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Histochemical Investigation of Cyanogenic Toxicity in the Thyroid Epithelial Cells and Follicles of Thyroid Gland in Adult Female Wistar Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Author TDA concept and designed the study, managed the experimental studies, managed the literature search, performed the laboratory analysis and interpretation of result, the statistical analysis and wrote the first draft of the manuscript. Authors RAB and OMO contribute to the concept and design of this work, contribute to the analyses and interpretation of result. Author OBA contributes to the definition of intellectual content. All authors read and approved the final manuscript.

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ABSTRACT

Aim: This study was aimed at understanding the earlier findings involving chronic, low-level cyanide exposure resulting from eating poorly processed cassava products associated with the development of goitre as seen in cassava endemic regions of Nigeria.
Study Design: 30F_1 female adult Wistar rats were divided into five (5) groups of 6 animals each. Groups 1 to 4 represented the treatment groups while group 5 was the

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control of the experiment. The cyanide treatment dose were; group1: 20mg/KgBW, group 2: 12mg/KgBW, group 3: 6mg/KgBW and group 4: 2mg/KgBW while the control group received 0.25M Sucrose.
Place and Duration of Study: The animal facility of College of Health Sciences, Osun State University, Osogbo, Osun State, Nigeria. The treatment duration was 30days.
Methodology: Animals were sacrificed by cervical dislocation. The blood samples were collected to determine Serum FT3, FT4 and TSH concentration. The thyroid gland was excised and processed for light microscopic examination; 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. These thyroid epithelial cells are crowded and enlarged projecting into the lumens of their respective follicles. Their interstitial tissue all had dilated blood vessels. Application of one-way ANOVA statistical analytical method showed that there were highly significant differences $P<0.05$ in the activities of G6PDH, LDH, ALP, MDA, SOD, FT3, FT4 and TSH when compared with those of the control group.
Conclusion: The results obtained from this study showed hyperthyroidism was effectively induced by cyanide.

Keywords: Cyanide; cassava; thyroid gland; goitre.

1. INTRODUCTION

The term cyanide is as old as man's civilization itself [1]. It exists freely and is highly ubiquitous in nature. Cyanide is among the potent toxic and deadly poisons and sources of potential human exposure to it are numerous [2]. The most common source of cyanide in human is via oral means, most of which is associated with diet [3]. Cyanide occurs naturally as *cyanogenic glycosides*, a phytotoxin, in many plants and plant products use as food among which is cassava root [4,5]. These *cyanogenic glycosides* release hydrogen cyanide, a cytotoxin when ingested and is often uttered as milligram releasable cyanide [6]. Cassava root is a major source of dietary carbohydrate for humans and animals in tropics and subtropics which have been estimated as staple food for 500 million people [7,8].

Cassava root is consumed in form food products and these products are available year around thus making cassava an important basic food for many rural households in Nigeria [9]. When these cassava products are poorly processed and are consumed over a period of time, toxicity will result. Cyanide toxicity has been reported in thyroid gland of experimental animals treated with cyanide on a short and long term basis [10,11], which was seen in the perceptive of inducing oxidative stress in the thyroid cells. In rats, cyanide toxicity causes increased lipid peroxidation, raised free radical and superoxide anion [12]. The cumulative effect of exposure from breakdown of cyanide glycosides in food plants acts by suppressing the trapping iodine by the thyroid cells thereby altering the synthesis of thyroid hormone and thyroid function [13] eventually resulting in goitre and cretinism [14].

Few publications exist regarding safe levels of cyanide in cassava products for humans. The Joint FAO/WHO Expert committee on Food Additives and contaminant (JECFA) concluded that in the absence of quantitative toxicological and epidemiological information, a general safe level of cyanogenic glycoside intake could not be determined but a level of up to 10mg HCN equivalent/Kg dry weight, as defined by the codex Alimentarius [15]. Hence, the effect

of cyanide above the safe level of 10mg HCN/Kg dry weight of any cassava products, may present health risk to the consumers [16,17].

This present study adopted chemical induction of cellular alterations through cyanide intoxication. The morphology of cells in the thyroid gland of rats under toxic condition was examined. This was aimed at understanding the earlier findings involving chronic low-level cyanide exposure resulting from eating poorly processed cassava products associated with the development of goitre as seen in cassava endemic region of Nigeria (Akoko area of southwest Nigeria) [18].

