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Evaluation of precursors of N-nitrosamine in wistar rat fed high fat diet

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ABSTRACT

The objective of this work was to investigate the possible formation of the heterocyclic N-nitrosomorpholine upon administration of nitrite and morpholine and also the significance of high fat diet on the metabolism of the nitrosamine, using spectrophotometric, cell fractionation, centrifugation, thin layer chromatographic, ultraviolet irradiation and *in vitro* and *in vivo* methods. The possible endogenous formation of NMOR was detected in urine collected 24hours after a combined oral administration of 20mg nitrite and 40mg of morpholine/kg through the process of thin layer chromatography. The retention factor (Rf) 0.664 and the purple chromatogram observed in the test urine using a detector NEDSA spray correlated with that of the standard thereby indicating the *in vivo* formation of nitrosamine. Also an *in vitro* study confirmed the formation of nitrosomorpholine at pH 7.4 in an appropriate phosphate buffer. The increase nitrite level after incubation of the liver microsomal fraction for 30minutes, arising from exposure to ultra violet light irradiation, confirmed the formation of nitrosomorpholine. The histopathology result of rat liver treated with concurrent administration of morpholine and $NaNO_2$ revealed heavy hemorrhage, inflammatory cells, fat deposition and general cytolysis which confirm the toxicity of nitrosomorpholine on the liver. It is concluded that the combined administration of nitrite and morpholine produced a nitrosamine whose metabolism to toxic species in the liver tissue is attributed to the biochemical and histopathologic lesions enhanced by a high fat diet.

Introduction

Nitrosamines are produced from nitrites and secondary amines, which often occur in the form of proteins. Their formation can occur only under certain conditions, including strongly acidic conditions such as that of the human stomach, high temperatures, as in frying, can also enhance the formation of nitrosamines. The presence of nitrosamines may be identified by the Liebermann's reaction. Under acidic conditions the nitrite forms nitrous acid (HNO_2), which is protonated and splits, into the nitrosonium cation $N{\equiv}O^+$ and water: $H_2NO_2^+ = H_2O + NO^+$. The nitrosonium cation then reacts with an amine to produce nitrosamine.[1,2] Food is the major source of human exposure to N-nitrosamines, others being smoking and endogenous synthesis such as through the reactions of nitrite from vegetable with dietary amines.[3]

Sodium nitrite forms carcinogenic nitrosamines in meats containing sodium nitrite when exposed to high temperatures. Alpha-tocopherol, ascorbic acid, and erythorbic acid all inhibit nitrosamine production by their oxidation-reduction properties. Ascorbic acid, for example, forms dehydroascorbic acid when oxidized, which when in the presence of nitrous anhydride, a potent nitrosating agent formed from sodium nitrate, reduces the nitrous anhydride into the nitric oxide gas.[4] The effects of nitrite (NO_2^-) are the same whether nitrite containing compounds are ingested or inhaled, or nitrite is produced *in vivo* from nitrate. Acute acquired methemoglobinemia is the most important adverse health effect caused by excessive nitrate or nitrite exposure.

Morpholine is cyclic amino ether as well as a secondary amine. The ether property of morpholine is typically inert. The secondary amine property involves in the most chemical reactions. Morpholine is a versatile chemical used as a solvent itself for resins, dyes, and waxes. Its alkyl derivatives (e.g. N-methylmorpholine, N-ethylmorpholine) are used as a catalyst for the production of polyurethane foams. Morpholine has a similar volatility with water. It is used as a pH adjustment additive in fossil fuel and steam systems as a corrosion inhibitor. Morpholine is absorbed after oral, dermal and inhalation administration. In rats it was distributed to all organs and eliminated rapidly. In rabbits, mice, rats and hamsters, almost all morpholine was excreted unchanged in the urine following its administration by any route, whereas guinea-pigs excreted 20% of the dose as *N*-methylmorpholine- *IV*oxide.

