



## Evaluation of level of nitrite *in vitro* in wistar rats administered precursors of n-nitrosamine

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### ABSTRACT

The aim of this study was to evaluate the level of nitrite in urine, protein concentration and nitrite level in the microsomal plus soluble fraction of liver in wistar rats administered with sodium nitrite and dimethylamine hydrochloride. Wistar rats were divided into three groups, the first group was given a single concurrent dose of 50mg/kg of dimethylamine hydrochloride and 62.05mg/kg of sodium nitrite, the second group was given 62.05mg/kg of sodium nitrite and control group was given water and food only *ad libitum*. The methods used were cell fractionation, homogenization, centrifugation and spectrophotometry. There was a significant increase ( $P < 0.05$ ) in the concentration of nitrite in urine of rat, in the protein concentration and nitrite level in the microsomal plus soluble fraction of liver in all the experimental groups compared to the control. After exposure to UV-light there was a decrease in the level of nitrite in all the groups, which indicates that nitrite may form nitroso compounds by reacting with a nitrosatable compound. This study shows the level of nitrite in urine of wistar rat, the level of protein and nitrite in microsomal plus soluble fraction of rats and the UV degradation of precursors of N-nitrosamine.

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### Introduction

Sodium nitrite and dimethylamine hydrochloride are toxic compounds and may inter react to form dimethylnitrosamine, a much more toxic compound and a powerful carcinogen in the gastrointestinal tract. Among these chemical carcinogens are N-nitrosamines and their precursors, nitrite and secondary amines. Various levels of carcinogenic volatile N-nitrosamines, nitrites and secondary amines are present in a wide variety of foods such as cured meat, smoked fish, dried malt and beer (Schothort and Somers, 2005; Flower *et al* 2006; Ostersahl, 1988). Most N-nitrosamines have been shown to be carcinogenic in laboratory animals (Preussmann and Stewart, 1984). Nitrite is known to be a precursor of toxic and carcinogenic N-nitrosamines (Bassir and Maduagwu, 1978) and induces cancer in experimental animals (Sen and Baddoo, 1997; Mirvish, 1995). After ingestion, residual nitrite can form traces of certain N-nitroso compounds in stomach (where the  $\text{pH} < 7$ ) on reacting with secondary amines which might also be present in the ingested food. Nitrite can also interact with haemoglobin by oxidation of ferrous ion ( $\text{Fe}^{2+}$ ) to ferric state ( $\text{Fe}^{3+}$ ) preventing or reducing the ability of blood to transport oxygen a condition known as methaemoglobinaemia (Philips, 1971; Tannenbaum, 1980). Nitrosamines are formed by the interaction of nitrous acid with secondary or tertiary amines. Secondary amines are known to occur in various foods such as vegetables, fish, cheese, mushrooms, fruits, wines and beer. Nitrites are found in the environment either naturally or from reduction of nitrates by microorganism or are added to food as preservatives. In the liver microsomal plus soluble fraction, enzymes are present which are responsible for reactions of the first and second phase of biotransformation of nitroso compounds. Biotransformation reactions are catalyzed by microsomal enzymes dependent on

cytochrome P-450, i.e. a set of hemoproteins catalyzing the activation of molecular oxygen and transfer of oxygen to lipophilic molecule of the xenobiotic (Rostkowska *et al.*, 1998). Urine is the major routes through which drugs, foreign compounds and their metabolites are eliminated from the body of both animals and human. At reasonable concentrations nitrite is rapidly and extensively excreted in urine and thus does not accumulate in tissues (EFSA Journal, 2009).

The objective of this study is to evaluate the level of nitrite in the urine of wistar rats, the protein concentration, the nitrite concentration in microsomal plus soluble fraction and the effect of UV irradiation on the tissue homogenate of rats administered with sodium nitrite and dimethylamine hydrochloride.

### Materials and methods

**Chemical and reagents:** Sodium nitrite ( $\text{NaNO}_2$ , Mol.wt 69), Dimethylamine hydrochloride ( $(\text{CH}_3)_2\text{NH.HCL}$ ), Mol.wt 81.55), were obtained from Sigma (USA). All the other chemicals and test kits used were of analytical grade.

