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N-Nitrosation of dimethylamine hydrochloride and its toxicology in the wistar rats fed different levels of dietary protein

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ABSTRACT

The present study investigated the in vivo and in vitro effects of orally administered sodium nitrite ($NaNO_2$) and dimethylamine hydrochloride (DMA–HCL) on liver of rats fed ad libitum with high protein diet (64%), normal protein diet (27%) and low protein diet (3.5%). Thirty Male Wistar rats were divided into three groups and kept for four weeks. Group one was given high protein diet, group two was given a normal protein diet, group three was given low protein diet, all the groups were administered with 3mg $NaNO_2$ and 20mg DMA-HCL/kg, using the application of spectrophotometric analysis, centrifugation, histolopathology, enzymatic as well as colorimetric methods. Liver function test, showed significant elevations ($P < 0.05$) in the AST, ALT, ALP and GGT activities in all the groups compared with the control animals. The histopathology examination exhibited periportal necrosis. Following UV exposure after in vitro incubation of rat liver microsomal plus soluble fraction with $NaNO_2$ plus DMA-HCL, nitrite concentration in the rats fed high protein was highest 4.033 and 0.470 µg/ml, compared to the control rats which was 0.052 and 0.00192 µg/ml before and after UV irradiation. Nitrite loss was significant ($p<0.05$) before and after UV exposure in all the groups, indicating that the UV-light has degraded the nitrosamine precursors, thereby inhibiting possible nitrosation. The study has revealed that in rats, a high protein diet enhances N-Nitrosation of dimethylamine hydrochloride, liver derangement and the metabolisms in vivo and in vitro of the resultant compound.

Key words: Dimethylamine hydrochloride, incubation, protein, Sodium nitrite, UV irradiation.

INTRODUCTION

Sodium nitrite ($NaNO_2$) is a pure white or slightly yellowish crystalline powder. It is very soluble in water and 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 [Walker, 1990].

Dimethylamine hydrochloride is a white to off–white crystalline free flowing powder, a secondary aliphatic amine (2^o amine), with a smell of ammonia and/or rotten fish at room temperature. At sufficiently high concentrations and/or exposure durations, animal studies reported severe nasal and lung 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 which include trimethylamine N-oxide [Friedman, 2002].

Nitrosamines are present in water, soil and air. They can be found in contaminated food, feeding stuff (where they create the highest risk to health), cosmetic products [Schothort and Somers, 2005], [Flower *et al* 2006], tobacco smoke, soil [Pan *et al* 2006], ground water [Tomkins *et al* 1995] drugs and pesticides. Nitrosamines are absorbed by skin, airways and the alimentary tract. Nitrosamine is found in many food stuffs especially beer [Serag and Edrees, 2011], fish by products and in meat and cheese products preserved with nitrite picking salts [Wang *et al* 2005], [Luque-Pérez *et al* 2001]. They can also be formed by frying or smoking [Franco and Hotta, 1999]. Toxicological effect of nitrosamines includes hepatotoxicity, growth retardation, impairment of reproductive functions and endocrine disturbances [Tricker *et al* 1991]. Nitrosamines can form in the gastric juice of the human stomach. This is commonly referred to as endogenous nitrosation [Choi, 1985]. Bacteria in the mouth chemically reduce nitrate, which is prevalent in many vegetables, to nitrite, which in turn can form nitrosating agents [Griesenbeck *et al* 2009]. Many foods contain amines that can react with nitrosating agents in the acidic stomach to form nitrosamines [Mirvish *et al* 1980] and [Khodadady *et al* 2012].

Several nucleophillic or anionic salts, for instance, nitrosyl iodide (I-NO) and nitrosyl thiocynate (SCN-NO) can form effective nitrosating agents when they are present with nitrite in aqueous and acidic solutions. Enhanced nitrosation of lipophillic secondary amines has been demonstrated in aqueous systems containing micelles and some carbonyl compounds [Keefer, 1973] and [Okun, 1977].