N-Nitrosomorpholine was formed in rodents following concomitant administration with nitrite or nitrous oxide and *in vitro* when added to human saliva.[5]

Skin application in rabbits, oral administration and skin application in guinea-pigs or inhalation in rats caused necrosis of kidney tubules and liver. Oral administration induced stomach and small intestine haemorrhages in guinea-pigs and rats, liver degeneration in rats and renal insufficiency in mice. Rhinitis, lower airway irritation and corneal oedema have been reported in workers exposed to morpholine.[5]

The objective of this research is to study the toxicological effect of concurrent administration of morpholine and nitrite, to determine the possible *in vitro* nitrosation of morpholine and to study the metabolism of nitrite and morpholine in urine of rat fed high fat diet.

Materials and methods

Chemical and reagents: Sodium nitrite ($NaNO_2$**,** Mol. wt 69), Morpholine C_4H_9NO, Mol. wt 87.12 were obtained from Sigma (USA). All the other chemicals and test kits used were of analytical grade**.**

Experimental Animals:

Healthy young adult male albino rats were used for all studies and were housed in individual stainless- steel cages.

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Only those certified free of infection by the Veterinary pathologist were employed and all animals were kept at room temperature (approximately 28°C).The animals were allowed to acclimatize to their environments.

Animal treatment: Animals were divided into seven groups and were given sodium nitrite [$NaNO_2$] and Morpholine orally through intubation.

Group A- received normal diet + 20mg $NaNO_2$ and 40mg of morpholine/kg.

Group B- received normal diet + 20mg of $NaNO_2$/kg.

Group C- received normal diet + 40mg of morpholine/kg.

Group D-received normal diet + distilled water (control).

Group E- - received high fat diet + 20mg of $NaNO_2$ and 40mg of morpholine/kg.

Group F- received high fat diet + 20mg of $NaNO_2$/kg.

Group G- received high fat diet + 40mg of morpholine/kg.

Diet composition

Table 1: High fat diet

Maize	40.00%
Soya bean	10.00%
Groundnut cake	20.00%
Palm kernel cake	5.00%
Fish meal	5.00%
Bone	4.00%
Groundnut oil	10.00%
Methionine	0.05%
Lysine	0.05%
Salt	0.20%
Premix	0.30%

Collection of urine samples

The rats were placed in a metabolic cage and 24hour urine samples were allowed to flow into a urine collector. 0.5ml of NaOH was added to inhibit nitrosation in urine during collection and storage before nitrosamine extraction.[6]

After the initial collection of the 24hour urine, the rats fed with the experimental diets for 30 days were given oral administration of nitrosamine metabolites (morpholine and nitrite) as designed. Each rat was then placed back in the standard techniplast metabolic cages. Food and nitrite-free water was freely available to the animals. A urine sample from each rat was collected after 24hrs. Samples were stored for maximum of 5 days and nitrosomorpholine (NMOR) was extracted with dichloromethane in a column

Extraction of morpholine and nitrite from urine samples

Nitrosamine present in the urine was extracted on Extrelut column. Each of the samples collected were made up to 20ml with distilled water (the volume the column could take). Glass column was packed with extrelut Art 11.738 and fitted to Kurder-danish evaporator. The 20ml urine sample was adsorbed on the extrelut for about 30 minutes, when the flow of the urine down the column equilibrated; this was gently eluted with 20ml of dichloromethane. When elusion of nitrosamine was completed, the column was removed. Antibump chips were added to the eluate following which it was evaporated to about 5ml on a water bath at about 80°C or on hot plates. (If there were water in the eluate, it would splash). The concentrate was removed and immediately allowed to cool or pass through air by using a micropipette placed inside to blow air gently across. To further concentrate to 1ml mark of the evaporator, it was allowed to cool on its own (or use rotary evaporator to fasten evaporation down to 0.5ml) until all the CH_2Cl_2 was removed.

Preparation of TLC plates

Thin layer chromatographic plates were prepared first by cleaning the glass plates properly and finally with acetone. 50g of silica gel were mixed with 100ml of distilled water in a 250ml conical flask and vigorously shaken for about 3minutes. This mixture was used to coat 5 glass plates (20 x 20 x 0.25cm) to a thickness of 0.5mm, arranged on a Shandon Unoplan Leveller. Plates were allowed to set in air for about 30 minutes and then dried at 110°C in an oven for 3 hours for activation before use. Plates were stored in TLC plate container and activated just before use.

