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Toxicity Evaluation of the Liver and *in vitro* Metabolism in Wistar Rat on Exposure to N-Nitrosamine Precursors

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Abstract: The aim of this study is to evaluate liver toxicity on exposure to n-nitrosamine precursors as well as the effect of ultraviolet light on n-nitrosamines and its precursors. Toxicological evaluation of the liver following single dose treatment of wistar rat with 8.2125 mg $NaNO_2$/adult rat and in rats given a combined dose of 50 mgDMA-HCl and 8.2125 mg $NaNO_2$ /adult rat showed a steady elevation of the liver function enzymes. Histopathological analysis of the liver showed hepatic necrosis in the chemical induced rats. Following UV exposure after *in vitro* incubation of rat liver microsomal plus soluble fraction with $NaNO_2$ and $NaNO_2$ plus DMA-HCl, nitrite concentration in the $NaNO_2$ incubation medium was 19.5 and 2.2 μg/mL before and after UV exposure respectively while the nitrite concentration in the $NaNO_2$ plus DMA-HCl incubation medium was 23.5 and 2.5 μg/mL, respectively. Nitrite loss was significant ($p<0.05$) between before and after UV exposure in all groups. UV exposure, thus degraded the nitrosamine precursors, nitrite and DMA-HCl, thereby inhibiting possible nitrosation. The high values of the activities of serum transaminases (AST and ALT), alkaline phosphatases (ALP) and gamma-glutamyltransferses (γ-GT), relative to control values are indicative of severe intrahepatic cell damage.

Key words: Dimethylamine hydrochloride, incubation, N-nitrosamine, Sodium nitrite, UV irradiation

INTRODUCTION

Secondary amines are common constituents of foodstuffs and can react with naturally occurring or added nitrite in acidic conditions or in the stomach to form N-nitrosamines (Bavin *et al*., 1984; Pignatelli *et al*., 1987). It appears well established that enough amines occur in human foods and in the environment and constitute health hazards through endogenous nitrosation (Haorah *et al*., 2001). The interest in nitrate consumption is due to the subsequent conversion of nitrates to nitrites, which are of greater concern in the formation of N-nitroso compounds (Griesenbeck, *et al*., 2009). The endogenous conversion of nitrate to nitrite is a significant source of exposure to nitrites; approximately 5% of ingested nitrates in food and water are converted to nitrite in the saliva (Choi, 1985).

Some of the target organs for n-nitrosamines-induced tumorigenesis are rich in specific cytochrome P-450 isoforms e.g., liver. In order to exert toxic and/or carcinogenic effects, most carcinogens need to be activated primarily by phase 1 drug-metabolizing enzymes including, cytochrome P450, cytochrome b5, arylhydrocarbon hydroxylase (AHH), N-nitrosodimethylamine N-demethylase 1 (NDMA-N-dl), NADPH-Cytochrome c reductase and the expression of cytochrome P4502EI (Sheweita *et al*., 2007). Sander and Bürkle (1969) reported the occurrence of oesophageal and hepatic tumours in rats fed N methyl-benzylamine or morpholine mixed with sodium nitrite in the diet.

It is known that nitrosation reactions can be influenced by inhibitors (vitamin C and E) or catalysts (metal ions, nucleophilic anions as chloride ions or iodide ions, carbonyl compound) (Tricker and Preussman, 1991). N-nitrosamines are extremely photosensitive and will decompose easily on exposure to UV light, particularly in the presence of strong acids to give the corresponding secondary amine and a good yield of nitrite (Luque-Paroez *et al*., 2001).The objective is to evaluate the toxicity of the liver on exposure to n-nitrosamine precursors and the possible inhibition of n-nitrosamine and its precursors on exposure to Ultraviolet light .

MATERIALS AND METHODS

Chemicals: Sodium Nitrite ($NaNO_2$, Mol.wt 69) was obtained from the Biochemistry laboratory, University of

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Ibadan while the pure Dimethylamine Hydrochloride (DMA-HCl, Mol.wt 81.55) was obtained from our Biochemical Toxicology Laboratory, Department of Biochemistry, University of Ibadan. All other reagents and chemicals were of analytical grade. This study was conducted in 2010.