Ascorbic acid, sulphur dioxide, α-tocopherol and gallic acid inhibited the N-nitroso compound formation in certain conditions [Mirvish, 1975]. CYP 2E1 is responsible for at least 60% of DNA methylation in rat hepatocytes. [Sheweita *et al* 2007] observed that b_5 is an essential component in CYP 2E1 catalysed oxidation of several substances.

Liver enzymes and function tests are used to determine if the liver is functioning normally or if it has injury or disease [Turek *et al* 1980]. The types of injury to the liver depend on the type of toxic agent, the severity of intoxication and the type of exposure, whether acute or chronic [Bartsch, 1996].The activity of the hepatic enzymes released into the blood by the damage liver is one of the useful tools in the study of hepatoxicity [Wallace, 2000].

A nitrosamine and its precursors are metabolized in the liver and to a lesser extent in the kidney [Biaudet and Mavell, 1994]. The toxicity of a substance depends on the dose and the higher the dose, the greater the toxic response [Smith *et al* 1967], [Kamm *et al* 1997]. An initial step in detecting liver damage is a simple blood test to determine the presence of certain liver enzymes in the blood. Under normal circumstances, these enzymes reside within the cells of the liver. But when the liver is injured, these enzymes are spilled into the blood stream [Fazilati *et al* 2013]. The most sensitive and widely used tests include: Aspartate Aminotransferase (AST or SGOT), Alanine Aminotransferase (ALT or SGPT), Alkaline Phosphatase (ALP) and Gamma – glutamyl transferase (γGT).

The formation of N-nitroso compounds in experimental animals *in vivo* has been demonstrated by identifying nitrosated products in the stomach contents [Braunberg, 1973], [Sander, 1972] or in the whole animal [Rounbehler *et al* 1977] after feeding relatively high doses of precursors. Formation *in vivo* has also been demonstrated in human subjects who ingested diphenylamine and nitrate, by detection of N-nitrosodiphenylamine in their stomachs [Sander *et al* 1969]. [Fine *et al* 1977] reported the formation of volatile nitrosamines *in vivo* as measured in the blood of a human subject who ingested a lunch consisting of spinach, cooked bacon and beer.

The Objective of this research is to investigate the effects of orally administered sodium nitrite ($NaNO_2$) and dimethylamine hydrochloride DMA-HCL *in vivo* and *in vitro* on liver of rats fed ad libitum with high protein diet (64%), normal protein diet (27%) and low protein diet (3.5%). The effect of dietary protein on Nitrosation of dimethylamine hydrochloride and the possible inhibition of n- nitrosamine precursors on exposure to ultraviolet light.

MATERIALS AND METHODS

Experimental animal: Thirty (30) Male experimental albino Wistar rats weighing between 70-100g were purchased from Biochemistry animal farm in university of Ibadan, Nigeria and were kept at room temperature (27^oC) in standard cages at the animal house of the Biochemistry Department, University of Ibadan. They were given different levels of dietary protein and water ad libitum and acclimatised for 4 weeks before being used for any research work.

Experimental group: The rats were divided into three groups, and were placed in different protein diets: Group one was given high protein diet, group two was given a normal protein diet and group three was given low protein diet. Sodium Nitrite and Dimethylamine hydrochloride (DMA-HCL) were administered orally at a single dose of 3mg $NaNO_2$/kg and 20mg of DMA-HCl /kg to each experimental group. Each group had their control animals which were fed a chow diet for rodent and water. All rats were starved overnight prior to administration of toxins. The weight of the rats was taken before and after oral administration.

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 10,000 rpm in a portable general laboratory centrifuge for about 5 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, 1957]. 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* 1960] and [Szasz 1969].

Experimental Diets: Fish was used as a source of protein, Carbohydrate was obtained from cornstarch, vegetable oil as source of fat and oil, Emvite multivitamin tablets as source of vitamin, 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.

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

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**.**

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.