Running of TLC plates

A starting line was marked at the edge of the plate with a sharp pencil and the finishing line (14cm from the base) was drawn right across the plate. (The pencil removes a fine line of adsorbent down to the glass, and the solvent flow is forced to stop when the solvent reaches the line). The edges of the plates were rubbed clear with a piece of cotton wool, before spotting to a width of about 0.5cm to give a sharper edge to the absorbent layer. The test extracts were spotted on the thin layer plates alongside the authentic morpholine solution in dichloromethane using a micropipette. The spots were arranged about 2cm centre to centre and individual spotting positions were noted. The spots were allowed to dry and the plates developed in the selected mobile phase (solvent mixture) to a depth of 0.5 - 1.0cm, n-Hexane - diethylether – dichloromethane (4: 3: 2) {Solvent system of Daiber and Preussmann}.The plates were immersed such as to obtain an ascending chromatographic separation. At the end of the run, the solvent was allowed to evaporate from the plates.

Location and identification of the nitrosamines

The developed plate was allowed to dry in air for about 5 minutes and a thin spray with either the NEDSA reagent or the diphenylamine/palladium Chloride reagent of Preussmann was applied.[7] The moist TLC plate was irradiated with short wave, UV light (240nm) for a minimum of 15 minutes. The test nitrosamine was identified with respect to the positions of the standard on the same plate. Areas containing nitrosamine appeared red purple spots with NEDSA reagent and as dark blue/violet sports with Preussmann reagent. Preussmann *et al* recommended that response of spots to both reagents should be positive before identification is made.[7]

Preparation of liver microsomal plus soluble fraction

Liver were removed from animals under Rompon and Sargatal 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. Liver tissue was homogenized with 4 volumes of 0.06 M phosphate buffer plus 0.15MKCl pH7.4 with a Teflon glass homogenizer. The homogenate was centrifuged at 800 x g to separate cell debri and nuclei and the resultant homogenate was centrifuged at 10,000 x g for 15 minutes in an MSE high speed refrigerated centrifuge. The resulting post mitochondria supernatant containing the microsomal plus soluble fraction was used for the *in vitro* studies.

Incubation assay

The complete incubation medium had a total volume of 6ml and contained NADP (0.25mM), glucose-6-phosphate (0.25mM), semicarbazide hydrochloride (10mM), $MgCl_2$ (20mM), 0.06M phosphate buffer, 0.15MKCl and 2.5ml of microsomal plus soluble fraction of liver homogenate. The concentrations of sodium nitrite and morpholine used were 5mM.

The liver homogenate in each groups were incubated with the complete microsomal incubation medium that had a total volume of 6ml and contained NADP (0.025mM) glucose -6-phosphate (2.5mM),$MgCl_2$ (20mM), 0.06 M Phosphate buffer

0.15m KCl and 2.5ml of microsomal protein. In Group A, the concentration of Dimethylamine hydrochloride and sodium nitrite incubated in the incubation mixture was 5mM and the sodium nitrite incubated in Group B was 5mM. The control was not incubated with DMA. HCl and $NaNO_2$. The incubation was carried out in a sealed test tube for 30 min at 37^0C in a water bath shaker. The reactions were terminated by the addition of 2ml of 5% trichloroacetic acid, the nitrite concentration were determined using Dymock and Montgomery.[8] After the incubation for 30 minutes, the test tube was exposed to UV-light for 15 minutes. The level of nitrite was determined using Dymock and Montgomery method.

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.

Data analysis

Statistical analysis was performed using the Microsoft excel 11.0 statistical package, all results were expressed as means ± S.D. Comparisons within each group were performed using Student's *t* test for paired and unpaired data.

Results

Table 2: Chromatographic analysis of urine following concurrent administration of 20mg of nitrite and 40mg of morpholine/kg

Substance streaked on TLC plates	RF Value	Colour under UV Light	Spray reagent Colour
Nitrosamine	0.664	Blue	Purple
Morpholine	0.897	Blue	Blue
Nitrite	0.270	Blue	Reddish-Brown
Test Urine 1	0.664	Blue	Purple
Test Urine 2	0.897	Blue	Blue
Test Urine 3	0.270	Blue	Reddish-Brown
Control	-	-	-

Table 3: Nitrite level in microsomal fraction of rats fed fat diet following incubation with 20mg of nitrite and 40mg of morpholine/kg.

