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Research Article

Sub-Cellular Correlation of Nitrite in Cassava (*Manihot Esculenta Crantz*) Leaves and Nitrosamine Toxicology in Wistar Rats

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ABSTRACT

The aim of this study was to determine the nitrite levels in the sub-cellular fractions of cassava (*Manihot esculenta crantz*) leaves containing chloroplast, mitochondria and in the cytosol respectively and to their contribution in the causation of liver toxicity in rats exposed to N-nitrosamine precursors. The design of this experiment consisted of three animal groups. The first group was administered with dimethylamine hydrochloride plus sodium nitrite (DMA.HCL + $NaNO_2$), the second group was administered with sodium nitrite ($NaNO_2$) only and the third group (control) was given distilled water. The methods used included cell fractionation, tissue homogenization and centrifugation, spectrophotometric analysis, enzymatic determination and histopathology. Nitrite levels were estimated at 6.08 ± 0.92, 4.06 ± 1.65 and 1.29 ± 1.66µg/200g of cassava leaf tissue in chloroplasmic, mitochondrial and cytosolic sub-cellular fractions respectively. Both the $NaNO_2$ dose regime and the combined dose of DMA.HCL and $NaNO_2$, at P- value 0.05, caused significant increases in GGT, ALP, AST and ALT levels in serum. The histopathological study of the rat liver for DMA.HCL + $NaNO_2$ administration showed severe portal and central venous congestion while the $NaNO_2$ administration revealed a mild periportal cellular infiltration. This study shows that there is a correlation of nitrite in the chloroplast, mitochondria and cytosol sub-cellular fractions of cassava leaves and administration of nitrite dietary level in cassava leaves and dimethylamine hydrochloride produced acute synergistic toxicity in the liver.

Keywords: Cassava, Chloroplast, Cytosol, Dimethylamine hydrochloride, Mitochondria, Nitrite.

INTRODUCTION

Nitrosamines are present in water, soil and air and they can be found contaminating food, feeding stuff, drugs, cosmetics, and pesticides.[1] There is evidence that nitroso-compounds may be generated *in vivo* from nitrites or nitrates and primary, secondary and tertiary amines in organs of people who apparently were not exposed to these compounds.[2] The formation of nitrosamines depends on the pH of the environment, alkalinity of amine precursor and temperature. The rate of formation of N-nitroso compounds from secondary amines increases proportionally with a decrease in alkalinity of the amines. Nitrosamines are formed by the interaction of nitrous acid as a nitrosating agent with secondary or tertiary amines. Nitrite is known to be a precursor of toxic and carcinogenic N-nitrosamines[3] and it induces cancer in experimental animals.[4,5] Nitrite can also interact with haemoglobin by oxidation of ferrous ion (Fe^{2+}) to the ferric state (Fe^{3+}) thereby preventing or reducing the ability of blood to transport oxygen in a condition known as methaemoglobenaemia.[6,7]

Cassava (*Manihot esculenta*) is extensively cultivated as an annual crop in tropical and subtropical regions for its edible starchy, tuberous root and a major source of carbohydrates. It is high energy food obtained with low inputs and little effort.[8,9] Cassava can be classified into two varieties namely, toxic or 'bitter' and non-toxic or 'sweet' varieties, based on cyanide content. Varieties yielding less than 50mg HCN/kg of fresh root are classified as non-toxic, whilst those yielding more are considered toxic.[10] Nitrate content is an important quality characteristic of vegetables, the concentration of nitrate in vegetables varies considerably, and may be up to 3-4g/kg fresh weight, and these levels could have potential health impacts, especially in cassava eating populations.[11] One of the toxicology implications of nitrate and nitrite ingestion in cassava-eating populations is that thiocyanate, which is present in high amounts in the stomach of such individuals, may act as catalyst for the nitrosation of amines in the stomach to form carcinogenic nitrosamines, arising from microbial conversion of nitrate to nitrite which acts as the nitrosating agent enzymatically or spontaneously.[12]

The liver is the primary site of chemical biotransformation. Serum tests are often used to detect the liver injury caused by certain chemicals. These tests are based upon identifying and measuring the serum activity of enzymes present within liver cells. Thus increased concentrations of these enzymes in serums normally indicate hepatocellular damage and leakage of these enzymes from liver cells into the blood stream.[13]

The objective of this study is to investigate the correlation of nitrite level in the chloroplast, mitochondria and cytosol sub-cellular fractions of cassava leaves, to evaluate the toxicity of the liver and the effects on the serum enzymes of wistar rats on exposure to sodium nitrite and dimethylamine hydrochloride (precursors of N-nitrosamine).

