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Evaluation of Level of Precursors of N-Nitrosamine *in Vitro* in Wistar Rats Fed Different Levels of Dietary Protein

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Abstract: *This study compares the level of Nitrite in urine, protein concentration and nitrite concentration in post mitochondrial fraction of rats fed different levels of dietary protein with concurrent administration of precursors of N-nitrosamine; dimethylamine hydrochloride (DMA-HCl) and sodium nitrite ($NaNO_2$). Thirty Male Wistar rats were divided into three groups and were kept for four weeks. Group one was given high protein diet (64%), group two was given a normal protein diet (27%) and group three was given low protein diet (3.5%). All the groups were administered with 3mg $NaNO_2$ and 20mg DMA-HCL/kg, using the application of spectrophotometric analysis, centrifugation, as well as colorimetric methods. Following administrations of the chemicals to the test animal groups, the concentration of 24 hours urinary excretion of nitrite was 7.417µg/ml in high protein fed rats, 2.063µg/ml in normal protein fed rats and 0.569 µg/ml in low protein fed rats. There was a significant difference ($p<0.05$) in the nitrite concentration in the group fed high protein diet with concurrent administration of $NaNO_2$ and DMA-HCl. The wistar rats fed with high protein diet, excreted 5.8 to 7 times more nitrite in urine than the severely protein deprived animals. The protein and nitrite concentration of the post mitochondrial fraction of liver was highest in rats that were fed high protein diet. This study has revealed that nutrition status affects metabolism of foreign compounds including nitrites and dimethylamine hydrochloride.*

.***Key words:*** *Dietary protein, Dimethylamine hydrochloride, metabolism, Sodium Nitrite, Urine.*

I. Introduction

Protein is an essential nutrient that is important to human health. Protein consists of chains of amino acids that are used by the body to grow muscles, hair, nails, skin and internal organs. Although protein is an essential nutrient, some researchers suggest that too much protein can increase the risk of developing heart disease, stroke, kidney stones and osteoporosis.[1,34,35]

Sodium nitrite ($NaNO_2$) is a pure white or slightly yellowish crystalline powder. It is very soluble in water and is hygroscopic. It is also slowly oxidized by oxygen in the air to sodium nitrate, $NaNO_3$. The compound is a strong oxidizing agent. It is used as a color fixative and preservative in meats and fish, it is also used in manufacturing diazo dyes, nitroso compounds and other organic compounds; in dyeing, printing textile fabrics, bleaching fibers and in photography etc.[2] Dimethylamine hydrochloride is a white to off–white crystalline free flowing powder, it has a molecular weight 81.55g, the melting point is 170^{o}C and it decomposes at its boiling point. It is soluble in water and has a pH of 6.0 -6.5 (5.0% sol.). Dimethylamine hydrochloride vapor causes irritation of the eyes, skin and respiratory tract in humans and animals which is manifested at lower concentrations as lacrimation and mild lesions in the nasal mucosa. At sufficiently high concentrations and/or exposure durations, animal studies reported severe nasal and lungs lesions and occasionally lesions of the liver, kidneys and testes. DMA is present in many foods including cabbage, celery, corn, fish, and coffee and is also formed endogenously by gut bacteria from DMA precursors including trimethylamine N-oxide.[3]

Nitrosamines are a major candidate class of carcinogens likely to be causally related to human cancer.[4] Nitrosamines act systemically and produce cancer in a wide variety of organs of many species at the (parts per million) ppm dietary level;[5] nitrosamines have been found in tobacco smoke, grains, and alcoholic beverages in concentrations of < 5 ppm.[6] Higher concentrations of nitrosamines, particularly dimethylnitrosamine, have been found in nitrite-preserved fish meals which were highly toxic to ruminants. Dimethylnitrosamine is formed during cooking of canned or smoked fish with nitrite. The average human daily intake of nitrite has been estimated as 22 pmoles, equivalent to I.5 mg of $NaNO_2$; levels in excess of this have produced acute methemoglobinemia in infants. Nitrosamines are formed chemically from the interaction of nitrites or oxides of nitrogen and secondary amines in acidic conditions.[7,8] Nitrosamines can also be formed in the gastric juice of the human stomach. This is commonly referred to as endogenous nitrosation. Bacteria in the mouth chemically

reduce nitrate, which is prevalent in many vegetables, to nitrite, which in turn can form nitrosating agents. Many foods contain amines that can react with nitrosating agents in the acidic stomach to form nitrosamines.[9]