### Composition of the Montgomery and Dymock Reagent for Nitrite Determination

**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.

a. **Solution B:** Naphthylethylenediamine (NEDA) solution. 0.4g Naphthylethylenediamine dihydrochloride was dissolve in one litre double distilled deionised water.

b. **Solution C:** 0.5% sodium carbonate solution. 0.5g  $\text{Na}_2\text{CO}_3$  was dissolved in 100ml double-distilled-deionised water.

### 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°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 62.05mg/kg of sodium nitrite per adult rat.
- A single concurrent dose of 50mg/kg of dimethylamine hydrochloride and 62.05mg/kg of sodium nitrite per adult rat.

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 animal were starved (fasted) over night prior to the administration of toxin compounds. The rats were sacrificed 24 hours after dosing.

### Collection of blood samples for serum preparation:

The animals were sacrificed by cervical dislocation and blood was collected in dry plastic or glass centrifuge tubes. The blood was allowed to clot and immediately centrifuged at 2500rpm for 15 minutes in a table top centrifuge and serum was collected for analysis.

### Collection of Urine

The rats were placed in metabolic cages with separate facilities where urine was collected every six hours and the 24 hours product pooled together in the end. Samples were analysed daily or stored in a refrigerator (at 10°C).

### Determination of Nitrite in Urine

The urine was collected after 24 hours and 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.

### Preparation of liver post mitochondrial fraction (10,000 x g fraction)

Livers were quickly removed from animals and the blood mopped. The tissues were weighed and immediately cooled in ice-cold 0.15Mkcl. Gall bladder and extraneous tissues were earlier discarded. Liver tissue was homogenized with 4 volumes of 0.06M phosphate buffer plus 0.15MKCL pH 7.4 using a Teflon glass homogenizer. The homogenate was centrifuged at 800xg for 15 minutes to remove cell debris and nuclei and the resulting supernatant was then centrifuged at 10,000 x g for 15 minutes in an MSE high speed refrigerated centrifuge. The resultant supernatant containing the microsomal plus soluble fraction was used for the *in vitro* studies. Protein concentration was determined using the method of Gornal *et al.*

### Incubation assay

The complete incubation medium had a total volume of 6ml and contained NADP (0.2mM), glucose-6-phosphate (0.2mM), MgCL<sub>2</sub> (20mM), 0.06M phosphate buffer, 0.15MKCL and 2.5ml of the microsomal plus soluble fraction of liver homogenate.

### Data analysis

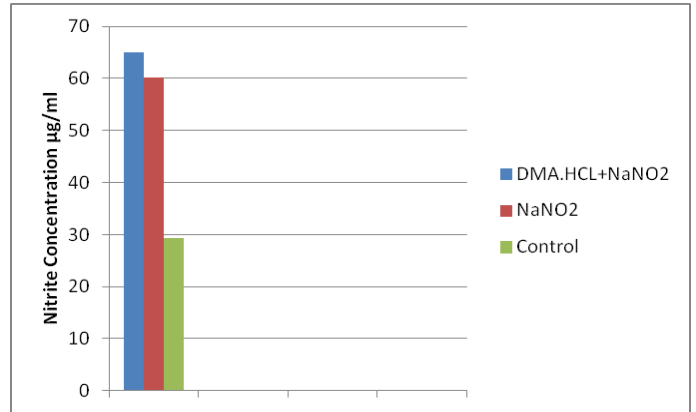
Data from treated and control animals were analyzed statistically using student's T-test and was expressed as mean  $\pm$  standard deviation. Significant assessed was at P<0.05 levels.

### Results

**Table.1: Nitrite concentration in urine of rats following oral administration with dimethylamine hydrochloride and sodium nitrite**

Group	Volume of urine (ml)	Concentration of Nitrite( $\mu$ g)
Control	2.2	29.33 $\pm$ 0.18
DMA.HCL+ NaNO <sub>2</sub>	4.6	65.05 $\pm$ 0.28
NaNO <sub>2</sub>	4.0	60.25 $\pm$ 0.94