Experimental animals: Forty-five (45) wistar albino rats weighing between 100-120 g were purchased from Covenant Farm (NIG.) enterprises beside Gbalosire estate molade via, Bishop Philips academy, Iwo road, Ibadan, Oyo state and housed in the Experimental Animal House, Department of Biochemistry, University of Ibadan. They were fed pellets and water, ad libitum and all test animals were acclimated to their environment and diet for one (1) week before experiment were begun.

Experimental group: Three groups of wistar albino rats with each group comprising of 10 rats were used for the investigation. Group one animals were given single oral dose of 8.2125 mg Sodium nitrite ($NaNO_2$) per adult albino rats. Group two animals were given a single oral dose of 8.2125 mg sodium nitrite ($NaNO_2$) and 50 mg Dimethylamine hydrochloride (DMA-HCl) per adult albino rat. Group three rats received only water and were used as control.

All experimental animals had free access to rat pellets and water ad libitum throughout the period of the experiment. 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: Twenty-four (24) hours after treatment with sodium nitrite ($NaNO_2$) and Dimethylamine Hydrochloride (DMA-HCl), all the rats were bled and 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 in an MSE portable general laboratory centrifuge for about 15 min at 3000 rpm. The supernatant sera were collected and stored in a refrigerator for a short time prior to analysis.

Gamma-glutamyl transferase (γ-GT) and Alkaline phosphatase (ALP) activities were determined in the serum samples using the method of Szasz (1969) and Englehardt (1970) respectively as described. The activities of Alanine amino transferase (ALT) and Aspartate amino transferase (AST) were evaluated base on the method of Reithman and Frankel, (1957) also as described.

Preparation of rat liver for histopathology: The livers of both the test and control rats were excised, dried with blotting paper, weighed and fixed in 10% formal saline prior to histology. Sections of livers were then prepared and stained with hematoxylin and eosin following fixation. Permanent amounts were examined by light microscopy and the results obtained were compared with control.

Preparation of rat liver microsomal plus soluble fraction (10,000×g fraction): Livers were removed from under urethane anaesthesia. Livers were immediately cooled with ice-cold 0.15 M KCl. Gall bladder and extraneous tissue were removed and the liver weighed after rinsing and blotting. Liver tissue was homogenized with 4 volumes of 0.06 M phosphate buffer plus 0.15 M KCl. pH 7.4 with a Teflon glass homogenizer. The homogenate was centrifuged at 10,000×g for 15 min in an MSE high speed refrigerated centrifuge. The resultant supernatant containing the microsomes plus soluble fraction was used for the in vitro studies.

Incubation assay: The complete incubation medium had a total of 6 mL and contained NADP (0.25 mM), glucose 6-phosphate (0.25 mM), $MgCl_2$ (20 mM), 0.06 M phosphate buffer, 0.15 M KCl and 2.5 mL of microsomal plus soluble fraction of liver homogenate. For the different experiments carried out, the concentration of sodium nitrite was 5 mM and DMA-HCl was 5mM.

The incubation was carried out in a shaking water bath, temperature 37ºC for 30 min. The reaction was terminated by adding 2 mL 5% TCA followed by exposure to UV irradiation (short wavelength) for a minimum of 15 min. Incubation medium containing boiled tissue for 30 min was used as control. Nitrite concentration before and after exposure to UV irradiation was determined according to Montgomery and Dymock (1961).

Statistical analysis: The data were expressed as mean±Standard deviation. $p<0.05$ were considered statistically significant for differences in mean.