Recently, on the basis of animal experiments, it was concluded that the monitoring of urinary levels of N-nitrosoamino acids such as NPRO[3] and N-nitrosohydroxyproline could be a useful procedure for the quantitative estimation of nitrosation *in vivo*.[10,11] Thus, more than 80% of a dose of NPRO administered to rats was excreted unchanged into urine within 24 hour; in a typical experiment, the simultaneous administration of 10µmol of each proline and nitrite to rats resulted in a significant increase in urinary excretion of NPRO (24 nmol/24 hour/rat).[12] Endogenous N-nitrosation was demonstrated in a male volunteer who ingested vegetable juice as a source of nitrate together with proline by quantitative monitoring of NPRO excreted into the urine. A significant increase of urinary excretion of NPRO occurred only after ingestion of nitrate and proline. The vegetable juice and proline used contained no detectable levels of preformed NPRO or nitrite. Ingestion of either of the precursors alone did not increase urinary excretion of NPRO; therefore, the NPRO excreted in the urine is most probably formed in the human body through the reaction of proline with nitrite, the latter being derived from nitrate.[13]

Dietary intakes of red and processed meat are of particular importance in the formation of fecal NOC.[14] Higher consumption of red meat (600 vs. 60 g/day), but not white meat, resulted in a three-fold increase in fecal NOC levels.[15] Studies have shown that the post mitochondrial fraction of liver tissue homogenates could decompose NDMA by oxidative dealkylation.[16,17,18]

The urine is one of the major routes of excretion of drugs, foreign compounds and their metabolites. The excretion of N-nitrosamines including DMA in the urine of humans and animals have been reported by researchers.[19,20]

This investigation has been designed to assess the effect of different levels of dietary protein intake on the excretory pattern of orally administered dimethylamine hydrochoride and sodium nitrite by comparing the urinary excretion of the unchanged nitrite (precursor of nitrosamine) in high protein, normal protein and low protein diet fed rats. Also to determine the protein and nitrite concentration in the post mitochondrial fraction in livers of rats fed different levels of dietary proteins.

II. Materials And Methods

Experimental animal

Thirty (30) Male experimental albino Wistar rats weighing between 70-100g were purchased from Biochemistry animal farm in the University of Ibadan, Nigeria and were kept at room temperature (27^{o}C) in standard cages at the animal house of the Biochemistry Department, University of Ibadan. They were given different levels of dietary protein which are shown in Table.1, water *ad libitum* and acclimatised for 4 weeks before being used for the research work.

Table 1: Composition of the Experimental Diets

	High protein (%)	Normal protein (%)	Low protein (%)
Protein	64	27	3.5
Cornstarch	22	59	81.5
Oil	8	8	8
Vitamin	2	4	4
Mineral Salt	4	2	3

Experimental group

The rats were divided into three groups and were placed in different protein diets. Group one was given high protein diet (64%), group two was given a normal protein diet (27%) and group three was given low protein diet (3.5%). Sodium Nitrite ($NaNO_2$) and Dimethylamine hydrochloride (DMA-HCL) were concurrently administered orally at a single dose of 3mg $NaNO_2$ and 20mg of DMA-HCl/kg to each experimental group. All rats were starved overnight prior to administration of toxins. The weight of the rats was taken before and after oral administration.

Experimental Diets

Fish was used as a source of protein, carbohydrate was obtained from cornstarch and vegetable oil was the source of fat and oil. Emvite multivitamin tablets was the source of vitamin and different salt formulations into a salt mixture was used as source of mineral salt. In preparation of the diet, the constituents were mixed together thoroughly to achieve homogeneity. They were then made into pellets and dried in the oven for complete dryness and removal of any trace of water. The food were then stored at room temperature in large

plastic containers and labeled according to the different groups as required. See Table 2 and 3 for vitamins and minerals mixtures used.

Table 2:Special Vitamin mixture

S/N	Vitamin	Proportion
1	Vitamin A Acetate	2500 i.u
2	Vitamin B	250 i.u
3	Vitamin C	600 mcg
4	Vitamin D	1300 mcg

Table 3: COMPOSITION OF MINERAL MIXTURE

S/N	Types of mineral	Composition
1	Calcium Carbonate	20g
2	Calcium Hydrogen phosphate	10.50g
3	Sodium chloride	8g
4	Di-potassium hydrogen phosphate	35g
5	Magnesium sulphate	8g
6	Manganese sulphate	0.51g
7	Copper sulphate	0.03g
8	Potassium iodide	0.08g
9	Zinc chloride	0.925g
10	Cobalt chloride	0.005g
11	Ferrous sulphate	17.85g

Chemicals and reagents

Sodium nitrite ($NaNO_2$**,** Mol.wt 69) Dimethylamine hydrochloride $(CH_3)_2NH.HCL$), Mol.wt 81.55), were obtained from Sigma (USA).