Values are mean  $\pm$  SD of 5 determinants



**Figure 1: Nitrite concentration in urine of rats following oral administration with DMA.HCL AND NaNO<sub>2</sub>**

**Table. 2: Concentration of protein in microsomal plus soluble fraction of rat**

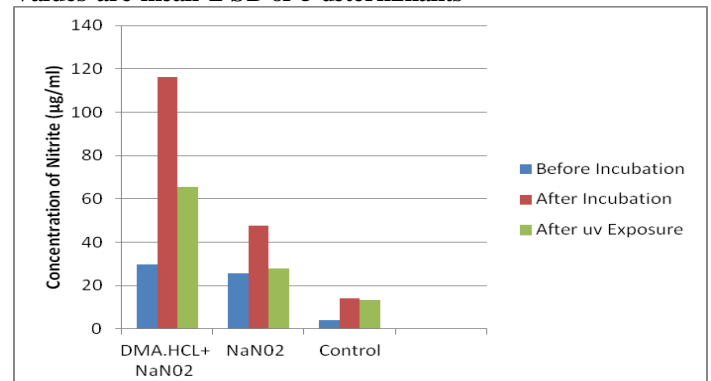
Animal Treatment	Weight of liver(g)	Protein Concentration ( $\mu$ g/ml)
DMA.HCL+ NaNO <sub>2</sub>	7.23	7.40 $\pm$ 0.36
NaNO <sub>2</sub>	7.25	6.66 $\pm$ 0.18
Control	7.56	3.72 $\pm$ 0.15

Values are mean  $\pm$  SD of 5 determinants

**Table.3: Nitrite level in microsomal plus soluble fraction of rat following incubation with DMA.HCL + NaNO<sub>2</sub> and NaNO<sub>2</sub>**

Animal Treatment	Nitrite Concentration Before Incubation ( $\mu$ gNO <sub>2</sub> /ml)	Nitrite Concentration After Incubation ( $\mu$ gNO <sub>2</sub> /ml)	Nitrite Concentration After Exposure to UV light ( $\mu$ gNO <sub>2</sub> /ml)
DMA.HCL+NaNO <sub>2</sub>	29.66 $\pm$ 1.03	116.35 $\pm$ 1.19	65.63 $\pm$ 1.38
NaNO <sub>2</sub>	25.61 $\pm$ 1.01	47.59 $\pm$ 0.64	28.03 $\pm$ 1.06
Control	22.02 $\pm$ 0.21	14.14 $\pm$ 0.19	13.50 $\pm$ 0.71

Values are mean  $\pm$  SD of 5 determinants



**Figure 2: Nitrite level in microsomal plus soluble fraction of rat following incubation with DMA.HCL + NaNO<sub>2</sub> AND NaNO<sub>2</sub>**

## Discussion

The concentration of nitrite in urine in group administered with DMA.HCl + NaNO<sub>2</sub> was more than those in group administered with NaNO<sub>2</sub> only (Table 1). When both were compared to the control group, there was significant difference at P < 0.05. There was a significant increase P < 0.05 in the concentration of protein in microsomal plus soluble fraction in the DMA.HCl + NaNO<sub>2</sub> and NaNO<sub>2</sub> group compared to the control (Table 2).

When the microsomal plus soluble fraction was incubated with DMA.HCl + NaNO<sub>2</sub> for group A and NaNO<sub>2</sub> only for group B, there was a significant increase (p < 0.05) in level of nitrite when each groups were compared with the control (Table 3). After 15 minutes exposure to UV light, there was a highly marked significant decrease in each group (Fig.2). This is as a result that nitrite may form nitroso compounds by reacting with a nitrosatable compound. 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. The high level of nitrite after incubation in each group indicates that the microsomal enzyme N-demethylase in the liver has metabolized NDMA.

The high nitrite concentration in the medium incubated with DMA-HCl plus NaNO<sub>2</sub> (Table 3) compared to the group incubated with NaNO<sub>2</sub> alone is because they are precursors of nitrosamine which can readily form nitrosamine. Rounbehler *et al.* (1977) demonstrated the formation of NDMA in mice after gavage administration of 50 ng each of sodium nitrite and dimethylamine hydrochloride.

## Conclusion

This study shows the increase in the level of nitrite in urine of rat administered with precursors of N-nitrosamine, the protein concentration and increase nitrite level in the microsomal plus soluble fraction of liver in wistar rats administered with sodium nitrite and dimethylamine hydrochloride. It also shows UV degradation of precursors of N-nitrosamine.

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