in size and number) projecting into the lumens of their respective follicles. The follicles showed variable density of colloid staining with scalloped edges (Figure 2b) when compared with the control group (Figure 1b).
The outcome from this work demonstrated that Cyanide toxicity significantly affected the activity of G6PDH, LDH, ALP, MDA, SOD (Table 1), and FT3, FT4, TSH (Table 2).

Discussion
In this study two parameters were investigated to describe morphologically the several structural changes associated with the follicular cells cytotoxicity response in the thyroid gland. While Haematoxylin and Eosin demonstrated cell morphology, the Periodic Acid-Schiff method demonstrated the integrity of the basement membrane and the colloid of the thyroid gland. These complimentary and

independent methods demonstrated structural changes in the thyroid gland of Wistar rats treated with potassium hexacyanoferrate. The photomicrograph of rats treated with 6mg/Kg BW of cyanide shows characteristic features of hyperplastic thyroid. Each of the follicular cells is large and columnar and the edges of the colloid are scalloped (Figure 2), indicating active removal of stored colloid for processing into thyroxine (36). This could explain the high values of FT4 followed by FT3 obtained from the experimental groups (Table 2). Total loss of cellular boundary and pale nucleus were observed in follicular cells of rats treated with 12mg/Kg BW of cyanide (Figure 3). They are lined by epithelial cells ranging between squamous and columnar which is regulated by TSH. These microscopic findings obtained from cyanide-induced rats emphasized characteristic features as seen in thyrotoxic hyperplasia, a thyrotoxicosis or hyperthyroidism condition (36, 37).

Follicular cells of the thyroid gland mainly function in the production and discharge of thyroid hormones. In this study, their function was evaluated by measuring the concentration of serum thyroid hormones (FT3 and FT4), and pituitary TSH. Elevated concentration of FT3 and FT4 with a depressed concentration of TSH sustained through all the experimental groups was observed. This finding showed significant difference on comparing with the control group which perfectly describes thyrotoxicosis, a hyperthyroidism condition (39) thereby supporting our histological findings. We further quantify for enzymes of carbohydrate metabolism and oxidative stress markers. These two influence cytotoxicity by inhibiting cellular enzymes of mitochondrial oxidative phosphorylation chain in cellular respiration and energy metabolism. They were studied as clinical indicators of cyanide cytotoxicity.

G6PDH, a cytosolic enzyme, has the role of starting off the pentose phosphate pathway. Within this pathway, G6PDH changes nicotinamide adenine dinucleotide phosphate ($NADP^+$) into its reduced form, NADPH. This eventually converts glucose-6-phosphate into a pentose sugar (ribulose-5-phosphate), a precursor of deoxyribonucleic acid, ribonucleic acid, and adenosine-triphosphate (38). In our study, G6PDH was investigated as an indicator of oxidative metabolism. From our result, G6PDH activity levels were increased with increase cyanide-induced concentration. This showed significant difference when compared with the control group. From literatures, thyrotoxicosis influences a catecholamine-induced inhibition of insulin release resulting in hyperglycemia (39).

LDH catalyzes the change of pyruvate to lactate with associated oxidation of NADH all through the final stage in anaerobic glycolysis (40). In our study, LDH activity levels were increased with increased cyanide-induced concentration which showed significant difference on likened to the control group. A move from aerobic to anerobic metabolism is seen as an effect of inhibition of mitochondrial electron transport chain activity induced by cyanide which finally results in lactic acidosis (41, 42, 43). This might inform the wide difference obtained in the LDH activity of cyanide-induced rats when compared with the control.

There is dearth of information on how follicular cells utilize energy to carry out their metabolic processes in hyperthyroidism condition. However, hyperthyroidism confers high basal metabolism rate due to the over activity of the thyroid gland. This might as well contribute to the high values of G6PDH and LDH obtained in cyanide-induced rats.

ALP primarily helps transport across cell membranes, inducing breaking down of ATP to ADP and inorganic phosphate, thereby creating free energy for metabolic process (44). The levels of ALP will not only serve as an indicator of membrane activity but also a regulatory measure. In our study, ALP activity levels were increased with increased cyanide-induced concentration. This also showed significant difference when compared with the control group. Elevated alkaline phosphatase is among the major laboratory findings associated with thyrotoxicosis (39) which our finding supported. This as well explains the loss of membrane integrity seen as reduce and total loss of cellular boundary reported in this study, (Figure 2 & 3).