Composition of the Montgomery and Dymock Reagent for Nitrite Determination

a. Solution A: Sulphanilic acid solution.
   27.2g of Potassium hydrogen sulphate and 3.46g of sulphanilic acid were dissolved in one litre double-distilled-deionised water.
b. Solution B: Naphthylethylenediamine (NEDA) solution.
   0.4g Naphthylethylenediamine dihydrohychloride was dissolve in one litre double distilled deionised water.
c. Solution C: 0.5% sodium carbonate solution.
   0.5g Na_2CO_3 was dissolved in 100ml double-distilled-deionised water.
   Except stated, all chemicals used were of analytical grade (Analar).

Collection of urine samples

The rats were administered with 3mg of sodium nitrite and 20mg of dimethylamine hydrochloride/kg. Then they were placed in metabolic cages with separate facilities for collection of urine. The rats were fed with the experimental diets and drinking water was given *ad libitum.* Urine was collected after 24 hours. The samples were analysed daily or stored in a refrigerator at 10^oC.

Determination of nitrite in urine

The 24-hour urine was clarified by swirling with activated charcoal and filtered through Whatman No. 1 filter paper. 1ml of the filtrate was analyzed for nitrite using the method of Montgomery and Dymock.[21]

Preparation of post mitochondrial fraction

Liver were removed from the animals under urethane anaesthesia. Liver were immediately cooled with ice-cold 0.15M KCL. Gall bladder and extraneous tissue were removed and the liver weighed after rinsing and blotting. The liver tissue was homogenized with 4 volumes of 0.06M phosphate buffer plus 0.15M KCL at pH 7.4 with a Teflon glass homogenizer. The homogenate was centrifuged at 10,000xg for 15 minutes in a high speed refrigerated centrifuge. Protein concentration was determined using the method of Gornal *et al.*[22]

Incubation Assay

The complete incubation medium had a total of 4ml and combined NADP (0.5mM), glucose 6-Phosphate (5mM), $MgCl_2$ (20mM), 0.06M phosphate buffer, 0.15M KCl and 2.5ml of post mitochondrial fraction of the liver homogenate. The concentration of sodium nitrite was 5mM and DMA-HCL was 5mM.
The incubation was carried out in a shaking water bath; temperature 37^{o}C for 30min, the reaction was terminated by adding 2ml 5% TCA. Nitrite concentration was determined according to Montgomery and Dymock.[21]

***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.

III. Results

There was a significant difference ($p<0.05$) in the nitrite level in urine in the group fed high protein diet with concurrent administration of nitrite and DMA-HCl. There was a significant difference ($p< 0.05$) in the protein and nitrite concentration of post mitochondrial fraction of rats fed high protein. The nitrite concentration was highest in the rats that were fed high protein diet, followed by the rats that were fed normal proteins and the lowest concentration was seen in the rat that were fed low protein diet.

Table 4: Comparative urinary excretions of Nitrite in 24hr Urine samples of rats fed different levels of dietary protein with or without a concurrent administration Nitrite and DMA-HCl.

	Absolute and Relative Nitrite Excretion (µg/ml)		
Dosage of Nitrite and DMA-HCL/kg	**High Protein (64%)**	**Normal Protein (27%)**	**Low Protein (3.5%)**
3mg $NaNO_2$ + 20mg DMA -HCl	7.417 ± 3.71	2.063 ± 0.005	0.569 ± 0.09
0.0mg $NaNO_2$ + 0.0mg DMA-HCl	2.063 ± 0.40	1.050 ± 0.20	0.230± 0.19