2. MATERIALS AND METHODS

2.1 Experimental Animals

All experimental procedures followed the recommendations provided in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Academies Press [19]. Thirty first filial (F1) generation in bred adult Female Wistar rats (*Rattus novergicus*) with an average weight of 200 g were secured from the animal facility of College of Health Sciences, Osun State University, Osogbo, Osun State, Nigeria. All animals were randomly assigned into five groups comprising six rats in each group. Group 1 to 4 represented the treatment groups while group 5 was the control of the experiment. The treatment groups received different doses of cyanide solutions; with group 1: 20mg/KgBW, group 2: 12mg/KgBW, group 3: 6mg/KgBW and group 4: 2mg/KgBW while the control group received 0.25M sucrose. This was adopted according to the protocol cited by Ogundele et al. [6].

2.2 Treatment Solutions and Mode of their 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: Sigma in order to obtain a final working solution of concentration 5 mg/ml of potassium hexacyanoferrate in 0.25 M sucrose solution. 5 g 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: Sigma) [20].

The animals were force fed orally using oral canula with a ball point at the tip. They were held with a gloved left hand such that the neck region was held by the fingers to still the neck while being fed with the canula. Treatment was done once daily at 07.00 o'clock just before the animals were fed. The treatment duration was 30 days. They were kept under standard laboratory condition of good lighting, moderate temperature and adequate ventilation in a hygienic environment and fed on standard rat chow containing proteins, carbohydrate, fats, vitamins, minerals and water ad libitum.

2.3 Histological, Biochemical and Histochemical Methods

The animals were sacrificed by cervical dislocation. Blood samples were collected by jugular venepucture into plain universal bottle 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. The specimens for routine histological investigations were fixed in formol saline and processed for paraffin wax embedding. Serial sections of 3μm thickness were stained with Haematoxylin and Eosin (H&E) [21] and

Periodic Acid Schiff (PAS) [22]. Those for quantitative histochemical evaluation 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 (-20ºC) and assayed within 48 hours. Through spectrophotometry, the activities of G-6-PDH, LDH, ALP, MDA and SOD were determined in the homogenate by the methods of Kletzien et al. [23], Wei Bhaar et al. [24], Babson et al. [25], Pasha and Sadasivadu [26] and Marklund & Marklund [27] respectively.

Serum blood was immunoenzymometric assayed for the concentration of FT3, FT4 and TSH by the methods of Wild [28], Lee et al. [29] and Fisher [30] respectively.

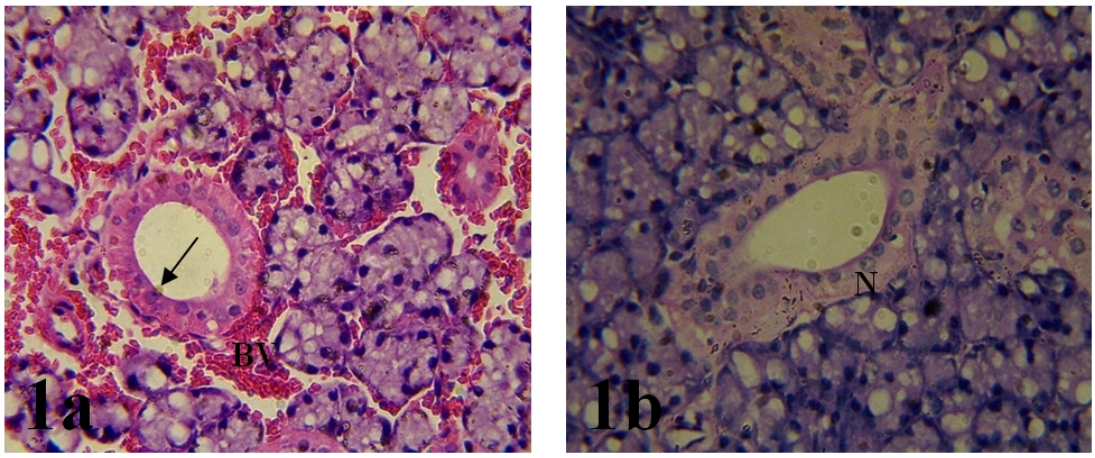
2.4 Statistical Analysis

Values were reported as mean ± SEM (standard error of the mean). Significance was determined statistically by application of one-way analysis of variance (ANOVA) using statistical software SPSS version 17 at 95% confidence interval. Differences between means were considered statistically significant at $P<0.05$.