RESULTS AND DISCUSSION

N-Nitrosamines are potent mutagenic and carcinogenic compounds in humans and animals and are widespread in the environment. Their existence has been confirmed in food products (Wang *et al*., 2005; Luque-Pérez *et al*., 2001) cosmetic products (Schothorst and Somers, 2005; Flower *et al*., 2006), tobacco smoke (Lee, 2007) soil (Pan *et al*., 2006), ground water (Fu and Xu, 1995; Tomkins *et al*., 1995; Tomkins and Griest, 1996), etc. In general, it is well known that *N*-nitrosamines are formed by a nitrosation reaction between secondary amines and nitrite and Dimethylamine Hydrochloride is a secondary amine.The toxicological evaluation of the liver showed a steady elevation of the enzymes in rats

Table 1: Effect of a single oral dose of 8.2125 mg $NaNO_2$/adult rat and a combined dose of 8.2125 mg $NaNO_2$/adult rat with 50 mgD MA-HCl/kg body weight

Drug administered (mg)	AST (U/l)	ALT (U/l)	ALP (U/l)	γGT (U/I)
$NaNO_2$	30.4±11.21	34.5±5.75	7.068±3.01	2.345±1.514
$NaNO_2$ + DMA-HCl	38.2±2.28	30.6±10.41	18.03±9.23	1.4925±1.1027
Control	13.25±10.44	25.67±1.53	0.69±0.59	0.94±0.1

Values are mean ± SD of 4 determinants

Table 2: Nitrite appearance following UV irradiation of liver microsomal plus soluble fraction of wistar albino rats at concentration of 5mM $NaNO_2$ and a combination of 5mM $NaNO_2$ and 5mM DMA-HCl

Compound administered	Nitrite conc. before UV irradiation after incubation (µg NO_2/mL)	Nitrite conc. after x incubation (µg NO_2/mL) after incubation (µg NO_2/mL)
$NaN0_2$	19.5	2.2
$NaNO_2$ + l DMA-HC	23.5	2.5
Control	0.33	0.012

Values are mean ± SD

induced with $NaNO_2$ (Table 1) and in rats with a combined dose of DMA-HCl with $NaNO_2$ (Table 1). It is known that these enzymes are mainly found on the liver in high concentration and whenever the enzymes are found in high amounts in the serum, it signifies that the liver has problem. The high values of the activities of serum transaminases, alkaline phosphatases and γ-GT, relative to control values are indicative of severe intra-hepatic cell damage due to the compound administered.

Histological evaluation showed hepatic necrosis in both nitrite induced group (Fig. 2) and DMA-HCl combined with $NaNO_2$ group (Fig. 1), thus showing acute damage. This was against the control group (Fig. 3) which showed no lesion. Thus the single dose, necrotizing to rat liver, produced acute hepatotoxicity when compared with the controls. Morphological changes observed in the livers during necrosis were similar in nitrite induced groups (Fig. 2) and nitrite plus DMA-HCl group (Fig. 1). In affected areas, there was congestion of the portal vessels, diffuse hydropic degeneration, periportal cellular infiltration by mononuclear cells as well as severe diffuse hepatic necrosis accompanied by diffuse mononuclear infiltration.

The nitrite concentration from the incubation medium with DMA-HCl plus $NaNO_2$ (Table 2) showed a high nitrite concentration and an easier room for nitrosamine formation as the high nitrite concentrationis a precursor to nitrosamine formation. Rounbehler *et al*. (1977) demonstrated the formation of NDMA in mice after gavage administration of 50 ng each of sodium nitrite and dimethylamine hydrochloride. Nitrite has been shown to cause methaemoglobinemia (Archer, 1981). Also there is circumstantial evidence in man that nitrite formed by reduction of nitrate is absorbed leading to elevated methaemoglobinemia levels in infants (Shuval and Gruener, 1972). Bingbing *et al*. (2008) reported that

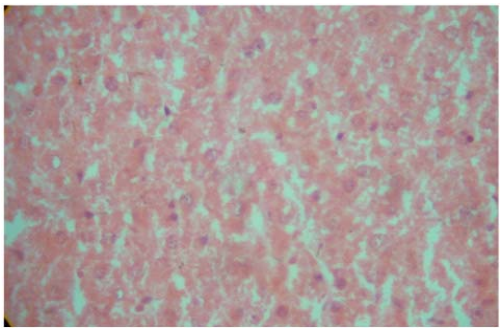

Fig. 1: Photomicrograph Hepatocyte of rats administered Dimethylamine hydrochloride (DMA-HCl) and Sodium nitrite ($NaNO_2$) showing congestion of the portal vessels, severe diffuse hepatic degeneration and necrosis. (Mag. ×40)