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Table 1: Nitrite Concentration in the sub-cellular fractions of the cassava leaf tissue

Chloroplast (µg)	Mitochondria (µg)	Cytosol (µg)
6.08 ± 0.92	4.06 ± 1.65	1.29 ± 1.66

Table 2: Distribution of Nitrite in the sub-cellular fractions of the cassava leaf tissue

Sub-Cellular Fractions	Chloroplast vs Mitochondria	Chloroplast vs Cytosol	Mitochondria vs Cytosol
Regression Equation	Y=7.34-0.54X	Y= -4.48+0.95X	Y=2.07-0.19X
Standard Error	1.85	13.50	1.55
Correlation Coefficient	0.69	0.91	0.54

Number of samples=30

Table 3: Sub-cellular Correlation of nitrite in 50g of cassava leaf on different days of consumption

Days	Chloroplast (µg)	Mitochondria (µg)	Cytosol (µg)	Total (µg)
1 day	6.08	4.06	1.29	11.43
7 days	42.56	28.42	9.03	80.01
28 days	170.24	113.68	36.12	320.04

Table 4: Serum enzyme activities in rats following oral administration with dimethylamine hydrochloride and sodium nitrite

Group	GGT (U/L)	ALP (U/L)	AST (U/L)	ALT (U/L)
Control	7.46 ± 1.40	5.45 ± 1.16	2.63 ± 1.12	27 ± 0.73
DMA.HCL+ $NaNO_2$	19.20 ± 0.36	18.42 ± 1.78	69.84 ± 1.77	35.83 ± 1.31
$NaNO_2$	13.82 ± 0.60	8.19 ± 0.26	44.90 ± 0.85	31.85 ± 0.59

Values are mean ±SD of 5 determinants

MATERIALS AND METHODS

Chemical and reagents: Sodium nitrite ($NaNO_2$, Mol.wt 69) Dimethylamine hydrochloride ($(CH_3)_2NH.HCL$), Mol.wt 81.55), were obtained from Sigma (USA). Others reagents such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and gamma- glutamyl transferase (GGT) were from Randox Laboratories Ltd, United Kingdom. All the other chemicals and test kits used were of analytical grade.

Experimental Animals: The experimental animals used in this work were healthy male albino rats (*Rattus norvegicus*) of the Wistar strain. They weighed between 180g and 200g, and were obtained from the animal house of Veterinary Physiology Department, University of Ibadan, where they had been fed commercial rat pellets *ad libitum* and allowed access to clean drinking water. Only those certified free of infection by the Veterinary pathologist were used. They were kept at room temperature (approximately 28^{o}C) and all test animals were acclimatized to their environment before experiments were begun.

Animal treatment: The doses of precursors of nitrosamine given to the rats were:

A single dose of 6.20mg $NaNO_2$/kg.

A concurrent dose of 50mg of dimethylamine hydrochloride and 6.20mg $NaNO_2$/kg.

Both compounds were dissolved in distilled water and administered orally by intubation using a cannula tubes. Control animals were given drinking water and food only. All animals were starved over night prior to the administration of toxin compounds. The rats were sacrified 24 hours after dosing.