3. RESULTS AND DISCUSSION

3.1 Histological Observations

The control group (group 5): the thyroid gland of the control rats administered with 0.25M sucrose was composed of follicles lined with a single layer of cuboidal follicular cells (Figure 1a and 1b). The follicular cells had vesicular nuclei and prominent nucleoli (Figure 1b).



(a) (b)

Figure 1. Photomicrographs of sections in the thyroid gland of rats from the control group (group 5) showing: (a) Normal thyroid follicle lined with simple cuboidal epithelium (arrow). There are blood vessels (BV) in the surrounding connective tissue stroma. H&E X 400 and (b) It shows that the thyroid follicle is lined with simple cuboidal follicular cells that have vesicular nuclei (N) with prominent nucleoli. PAS X 400

The experimental groups (Group 1- 4): 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 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. Their interstitial tissue all had dilated blood vessels. The follicles showed variable density of colloid staining. In addition to the described features, some thyroid follicles showed presence of desquamated epithelial cell inside the follicle (Figure 2a). While some coalesced together to form a large cyst (Figure 2b), some revealed focal involuted follicles with minimal amount of colloid (Figure 2b). The wall of some follicles was interrupted (Figure 3a). The thyroid epithelial cells of some follicles present atypical nuclear features (Figure 3b). The cytoplasm of some affected thyrocytes was vacuolated (Figure 5a and 5b).

3.2 Biochemical Observations

Result obtained from the present study showed Cyanide toxicity significantly affected the activity of G6PDH, LDH, ALP, MDA and SOD (Table 1). Cyanide also significantly affected the activity of FT3, FT4 and TSH (Table 2).

Table 1. Quantitative histochemical result

Treatment dose	G6PDH (U/L)	LDH (U/L)	ALP (U/L)	SOD (unit/ml)	MDA (nmol/dl)
20mg/Kg	3213.50±1.08*	282.50±3.26*	919.17±1.70*	5.24±0.55*	0.010±0.0003*
12mg/Kg	3063.50±1.23*	278.33±1.28*	886.17±2.48*	8.72±0.43*	0.008±0.0006*
6mg/Kg	4048.50±1.09*	255.33±1.26*	749.50±1.61*	13.38±0.62*	0.007±0.0003*
2mg/Kg	4841.50±0.99*	233.50±2.09*	620.67±3.50*	32.87±0.73*	0.004±0.0004*
0.25M Sucrose	3230.50±61.77	161.50±1.26	222.67±1.17	3.30±0.59	0.002±0.0006

Mean ± S.E.M = Mean values ± Standard error of mean.
*Analysis was done using one way ANOVA. Cyanide toxicity: significance from control group, *P<0.05. Cyanide toxicity significantly affected the activity of G6PDH (P=0.00), LDH (P=0.00), ALP (P=0.00), SOD (P=0.00) and MDA (P=0.00).*

Table 2. Thyroid hormones assay

Treatment dose	FT3 (Pg/ml)	FT4 (Pg/ml)	TSH (µlU/ml)
20mg/Kg	4.83±0.39*	6.73±1.50*	0.23±0.02*
12mg/Kg	3.13±0.33*	9.99±1.84*	0.23±0.01*
6mg/Kg	5.40±0.59*	16.76±1.12*	0.09±0.03*
2mg/Kg	2.20±0.08*	4.12±0.44*	0.03±0.01*
0.25M Sucrose	3.19±0.30	11.77±1.54	0.17±0.03