Group	Before incubation	After incubation	After exposure to UV light
Morpholine + $NaNO_2$	2.45 ± 0.17	2.03 ± 0.36	2.32 ± 0.66
$NaNO_2$	1.38 ± 0.18	1.34 ±0 .15	1.53 ±0.21
Control	0.78 ± 0.02	0.59 ± 0.41	0.67 ±0.07

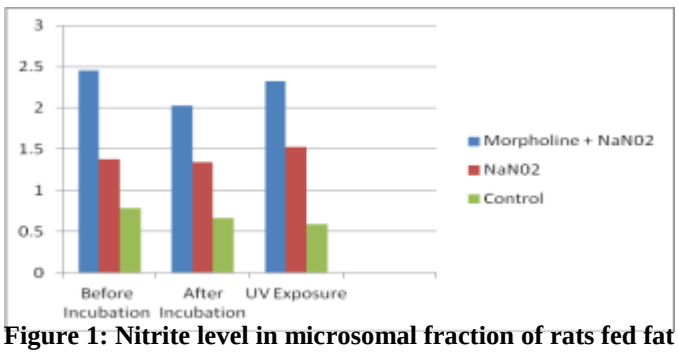

Figure 1: Nitrite level in microsomal fraction of rats fed fat diet following incubation with 20mg of nitrite and 40mg of morpholine/kg.

Table 4: Nitrite level in microsomal fraction of rats fed normal diet following incubation with 20mg of nitrite and 40mg of morpholine/kg.

Group	Before incubation	After incubation	After exposure to UV light
Morpholine + $NaNO_2$	1.63 ± 0.46	1.41 ± 0.25	1.71± 1.01
$NaNO_2$	1.29 ± 0 .44	1.21 ± 0.39	1.54± 0.35
Control	0.78 ± 0.41	0.59 ± 0.02	0.67 ±0.07

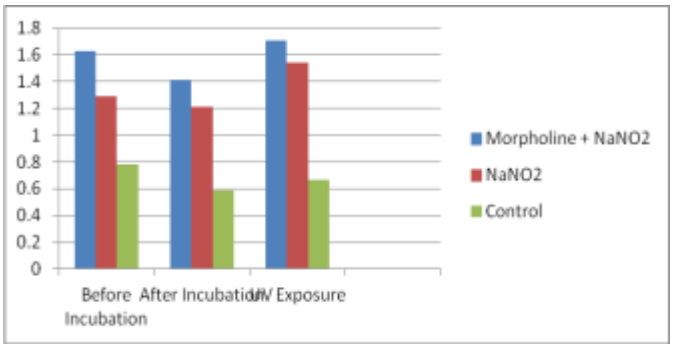

Figure 2: Nitrite level in microsomal fraction of rats fed normal diet following incubation with 20mg of nitrite and 40mg of morpholine/kg.

Histopathology

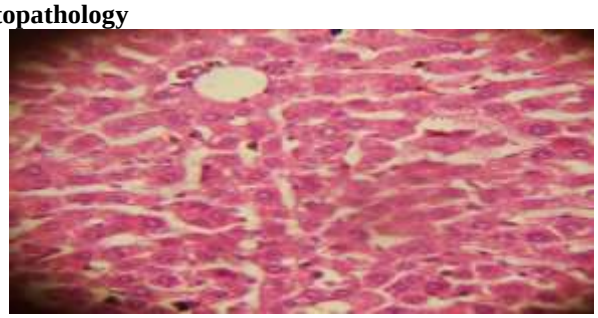

Figure 3: A photomicrograph of a liver section of fat fed rat administered 20mg of $NaNO_2$ and 40mg of morpholine/kg showing fat deposit displacing the nuclei (hyperplastic nuclei).

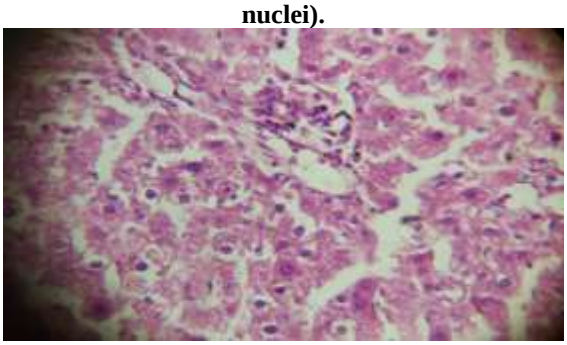

Figure 4: A photomicrograph of a liver section of fat fed rat administered 20mg of $NaNO_2$/kg showing fat deposition replacing the cytoplasm and inflammatory cells, nuclei displacement by fat.