Preparation of liver microsomal plus soluble fraction (10,000xgfraction): Livers were removed from the animals under urethane anaesthesia. Livers were immediately cooled with ice-cold 0.15M KCL. Gall bladders and extraneous tissues were removed and the livers weighed after rinsing and blotting. The liver tissue was homogenized with 4 volumes of 0.06M phosphate buffer plus 0.15M KCL pH 7.4 with a Teflon glass homogenizer. The homogenate was centrifuged at 10,000xg for 15 minutes in a high speed refrigerated centrifuge. The resultant supernatant contained the microsomes plus soluble fraction and was used for the *in vitro* studies.

INCUBATION ASSAY

The complete incubation medium had a total of 4ml and combined NADP (0.5mM), glucose 6-Phosphate (5nM), $MgCl_2$ (20nM), 0.06M phosphate buffer, 0.15M KCl and 2.5ml of microsomal plus soluble fraction of the liver homogenate. For the different experiment carried out, 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 followed by exposure to UV irradiation (short wavelength) for a minimum of 15 min. Incubation medium containing boiled tissue for 30min was used as control. Nitrite concentration before and after exposure to UV irradiation was determined according to Montgomery and Dymock [1961]

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

Table 2: Serum enzyme activities in rats fed different levels of dietary Protein following concurrent oral administrations of 3mg $NaNO_2$ and 20mg DMA-HCl/kg.

Model Test Diet	ALT(U/L)	AST(U/L)	GGT(U/L)	ALP(U/L)
High protein	78.20 ± 3.13	138.60 ± 2.70	77.51± 5.05	43.24 ± 0.93
Normal protein	24.86 ± 0.5	128.05 ± 8.5	27.12 ± 2.56	20.97 ± 6.0
Low protein	20.2 ± 4.3	100.00 ± 0.5	6.90 ± 2.6	12.5 ± 8 .3