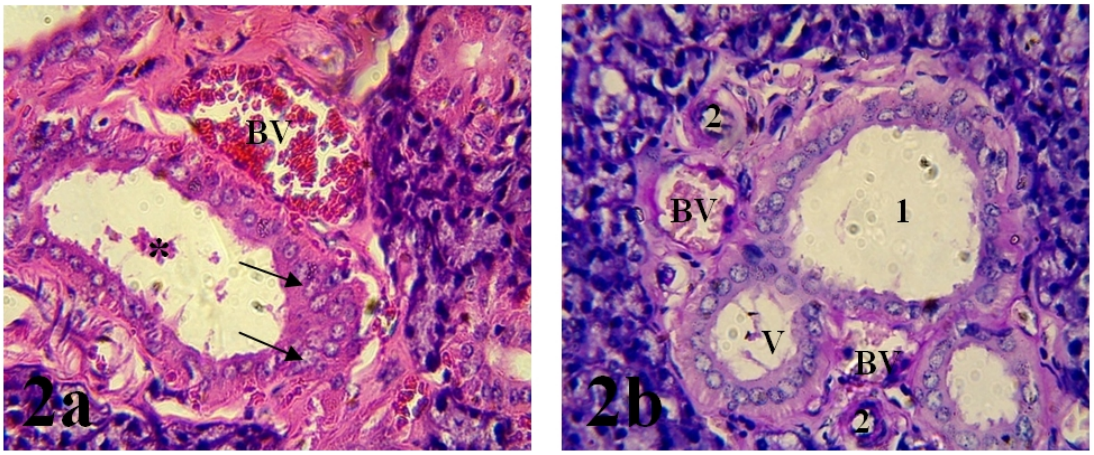
Mean ± S.E.M = Mean values ± Standard error of mean.
*Analysis was done using one way ANOVA. Cyanide toxicity: significance from control group, *P<0.05. Cyanide significantly affected the activity of FT3 (P=0.00), FT4 (P=0.00) and TSH (P=0.00).*

3.3 Discussion

A safety concern among cassava products consumers arises from the presence of *cyanogenic glucosides* which upon hydrolysis produces cyanohydrins that further breaks down to release hydrogen cyanide [31]. These food substances despite being processed to remove their cyanide composition are found to contain it to certain level. Since the traditionally developed methods of processing cassava products have been found to be grossly inadequate in the removal of cyanogens resulting into cyanogenic toxicity irrespective of the roots being of a low or high cyanide variety [32,33].