Collection of blood samples for serum preparation: The rats were sacrificed within 24 hours after the oral dose of sodium nitrite and Dimethylamine Hydrochloride, all the rats were sacrificed by cervical dislocation. Blood was collected with the capillary tubes from the eyes. The blood was collected in dry plastic or glass centrifuge tubes. The blood was allowed to clot and immediately transferred to an ice water bath prior to centrifugation. The clotted blood samples were centrifuged at 2000 rpm in a portable general laboratory centrifuge for about 15 minutes. The resultant supernatant sera were collected and preserved in a refrigerator at 4^0C for a short time. The activity of Alanine amino transferase (ALT) and Aspartate Amino transferase (AST) were estimated using the method of Reitman and Frankel.[14] The activities of Alkaline Phosphatase (ALP) and Gamma – glutamyl Transferase (γ-GT) were determined in the serum samples using the method of Klein *et al* and Szasz.[15,16]

Histopathological analysis: Liver samples were immediately collected and fixed in 10% buffered formal saline solution for a period of at least 24 h before histopathological study. Samples were then embedded in paraffin wax and five-micron sections were prepared with a rotary microtome. These thin sections were stained with hematoxylin and eosin (H&E), mounted on glass slides with Canada balsam (Sigma, USA) and observed for pathological changes under a binocular microscope.

Extraction of Sub-cellular Fraction of Chloroplast, Mitochondria and Cytosol from Cassava Leaves: Fresh cassava leaves (sweet species) were obtained from a farm in University of Ibadan, washed and cut into small pieces. 50g -80g from each of the cultivars was homogenized in 250ml -350ml of 0.1% orthophosphoric acid (0.2µl) pH 7.4 at 4^0C was blend for 3 minutes. The homogenate was filtered at 4^0C through four layers of muslin. The filtrate was centrifuge at 2960xg for 5 minutes at 4^0C. Chloroplast formed a soft green pellet at the bottom of the centrifuge tube. The supernatant was centrifuged at 17,700g for

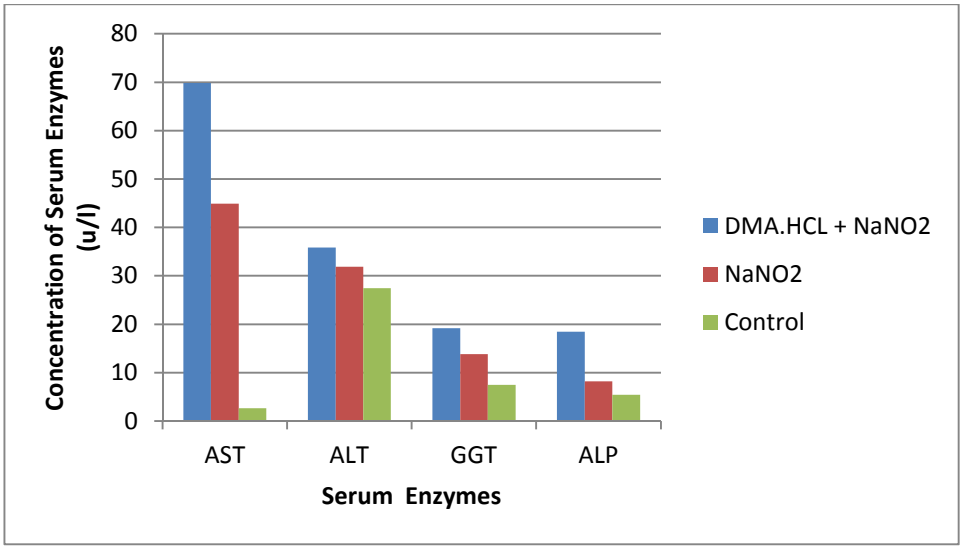

Figure 1: Serum enzyme activities in rats following oral administration with dimethylamine hydrochloride and sodium nitrite

Histopathology Section of the Liver of Rats

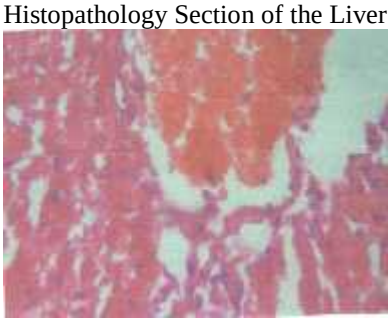

Figure 2: Photomicrograph of Hepatocyte of rat following oral administration of 50mg of DMA.HCL and 6.2mg $NaNO_2$/kg and showing a very severe portal and central venous congestion. (Mag. x 40)