Values are mean ±SD of 5 determinants

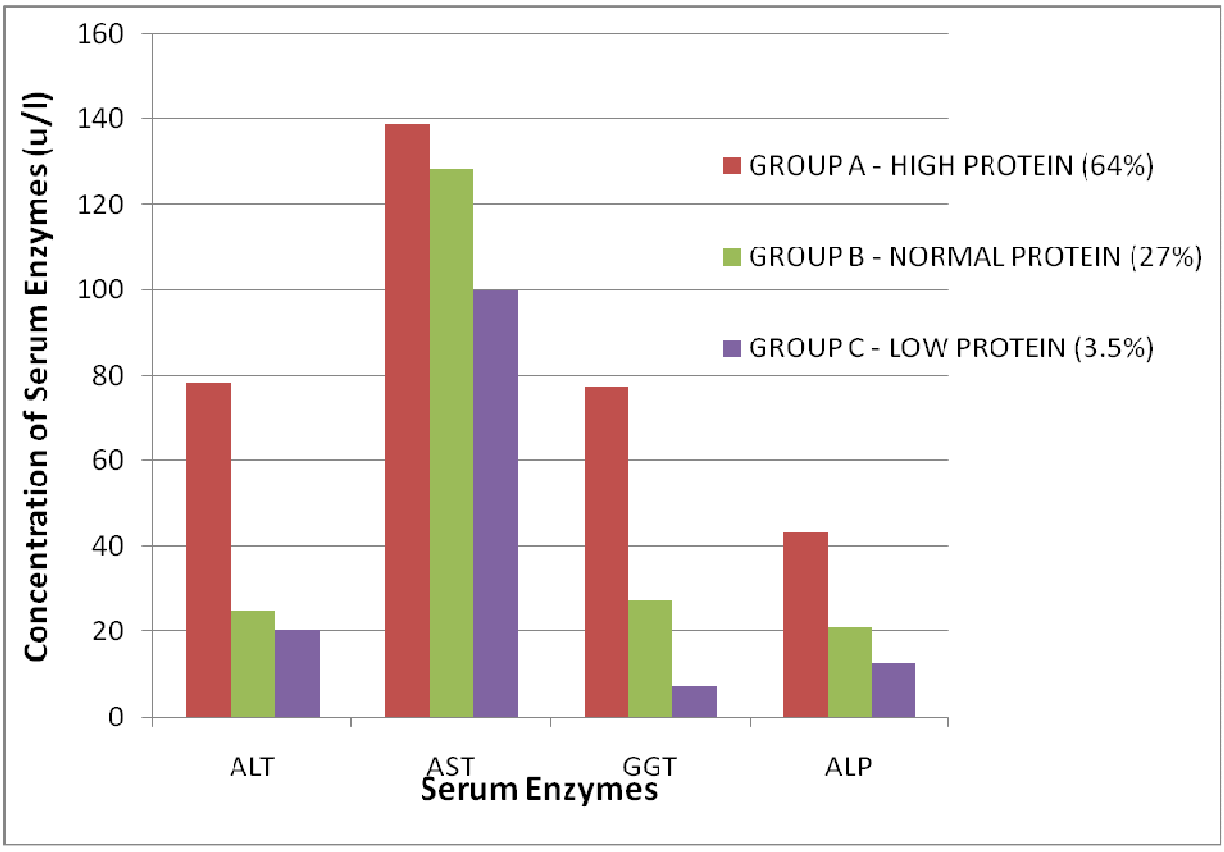

Figure 1: The activity of serum enzymes in rats fed different levels of dietary protein following concurrent oral administrations of 3mg $NaNO_2$/kg and 20mg DMA-HCl/kg.

Table 3: Serum enzymes activities in rats fed different levels of dietary protein without oral administrations of $NaNO_2$ and DMA-HCl.

Model Test Diet	ALT(U/L)	AST(U/L)	GGT(U/L)	ALP(U/L)
High protein	56.00 ± 1.66	119.20 ± 3.11	27.70 ± 0.30	17.13± 0.80
Normal protein	21.83 ± 2.23	113.42 ± 2.43	15.16 ± 0.04	6.90± 1.39
Low protein	17.81 ± 2.51	79.25 ± 2.62	5.15 ± 0.06	4.15 ± 0.01