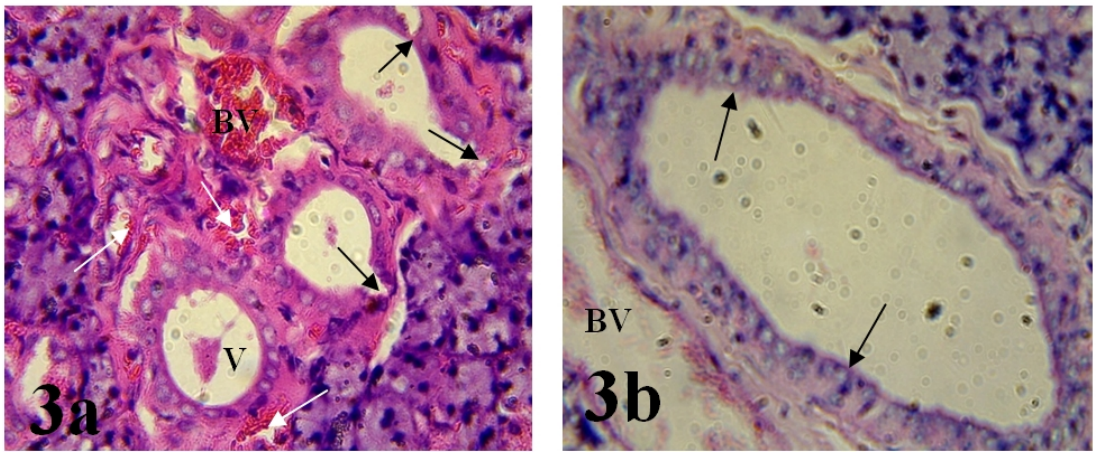
3.3.1 Histological findings

Two histological methods were studied to describe possible morphological changed of thyroid tissue in response to cyanide toxicity. H & E demonstrated cell morphology while PAS demonstrated the integrity of the basement membrane and the colloid. These methods revealed marked distortion of follicular structure in rats treated with cyanide. Each of the follicular cells is large and columnar. They show characteristic of hyperplastic thyroid. Dilated blood vessels were observed frequently in their interfollicular connective tissue indicating increase vascularity and blood flow to the thyroid gland. Specifically, light microscopic examination of group 1 had desquamated epithelial cells in their lumen. These changes could be attributed to cellular distension which resulted in cellular disruption and collapse with subsequent collapse of the follicles (Figure 2a). Distended follicles are coalesced to form a large cyst (Figure 2b). Light microscopic examination of group 2, thyroid tissue revealed follicles with interrupted follicular wall (Figure 3a). Light microscopic examination of group 3 revealed vast vacuolated colloid (Figure 4a and 4b), indicating active removal of stored colloid for processing into thyroxine [31]. This could explain the high values of FT4 followed by FT3 obtained from this experimental group (Table 2). Follicles in group 4 showed epithelium forming papiliferous projections into the lumen with lining epithelial cells showing degenerative changes such as vacuolated cytoplasm. Loss of cellular membrane and pale nucleus were as well observed (Figure 5b). While the control group showed normal thyroid tissue architecture.



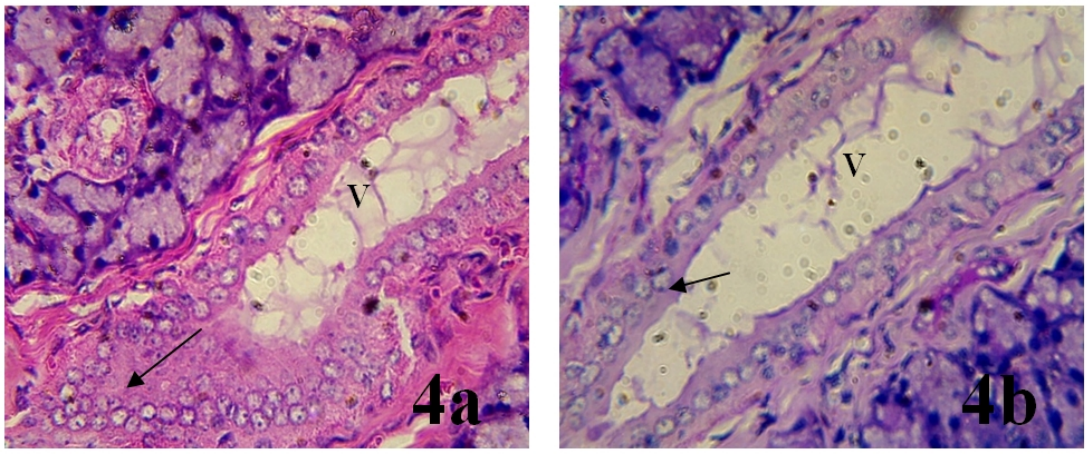
(a) (b)

Figure 2. Photomicrographs of sections in the thyroid gland of rats from the experimental group (group 1) showing: (a) The thyroid follicle is enlarged with presence of desquamated epithelial cells inside the follicle (*). It shows marked epithelial hyperplasia and lined by tall columnar cells (arrows). There is a dilated blood vessel (BV). H&E X 400 and (b) It shows thyroid follicles with variable activity as some follicles are markedly distended (1) and other follicles appear involuted and are small in size with minimal amount of colloid (2). The large follicles have coalesced to form a large cyst. The follicles are all lined with follicular cells having vesicular nuclei and vacuolated colloid (V). There are also dilated blood vessels (BV). PAS X 400