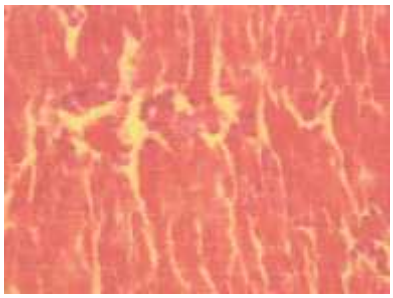

Figure 3: Photomicrograph of Hepatocyte of rat following oral administration of 6.2mg $NaNO_2$/kg and showing the portal areas markedly infiltrated by mononuclear cells. (Mag. x 40)

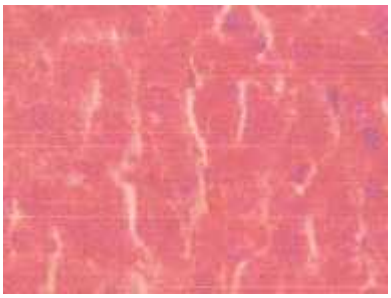

Figure 4: Photomicrograph of liver section of control rats showing no visible legion. (Mag. x 40)

15minutes in a cold centrifuge. A policeman rod was used to re-suspend the pellet in a small volume of 1% orthophosphoric acid and diluted to a final volume of 10ml with the acid. The supernatant was centrifuged at 19400xg for 10 minutes. The pellet was gently suspended in a small volume of 1% cold orthophosphoric acid. (The supernatant is the cytosol extract and the pellet is the mitochondria extract.[17]) The extracts were stored at 4^{O}C and the amount of nitrite and nitrate present in the different sub-cellular extract were determined.[18]

STATISTICAL ANALYSIS

Data were analysed using student's T-test analysis and was expressed as mean ± standard deviation. A level of $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The mean values of nitrite obtained in the chloroplast, mitochondria and cytosol sub-cellular fraction were 6.08± 0.92, 4.06 ±1.65 and 1.29±1.66µg respectively (Table 1). There was a significant deviation between the three sub-cellular fractions with chloroplast showing the highest concentration.

There was a strong positive correlation relationship when chloroplast sub-cellular fraction was compared to the cytosol sub-cellular fraction. There was a positive correlation relationship when chloroplast and mitochondria were compared to mitochondria and cytosol sub-cellular fraction respectively (Table 2). These values fall into the normal range because according to the Joint Expert committee on food Additives (JECFA), established acceptable daily intake of sodium nitrite is 0-0.07mg/kg body weight. This indicates that nitrite level in cassava is safe if minimum amount of cassava leaf are consumed. If this amount is consumed for 7 days and for 28 days the total concentration of nitrite in the sub-cellular fractions will be 0.08mg and 0.320mg/kg (Table 3) and this can lead to toxicity in the system.

ALP, AST, ALT and GGT levels are widely used in animal's studies to diagnose and observed the development of hepatocarcinogenesis. In the present study, the values of the analytes showed sharp significantly

increase at P-value < 0.05 in the group administered dimethylamine hydrochloride plus sodium nitrite and sodium nitrite, respectively as compared with that of the normal control rats (Table 4).

The photomicrography of liver section in DMA.HCL + $NaNO_2$ group and $NaNO_2$ group shows that the rat's livers in these two groups were damaged when compared to the control. DMA.HCL + $NaNO_2$ group shows very severe portal and central venous congestion (Fig.2), while $NaNO_2$ group shows markedly infiltration by mononuclear cells and periportal cellular infiltration (Fig.3).

Nitrites have been observed by researchers to occur widely in foods of animals and plant origin as well as community water supply.[19] Nitrite levels in food are very low (generally well below 10mg/kg and rarely exceed 100mg/kg). Exceptions to this are vegetables that have been damaged, poorly stored, or stored for extended periods as well as prickled or fermented vegetables.[20]

CONCLUSION

This study indicates low level of nitrite in sub-cellular fractions containing chloroplast, mitochondria and cytosol of cassava leaves. It also shows that dimethylamine hydrochloride and sodium nitrite (precursors of N-nitrosamine) can cause acute synergistic toxicity in the liver and could also make the serum enzymes level to rise due to the damage that it has done to the liver.

It is concluded that frequent consumption of vegetables whose nitrite contents are high by cassava-eating people might put them at risk of developing stomach cancer and other possible result of nitrite toxicity.

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