Values are mean ± SD of 5 determinants

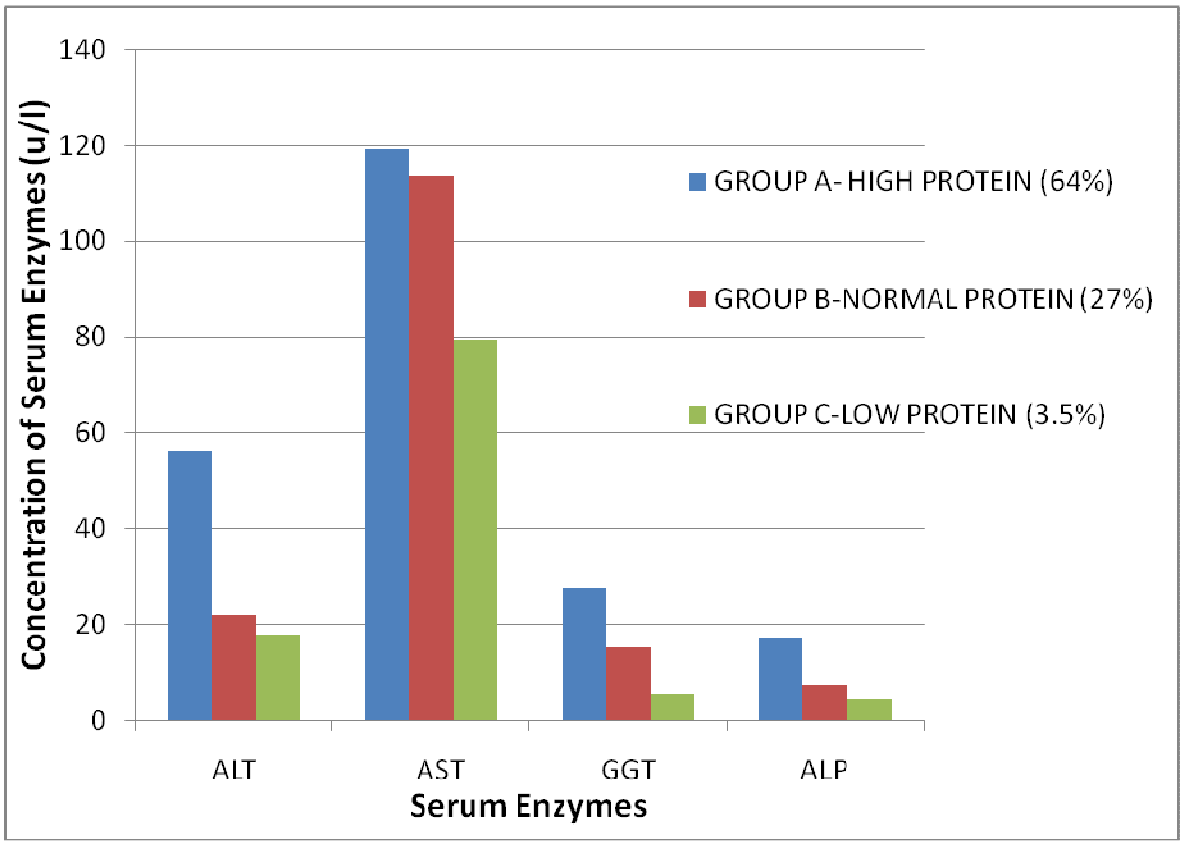

Figure 2: The activity of serum enzymes of rats fed different levels of dietary protein without oral administration of $NaNO_2$/kg and DMA-HCl.

Table 4: Nitrite appearance following UV irradiation of liver microsomal plus soluble fraction of wistar albino rats at concentration of 5mM $NaNO_2$

Model Test Diet	Nitrite concentration before UV irradiation after Incubation (µgNO_2/mL)	Nitrite concentration after UV irradiation (µgNO_2/mL)
High protein	3.105	0.126
Normal protein	2.306	0.718
Low protein	1.873	0.907
Control	0.052	0.00192

Values are mean ± SD of 5 determinants

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

Model Test Diet	Nitrite concentration before UV irradiation after Incubation (µgNO_2/mL)	Nitrite concentration after UV irradiation (µgNO_2/mL)
High protein	4.033	0.470
Normal protein	3.201	0.710
Low protein	2.542	1.247
Control	0.052	0.00192

Values are mean ± SD of 5 determinants

Histopathology sections of the liver of rats fed with different levels of dietary proteins

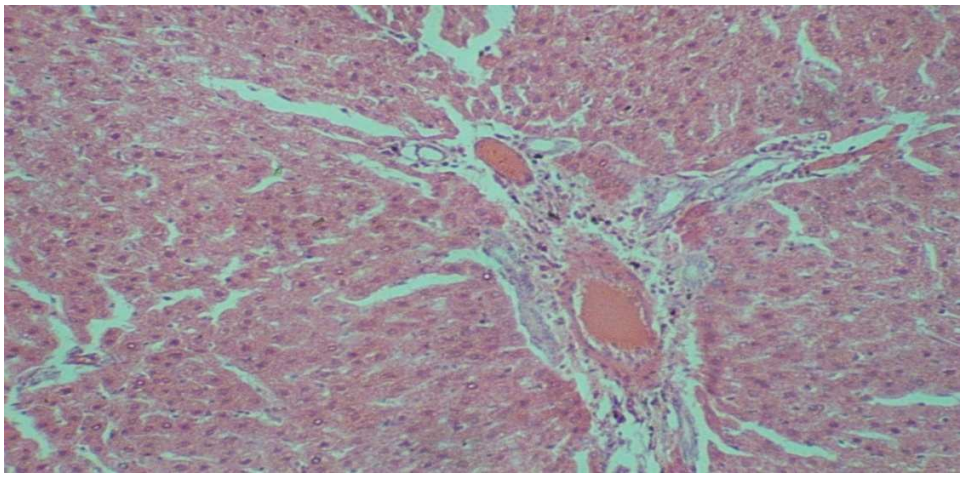

Figure.3: Photomicrograph of hepatocytes of high protein fed rats following oral administration of concurrent doses of 3mg $NaNO_2$/kg and 20mg DMA-HCL/kg and showing periportal necrosis of hepatocytes with mononuclear cellular infiltration. (Mag. x40)