MDA is a natural result of peroxidation of unsaturated fatty acids with three or more

double bonds. Increase levels of superoxide anions and ROS will in turn enhance lipid peroxidation. ROS causes damage to crucial cellular structures. They respond with membrane lipid thus destroying the cell membranes (45). The cell membrane according to the model proposed by Singer and Nicolson, (1972) (46) present a two dimensional sea bed of lipids with floating ice bergs of proteins. This explains the vital role lipid plays in maintaining membrane integrity. In this study, cytotoxicity might have contributed lipid peroxidation indicated by increase values of MDA assay from the homogenate of the experimental groups with increase concentration of cyanide administered which showed significant difference on comparing with the control group, thereby, supporting the findings of Hariharakrishan *et al.* (2009) (11). In correlation with the histological appearance (Figure 2b & 3b), the effect of lipid peroxidation might account for the reduced and/ total loss of membrane integrity observed. Literature also affirmed that hyperthyroidism result in increase MDA level owing to likely changes in the cellular respiration of intended tissues (47, 48).

SOD is an intracellular oxygen radical-scavenging enzyme that catalyses the dismutation of superoxide anion radical to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). ROS and superoxide anion are toxic because of their high reactivity potential which is disruptive to cells and tissues (49). Follicular cells contain variety of substances among which is SOD, that protects them from this harmful effect. We observed a decreased SOD activity with increase in concentration of cyanide in this study. This finding clearly showed significant difference on comparing with the control group which also supported the findings of Hariharakrishan *et al.* (2009) (11). According to Lampka et al. (2006), increase production of ROS and alteration in the antioxidant defense system are indices of hyperthyroidism induced oxidative stress (50). This might inform the outcome of our findings. Asayama and Kato, (1990), as well proposed that thyroid hormone-induced oxidative stress in intended tissues causes hyperthyroidism problems (51), suggesting oxidative stress was effectively induced by cyanide cytotoxicity.

Conclusion

The results from this study showed that hyperthyroidism was effectively induced by cyanide cytotoxicity which has the potential of leading to goitre formation.

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Table 1:Quantitative histochemical result

Treatment dose	G6PDH (U/L)	LDH (U/L)	ALP (U/L)	SOD (unit/ml)	MDA (nmol/dl)
6mg/Kg	4876.33 ±2.5*	1369.00±38.4*	1929.17±18.1*	69.67±1.5*	0.0080±0.0003*
12mg/Kg	4938.50±3.8*	1470.50±51.7*	2648.17±19.2*	59.50±1.3*	0.0127±0.0015*
0.25M Sucrose	4305.17±210.5	182.33±2.7	326.67±1.7	29.83±1.7	0.0137±0.0013

Number of subjects (n) = 6

Mean ± S.E.M = Mean values ± Standard error of mean.

Analysis was done using one way ANOVA. Cyanide cytotoxicity: *significance (P< 0.05) from control group. Cyanide toxicity significantly affected the activity of G6PDHR(=0.00), LDH (P=0.00), ALP (P=0.00), SOD (P=0.00) and MDA (P =0.00).

Table 2: Thyroid hormones assay result

Treatment dose	FT3 (Pg/ml)	FT4 (Pg/ml)	TSH (µIU/ml)
6mg/Kg	7.71±1.3*	22.09±6.1*	0.15±0.0*
12mg/Kg	4.65±0.4*	11.81±0.8*	0.17±0.0*
0.25M Sucrose	1.03±0.3	9.38±0.6	0.21±0.7

Number of subjects (n) = 6

Mean ± S.E.M = Mean values ± Standard error of mean.

Analysis was done using one way ANOVA. Cyanide cytotoxicity: *significance (P<0.05) from control group. Cyanide significantly affected the activity of FT3 (P=0.00), FT4 (P=0.00) and TSH (P=0.00).