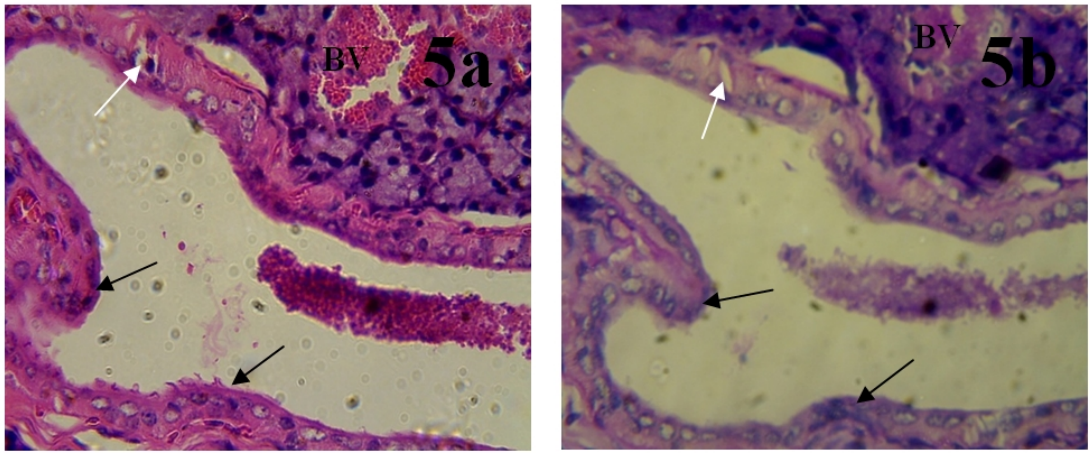
(a) (b)

Figure 3. Photomicrographs of sections in the thyroid gland of rats from the experimental group (group 2) showing: (a) It reveals follicles with interrupted follicular wall (black arrows) and vacuolated colloid (V). There is a dilated blood vessel (BV) and interstitial haemorrhage (white arrows). H&E X 400 and (b) It shows an enlarged thyroid follicle line by tall columnar epithelial cells (arrows). There is also marked epithelial hyperplasia with atypical nuclear features (arrows). PAS X 400



(a) (b)

Figure 4. Photomicrographs of sections in the thyroid gland of rats from the experimental group (group 3) showing: 4a & 4b. It reveals an enlarged follicle. The lining epithelium is intensely hyperplastic. It is composed of tall columnar cells which have prominent vesicular nuclei and nucleoli (arrows). There is some colloid but it is relatively vacuolated with their lumen containing strands of pale-staining secretion (V). 4a (H&E X 400), 4b (PAS X400)



(a) (b)

Figure 5. Photomicrographs of sections in the thyroid gland of rats from the experimental group (group 4) showing: a & b. It reveals an enlarged follicle with marked epithelial hyperplasia. It is lined by tall columnar epithelial cells having prominent vesicular nuclei. The epithelium form papiliferous projections into the lumen (black arrows). Some of the lining cells show degenerative changes such as vacuolated cytoplasm (white arrows). The lumen shows strands of pale-staining secretion. There is a dilated blood vessel (BV). 5a (H&E X 400), 5b (PAS X 400)

These findings emphasized that cyanide-induced microscopic feature of thyroid gland mimic recorded cases of Grave's disease with characteristic features as seen in thyrotoxic hyperplasia, a thyrotoxicosis or hyperthyroidism condition [34,35]. Surprisingly, literatures dealing with cyanide-induced microstructure of the thyroid epithelial cells and follicles remain scanty. Histological findings from this study showed hyperthyroidism was effectively induced by cyanide.

3.3.2 Thyroid hormones findings

Follicular cells of the thyroid are designed for hormone synthesis and secretion. Thyroid hormones (TH) are needed for the normal function of most tissues of the body [36]. Serum levels of thyroid hormones, including T3, T4 and TSH are commonly used as reliable indicators of the thyroid function in humans and experimental animals.

In this study, the function of thyroid epithelial cells was evaluated by measuring the levels of serum thyroid hormones such as free T3 and free T4, and pituitary TSH. There was elevated concentration of free T3 and free T4 with a depressed concentration of TSH sustained through all the experimental groups. This finding showed significant difference on comparing with the control group. This result perfectly describes thyrotoxicosis or a hyperthyroidism condition [37], which supported our histological findings. Clinical hyperthyroidism, also called thyrotoxicosis, is caused by the effects of excess thyroid hormone and can be triggered by different disorder. The result also showed that the effect was more in experimental group 3 followed by group 2, 1 and 4 respectively as seen in Table 2.

The reason for variations observed in this result is suggestive of varying tolerance ability of the rats. Since the production of TSH, T3 and T4 are under the control of the hypothalamus through the thyroid gland. However, results obtained from microanatomical structures of the thyroid tissues of the treated groups shows significant damage to the thyroid epithelial cells and follicles when compared with the control group.