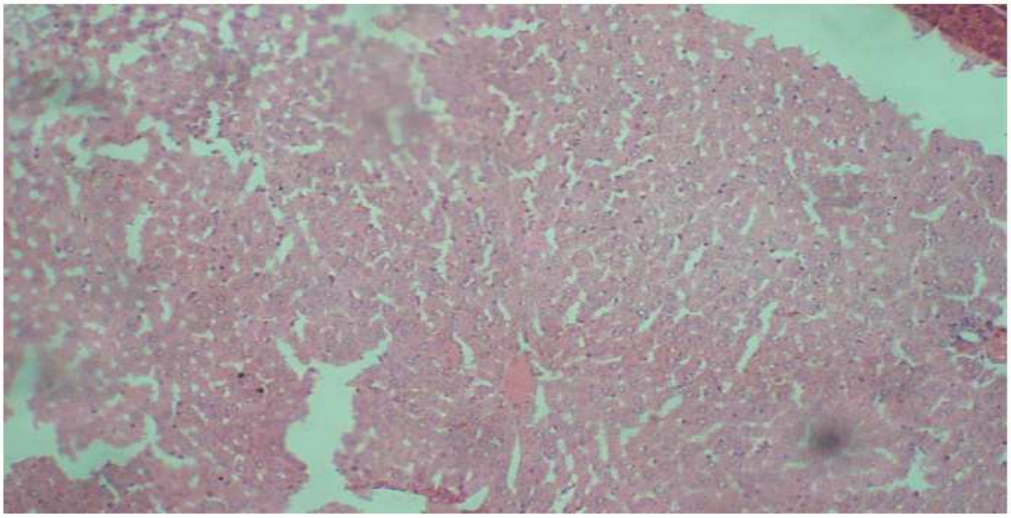

Figure.4: Photomicrograph of hepatocytes of normal protein fed-rats following oral administration of concurrent doses of 3mg $NaNO_2$/kg and 20mg DMA-HCl/kg and showing mild diffuse vacuolar degeneration of hepatocytes, with portal congestion. (Mag. x40)