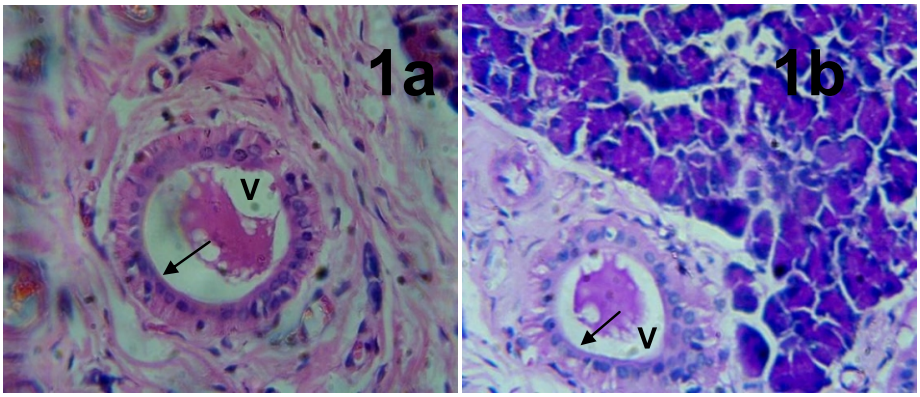

Figure 1: Photomicrographs of sections in the thyroid gland of rats from the control group (group 3) shows normal thyroid follicle lined with simple columnar epithelium (arrows) 1a & 1b. Their colloid is vacuolated with scalloped edges with lumen containing stained secretion (V). 1a (H&E X400), 1b (PAS X400)

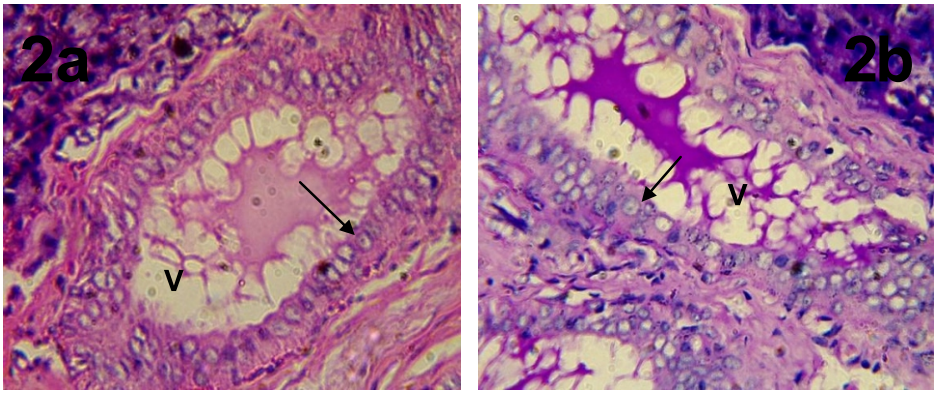

Figure 2: Photomicrographs of sections in the thyroid gland of rats from the experimental group (group 1) shows an enlarged follicle 2a & 2b. The lining epithelium is intensely hyperplastic. It is composed of tall columnar cells which have prominent vesicular nuclei and some bearing nucleoli (arrows). There is some colloid but it is relatively vacuolated and scalloped edges with their lumen containing strands of pale staining secretion (V). 2a (H&E X 400), 2b (PAS X400).

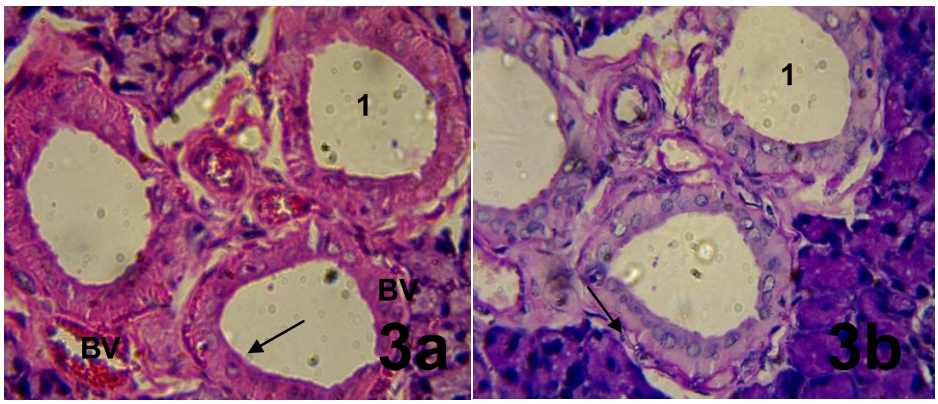

Figure 3: Photomicrographs of sections in the thyroid gland of rats from the experimental group (group 2) shows thyroid follicles are markedly distended (1) 3a & 3b. The columnar follicular cells show loss of cellular boundary and pale nucleus with vesicular nuclei (arrows). There are also dilated blood vessels (BV). 3a (H&E X400), 3b (PAS X400).