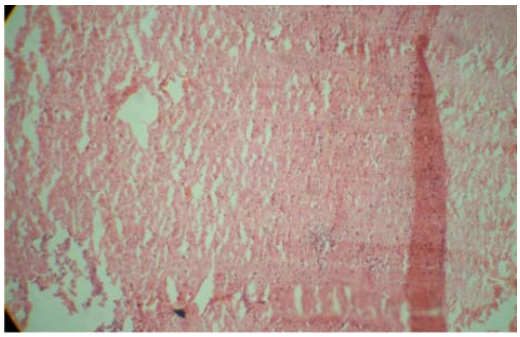

Fig. 2: Photomicrograph of Hepatocyte of rats administered with 8.2125 mg $NaNO_2$ only showing there is severe diffuse hepatic necrosis accompanied by diffuse mononuclear cellular infiltration. (Mag. ×40)

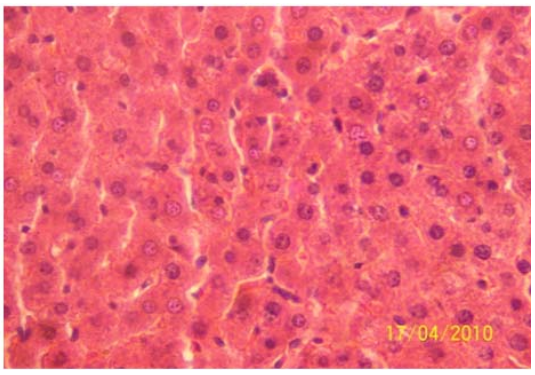

Fig. 3: Photomicrograph of liver of control rats showing no visible lesion. (Mag. ×40)

nitrosamines such as NDMA, NDEA could be completely degraded under the direct UV irradiation. When exposed to ultraviolet light, n-nitrosamine decompose either to aldehydes, nitrogen and nitrous oxide or quantitatively to

amine and nitrous acid depending on the wavelength used (Polo and Chow, 1976). Incubation of 10,000×g liver fraction with concentrations of 5 mM of $NaNO_2$ alone and 5 Mm $NaNO_2$ combined with 5Mm DMA-HCl resulted in loss of nitrite on exposure to UV light (Table 2). This was true in all groups studied. There was a significant difference in nitrite concentration between before and after exposure to UV light after incubation but there was no significant difference in amount of nitrite that disappeared among the different animal groups. It thus appears that UV exposure prevents nitrosation via reduction of the nitrite concentration. Another question might be the UV exposure on other parts of the liver fraction apart from nitrosation. 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. UV exposure thus degraded the n-nitrosamine precursor, nitrite and DMA-HCl, thereby inhibiting possible nitrosation.

This research showed the severe intra-hepatic cell damage caused by n-nitrosamine precursors as there was hepatic necrosis.The study also showed ultra-violet degradation and inhibition of n-nitrosamines and its precursors.

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ABBREVIATION

ALT	: Alanine aminotransferase
ALP	: Alkaline Phosphatase
AST	: Aspartate aminotransferase
DMA-HCl	: Dimethylamine hydrochloride
Kcl	: Potassium chloride
Kg	: Kilogram
Mg	: Milligram
$MgCl_2$	: Magnesium chloride
Mol.wt	: Molecular weight
Mm	: Millimolar
$NaNO_2$	: Sodium nitrite
NDMA	: N-nitrosodimethylamine
NDEA	: N-nitrosodiethylamine
NADP	: Nicotineamide dinucleotide phosphate
UV	: Ultraviolet
TCA	: Trichloroacetic acid
µg	: Microgram
γ-GT	: gamma-glutamyl transferase

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