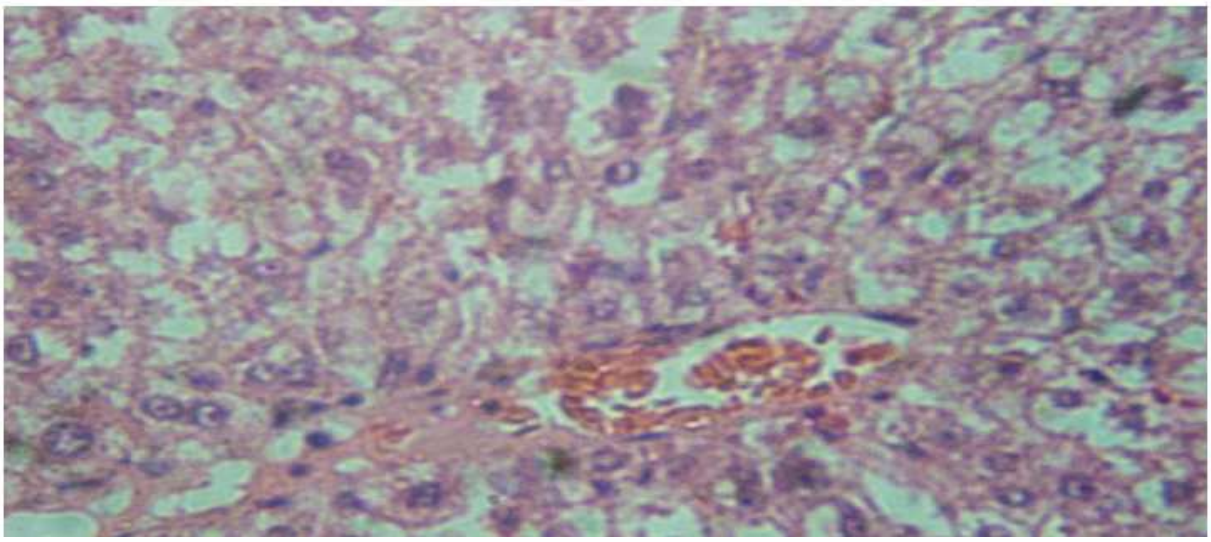

Figure.5: Photomicrograph of hepatocytes of low protein fed-rats following oral administration of concurrent doses of 3mg $NaNO_2$/kg and 20mg DMA-HCl/kg showing very mild diffuse vacuolar degeneration of hepatocytes, with mild portal congestion. (Mag. x40)

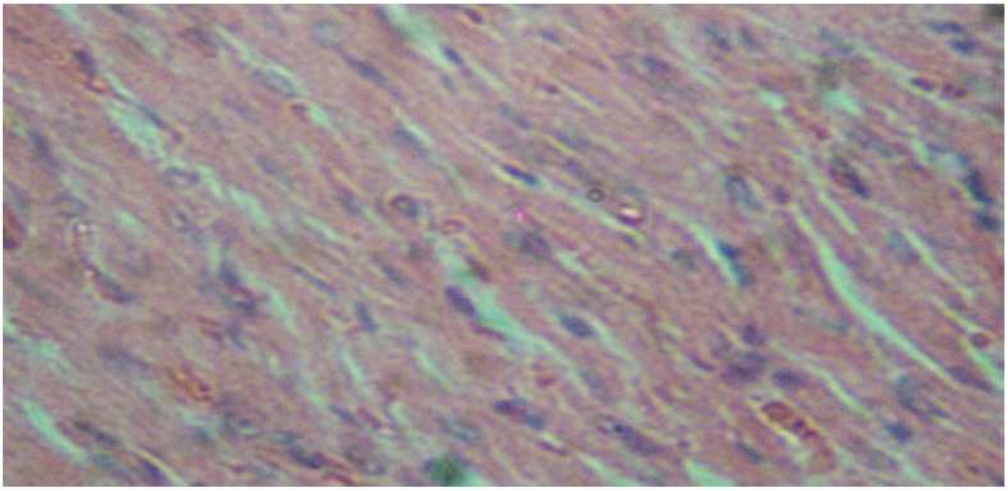

Figure.6: Photomicrograph of hepatocytes of control rats showing no visible lesions. (Mag. x40)

DISCUSSION

The Biotransformation of Foreign chemical compounds including Nitrite and dimethylamine hydrochloride (DMA-HCl) in the Liver has been shown to be influenced by nutritional status [Swann *et al* 1971], [Maduagwu *et al* 1983]; [Maduagwu, 1989]. Low protein diet ameliorates the toxic effect of Nitrosamines by slowing down hepatic metabolism of compounds into harmful products and invariably enhancing its elimination from the body. Without consumption of protein, concentration of plasma amino acids do not increase and net protein balance in the body remains negative [Kazemzadeh *et al* 2013].

The nutritional status in Nitrite 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. This should be expected since both structural changes in liver (which is the main organ of metabolism) and its attendant clinical manifestations have been observed during protein energy malnutrition. In this connection, nutrition status has been shown to affect metabolism of foreign compounds including nitrites and DMA [Swann *et al* 1971].