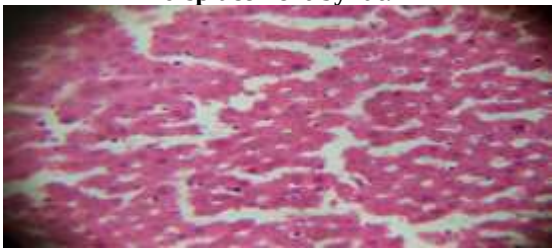

Figure 5: A photomicrograph of a liver section of fat fed rat administered 40mg of morpholine/kg showing fat deposit on hepatocytes cytoplasm, the fat choked and displaced nuclei to the edges of the cells

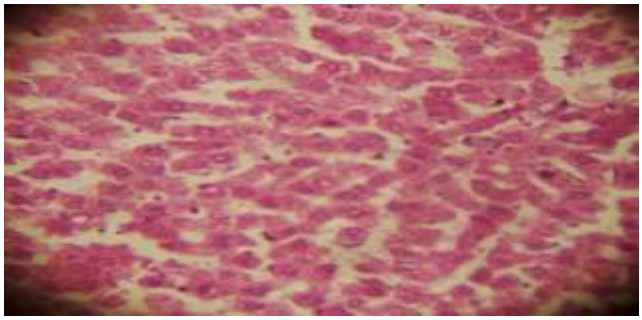

Figure 6: A photomicrograph of a liver section of control rat showing no visible legion.

Discussion

The result showed that both unchanged nitrosamine and its precursors (morpholine and nitrite) were present in the urine (Table 1). Identification was done by response to NEDSA spray reagent (colour) and RF values against known compounds.

The possible endogenous formation of NMOR was observed in the urinary analysis of rats administered with the precursors through the process of thin layer chromatography. Nitrosamine was observed in the urine of group 1 animals observed under UV lamp. The Rf values and color after NEDSA spray of the test urine correlate with that of the standard indicating the *in vivo* formation of nitrosamine.

The urine of rats concurrently administered morpholine and nitrite was analyzed and the RF value of 0.664 and purple color observed after NEDSA spray correlates with the standard nitrosamine compound.

There was a significant increase ($p<0.05$) in nitrite concentration in the microsomal plus soluble fraction in rat fed high fat diet (Table 3) compared to those fed normal diet (Table 4).

There was a significant decrease ($p >0.05$) after incubation and there was a significant increase ($p< 0.05$) when exposed to UV light in the microsomal plus soluble fractions of rat fed fat diet. This indicates the formation of N-Nitrosomorpholine (NMOR) after incubation and its degradation. Earlier reports showed that there was increased nitrite concentration under UV irradiation due to N –N fission of N –nitrosamines.[9,10]

The histopathological result showed fat deposit displacing the nuclei (hyperplastic nuclei) in fat fed rat administered 20mg of $NaNO_2$ and 40mg of morpholine (Figure 3). Fat deposition replacing the cytoplasm and inflammatory cells, nuclei displacement by fat was seen in fat fed rat administered 20mg of $NaNO_2$/kg (Figure 4). The fat fed rat administered 40mg of morpholine showed fat deposit on hepatocytes cytoplasm, the fat choked and displaced nuclei to the edges of the cells (Figure 5). The liver section of the control rat showed no visible legion (Figure 6).

Conclusion

Administration of all the nitrosamine precursors namely; morpholine, nitrite, and combination of both in the two groups presented in this study i.e. those fed normal and high fat diet respectively as shown in the result triggered hepatocellular toxicity but with intense effect on the latter group indicating that high fat diet is a booster of hepatocellular carcinoma. There was a formation of the N-nitrosomorpholine in vivo and in vitro after the oral administration of the precursors of N-nitrosamine and the increase in nitrite concentration of microsomal fraction after exposure to UV light also confirmed the formation of Nitrosomorpholine.

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