Values are mean ± SD of 5 determinants

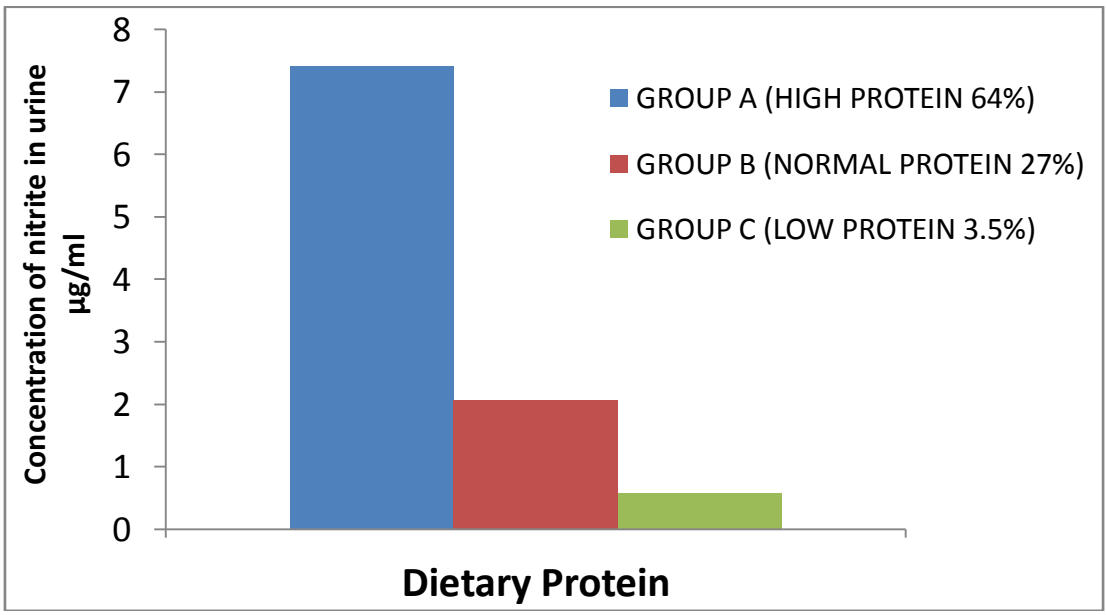

Figure 1: The level of nitrite concentration in urine of rat fed different levels of dietary protein following concurrent oral administrations of 3mg $NaNO_2$ and 20mg DMA-HCl/kg.

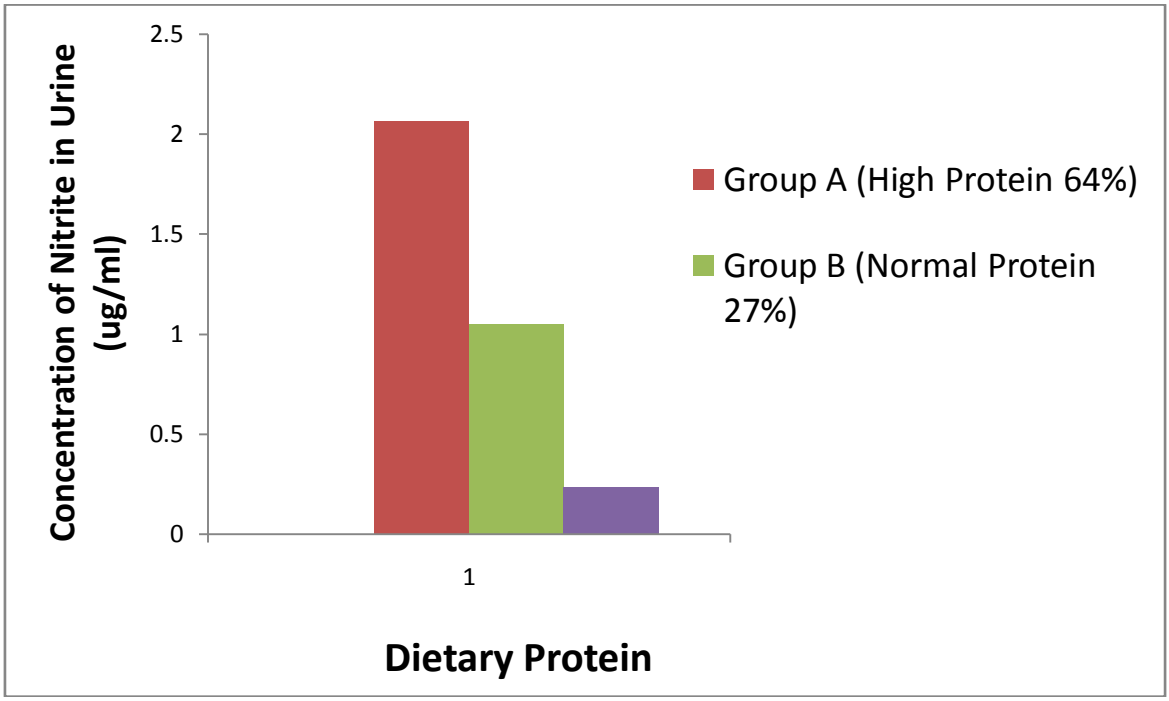

Figure 2: The level of nitrite concentration in urine of rat fed different levels of dietary protein without administration of $NaNO_2$ and DMA-HCl.

Table 5: Protein concentration in liver microsomal plus soluble fraction of wistar albino rats fed different levels of dietary protein.