Liver function test, showed significant elevations ($P < 0.05$) in the AST, ALT, ALP and GGT activities in the rats fed with high protein diet compared with normal protein and low protein diet fed rats, there was also a significant difference ($P < 0.05$) in the rats fed with normal protein and low protein diet following concurrent administrations of $NaNO_2$ and dimethylamine hydrochloride DMA-HCl/kg (Table 2) compared with the control group (Table 3). The activity of the liver enzymes were significantly increased in the rats fed with high protein diet, which is consistent with the findings of Williams et al which reported that the activities of AST and ALT per gram of liver and per gram of soluble protein were significantly increased in fish fed the high-protein diet. The activities of both enzymes in the liver were significantly higher in fish with a higher protein gain [Williams *et al* 1949].

When dietary protein fed to experimental animal was increased from 8 to 16%; liver weight and percentage nitrogen also increased significantly with increasing level of dietary protein. The activity of ALT increased linearly with increasing levels of dietary protein.

The result of Histopathology examination on livers of rats fed with high protein diet showed periportal necrosis of hepatocytes with mono nuclear cellular infiltration (Fig.3). In the normal protein diet fed rats, it showed mildly diffuse vacuolar, degeneration of hepatocytes, with mild portal congestion (Fig.4). The low protein diet fed rats showed very mild diffuse vacuolar degeneration of hepatocytes and very mild portal congestion (Fig. 5) and there was no visible lesion in the control rats (Fig.6). In the incubation medium the result shows significant difference ($p<0.05$) in the concentration of nitrite in liver microsomes in the high protein, normal protein and low protein diet fed rats that were given nitrite/DMA-HCL compared to the control rats and could form nitrosamine easily as the high nitrite concentration is a precursor to nitrosamine formation. There was a decrease in the metabolism in the low protein diet fed rats than the high protein and the normal protein diet fed rats. Incubation of 10,000xg liver fraction with concentrations of 5mM of $NaNO_2$ alone and 5mM $NaNO_2$ combined with 5nM DMA-HCL resulted in loss of nitrite on exposure to UV light (Table 4 & 5). This was true to all test groups studied. [Bingbing *et al* 2008] reported UV irradiation combined with ozonation (UV/O_3) inhibit the regeneration of NDMA, amine and nitrous acid after degradation depending on wavelength used. There was a significant difference in nitrite concentration between before and after exposure to UV light after incubation. The appearance of high nitrite concentration from DMA-HCL combined with $NaNO_2$ incubation medium over $NaNO_2$ alone incubation medium shows that DMA-HCL undergoes a reaction or metabolism that contributes to high nitrite formation, metabolism, and excretion.

After the ultra-violet light exposure, the concentration of metabolism in the high protein, normal protein and low protein diet fed rats were reduced (Table 4), indicating that the UV-light has degraded the nitrite. This is in consistent with the experiment done by [Suschek *et al* 2003]; [Usunobun *et al* 2011] which show that UV-light converts nitrite to nitric oxide (NO) because of the photodecomposition of nitrite solutions. This study has revealed that a high protein diet enhances N-Nitrosation of dimethylamine hydrochloride, its liver toxicity and metabolisms both *in vivo* and *in vitro*. It also showed the possible inhibition of n- nitrosamine precursors on exposure to ultraviolet light.

ABBREVIATION

ALT : Alanine aminotransferase
ALP : Alkaline Phosphatase
AST : Aspartate aminotransferase
DMA-HCl : Dimethylamine hydrochloride
Kcl : Potassium chloride
Kg : Kilogram
M : Molar
Mg : Milligram
$MgCl_2$: Magnesium chloride
Mol.wt : Molecular weight
Mm : Millimolar
NaNO2 : Sodium nitrite
NDMA : N-nitrosodimethylamine
NDEA : N-nitrosodiethylamine
NADP : Nicotineamide dinucleotide phosphate
RPM : Revolution per minute
SD : Standard deviation
TCA : Trichloroacetic acid
UV : Ultraviolet
µg : Microgram

µL : Microlitre
γ-GT : gamma-glutamyl transferase

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