3.3.3 Quantitative histochemical findings

One of the major functions of thyroid hormone secreted from the thyroid gland is the acceleration of the basal metabolic rate and the energy metabolism of tissues in several mammalian species [38].

Glucose-6-phosphate dehydrogenase (G6PDH), being a cytosolic enzyme, catalyzes the initial step of the pentose phosphate pathway which is a rate limiting step in glucose metabolism as to whether it follows glycolysis or amino acid synthesis. G6PDH converts nicotinamide adenine dinucleotide phosphate ($NADP^+$) into its reduced form, NADPH, and glucose-6-phosphate is then converted into a pentose sugar (ribulose-5-phosphate). The 5-carbon sugar is a precursor of DNA, RNA, and ATP [39]. G6PDH protects erythrocytes from oxidants such as H_2O_2 [40]. In our study, G6PDH activity levels were increased with decrease cyanide-induced concentration. This showed significant difference when compared with the control group. The result obtained from those administered with 20mg/Kg BW and 12mg/KG BW is suggestive of varying tolerance ability of the rats.

Lactate dehydrogenase (LDH) is an enzyme found in almost all body tissues. It catalyzes the conversion of pyruvate to lactate with concomitant oxidation of NADH during the last step in anaerobic glycolysis [41]. It converts pyruvate, the final product of glycolysis to lactate when oxygen is absent or in short supply. In our study, LDH activity levels were increased with

increased cyanide-induced concentration. This showed significant difference when compared with the control group.

Alkaline phosphatase (ALP) is a group of catalytic proteins found in all body tissues sharing the capacity to hydrolyze phosphate esters in alkaline medium [42]. ALP has been found to act opposite kinases, a function shared with endonucleases. ALP mainly facilitates transport across cell membranes, causing the breakdown of ATP to ADP and inorganic phosphate, thereby making free energy available for metabolic process [43]. ALP has also been described as an enzyme with regulatory effect. It is capable of initiating DNA cleavage thus, determining the life span of the cell. 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.

The increased glucose turnover (hyperglycaemic condition) reported in thyrotoxicosis tends to occur because of a catecholamine-induced inhibition of insulin release and increased glycogenolysis [40] due to increased metabolic rate and peripheral glucose utilization. From literatures, laboratory findings including hyperglycemia, hypercalcemia, elevated alkaline phosphatase, leukocytosis and elevated liver enzymes are associated with thyrotoxicosis [37], among which our findings supported.

Malondialdehyde (MDA) is a natural product of peroxidation of unsaturated fatty acids with three or more double bonds. In this study, values of MDA assay from the homogenate of the experimental groups increases with increase in concentration of cyanide administered. This finding showed significant difference on comparing with the control group, thereby supporting our earlier findings. It is well known that MDA is a terminal product of lipid peroxidation. So the content of MDA can be used to estimate the extent of lipid peroxidation. Lipid peroxidation indirectly reflects the status of the metabolism of free radicals, the degree to which the tissue cells are attacked by free radicals. The high increase in the level of MDA and hydroperoxides in hyperthyroidism might be due to the possible changes in the cellular respiration of target tissues, which are undoubtedly related to any alteration in the thyroid function, knowing the major role of the thyroid hormones in the control of the mitochondrial respiration rate [44].

Superoxide dismutase (SOD) is an important intracellular oxygen radical-scavenging enzyme. SOD catalyses the dismutation of superoxide anion radical to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). We observed a decreased SOD activity with increase in concentration of cyanide in this study. This may be informed by the capacity of SOD in catalysing the dismutation of superoxide radicals; the more the radicals, the more the catalytic reaction of SOD resulting in to a lesser concentration of SOD and vice-versa. This finding clearly showed significant difference on comparing with the control group, thereby supporting our earlier findings.

From literatures, clinical and experimental studies reported that oxidative stress accompanying hyperthyroidism is caused by increased synthesis of reactive oxygen species (ROS) and changes in the antioxidant defense system [45]; suggesting oxidative stress was effectively induced.

4. CONCLUSION

The results obtained from this study showed hyperthyroidism was effectively induced by cyanide.

CONSENT

Not applicable.

ETHICAL APPROVAL

Authors declared that "Principles of laboratory animal care" were followed and specific national laws where applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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