Model Test Diet	Weight of Liver (g)	Protein Concentration (µg/ml)
High protein	3.58	9.418 ± 0.032
Normal protein	3.49	2.139 ± 0.822
Low protein	2.68	0.432 ± 0.046

Table 6: Nitrite concentration in liver microsomal plus soluble fraction of wistar albino rats at concentration of 5mM $NaNO_2$ and 5mM DMA- HCL (Combined)

Model Test Diet	Weight of Liver (g)	Nitrite Concentration(µg/ml)
High protein	3.58	4.033 ± 0.52
Normal protein	3.49	3.201 ± 0.059
Low protein	2.68	2.542 ± 0.023

IV. Discussion

Nitrosamines are present in water, soil and air.[23] They can be found in contaminated food, feeding stuff (where they create the highest risk for health), drugs, cosmetics products and pesticides.[24,25] Nitrosamines are absorbed by skin, airways and the alimentary tract.

Ingested nitrite is readily absorbed from the upper intestine in both rats and humans, but little absorption takes place from the stomach, distal ileum, caceum or proximal colon.[26] Absorption is rapid and in humans, nitrate concentrations in body fluids (serum, saliva, urine) peaks within 1-3 hours after ingestion in food or water.[27]

The nutritional status in nitrite, nitrate and nitrosamine intake would play an important role in the determination of the short and long term effects arising from their ingestion in food materials. Nutrition status has been shown to affect metabolism of foreign compounds including nitrites and DMA.[28,29,30]

Dietary Protein altered urinary nitrite outputs in wistar rats fed with 64% protein diet; they excreted 5.8 times more than the normal protein fed rats and 7 times more nitrite in urine than the severely protein deprived animals (as shown in Table.4 and Figure 1). This observation is consistent with reports by Montesano *et al.* [31] The result of urinary excretion showed an excretion of nitrite in rats fed with high protein diet, normal Protein diet and low protein diet. Even though, is not a normal constituent of urine, but was obtained in urine when $NaNO_2$ and DMA-HCL were administered to the animals. This showed that nitrite would be excretable in the urine when its concentration in circulation is sufficiently high. It also indicate that nitrite of non-bacterial origin

can be excreted in the urine.[30] Urinary excretion in response to $NaNO_2$ load was higher for animals on high protein diet.

The results of this study showed that there was a significant difference ($p<0.05$) in the nitrite level in urine in the group fed high protein with concurrent administration of $NaNO_2$ and DMA-HCL. The nitrite level in the high protein fed rats was the highest, followed by the rats fed with normal protein diet and the least was the rats fed with low protein diet. There was a significant difference ($p< 0.05$) in the protein concentration of post mitochondrial fraction of rats fed high protein compared to the rats that fed normal protein diet and low protein diet (Table.5). In the incubation assay the result shows significant difference ($p<0.05$) in the concentration of nitrite in the post mitochondrial fraction in the high protein fed rats compared to the normal protein fed rats and low protein diet fed rats (Table.6). Low-protein diets generally decrease monooxygenase activity in rat liver microsomes. The liver carcinogen dimethylnitrosamine, which must be activated metabolically, is almost without effect in protein-deficient rats. Phase II reactions may also be affected by dietary protein levels.[32,33]

V. Conclusion

This study has revealed that dietary protein alters urinary nitrite outputs in wistar rats fed with 64% protein diet; they excreted 5.8 times to 7 times more nitrite in urine respectively than the severely protein deprived animals. It also shows the significance of DMA-HCl and $NaNO_2$ by comparing the urinary excretion of the unchanged nitrite in high protein, normal protein and low protein diet fed rats. It also assessed the effect of different levels of dietary protein on protein concentration and nitrite concentration in post mitochondrial fraction of the liver homogenate. Hence nutrition status has been shown to affect metabolism of foreign compounds including $NaNO_2$ and DMA-HCL.

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Abbreviation

DMA-HCl : Dimethylamine hydrochloride
i.u. : International units
KCl : Potassium Chloride
Kg : Kilogram
M : Molar
mcg : Microgram
Mg : Milligram
$MgCl_2$: Magnesium chloride
mM : Millimolar
NADP : Nicotineamide dinucleotide phosphate
Na_2CO_3 : Sodium Carbonate
$NaNO_2$: Sodium Nitrite
$NaNO_3$: Sodium Nitrate
NDMA : N-nitrosodimethylamine
NDEA : N-nitrosodiethylamine
NEDA : Naphthylenediamine
NOC : Nitrosocompound
NPRO : Nitrosoproline
ppm :Parts per million
RPM : Revolution per minute
SD : Standard deviation
TCA : Trichloroacetic acid
UV : Ultraviolet
µg : Microgram
µL : Microlitre

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