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# Histopathologic, biochemical and genotoxic investigations on chronic sodium nitrite toxicity in mice

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ABSTRACT

The aim of this study was to investigate the effects of long term Sodium nitrite ($NaNO_2$) consumption. Swiss albino mice were given $NaNO_2$ (0, 10 and 20 mg/kg/day) as mixed in feed for 8 months. At the end of treatments, animals were sacrificed and selected organs were processed for histopathologic, imunohistochemical, biochemical and genotoxic investigations. Mild to moderate degenerative changes were observed in liver, kidney, intestine, lung and spleen of $NaNO_2$-given mice. Inducible nitric oxide synthase and nitrotyrosine activities increased in liver and kidney of $NaNO_2$-given mice. Proliferating cell nuclear antigen activity increased in liver. Apoptotic cell death was observed in livers of the treatment groups. Liver malondialdehyde level was higher in the treatment groups while no change was seen in kidney. Nitric oxide levels in both liver and kidney of the treatment groups were lower than those of the control group. In genotoxic investigations, the number of chromosome and chromatid breaks, chromatid association, and polyploidy increased while mitotic index decreased in $NaNO_2$-given mice. The results showed that $NaNO_2$ would cause histopathologic changes, nitrosative tissue damage, and lipid peroxidation in liver and kidney, as well as induce chromosomal aberrations even if it was given at low levels for long time.


## 1. Introduction

Nitrate and nitrite compounds are important environmental toxicants and pose important health risks. High levels of these chemicals can also be found in water resources thereby causing major risks for consumers (De Roos et al., 2003). Several forms of these chemicals are widely used as food additives. As a source of color and flavor preservation, they are routinely added especially to cured meat products (Honikel, 2008).

Intentional or accidental toxicities and death due to nitrate and nitrite have been described in human and animals elsewhere (Worth et al., 1997; Yu et al., 2002; Ozmen et al., 2003; McKenzie et al., 2004). Methemoglobinemia is the well-known result of acute nitrate and nitrite toxicities. Other toxic effects of nitrate and nitrite compounds are due to mostly *N*-nitrosamines which are formed during the metabolic conversion of these substances. Nitrosamines are known to be a large group of chemicals that have the ability to induce oxidative stress and to cause cancer (Ahotupa et al., 1987; Tricker and Preussmann, 1991). Important sources of nitrosamines include smoked fish and meat products, dried foodstuffs by combustion gases, pickled and salt preserved foods, and foodstuffs with fungal contamination (Howe et al., 1986). Endogenous production of nitrite and eventually nitrosamines from ingested nitrate compounds by bacterial action can also take place (Bryan, 2006).

Although there are many investigations studying the effects of nitrate and nitrite compounds on health, most of these have been programmed as an acute toxicity study with considerably high dosage use. However, total intake of these substances is quite low in daily consumption for an average person. According to Özçelik (1982), total daily intake of nitrate and nitrite for a person is about 50–120 mg and 2–5 mg, respectively. Total amount of daily nitrite intake was also referred as 8–16 mg for a 60 kg person (Tan, 2003). However, it can be easily estimated that these amounts may show great variations among different countries based on the geographic or traditional differences. Therefore, the aim of this study was to investigate histopathologic, biochemical, and genotoxic effects of

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low level consumption of sodium nitrite ($NaNO_2$) in mice which might reflect the case in human.

## 2. Materials and methods

### 2.1. Animals and treatment

Swiss albino mice ($n = 30$) initially weighing 10–13 g at 3 weeks of age were housed as 3 groups (Control, group I and group II), each containing 10 animals, in individual cages in a room at a certain temperature (20–22 °C), humidity (60–70%) and 12:12 h light:dark cycle. While mice in control group were fed with a standard mouse feed twice a day, mice in group I and group II were given 10 mg/kg/day and 20 mg/kg/day $NaNO_2$, respectively. $NaNO_2$ was admixed to the standard mouse feed prior to the feeding started. Following 8 months of treatment period, all mice were sacrificed and necropsy was performed. All animal procedures were conducted in accordance with the guidelines of the Institutional Animal Ethics Committee.

### 2.2. Histopathologic investigations

At necropsy, tissue samples were collected for histopathologic evaluation. Tissues were fixed in 10% buffered formaldehyde solution and embedded in paraffin. Five μm thick sections were cut, then stained with hematoxylin and eosin (H&E), and observed under a light microscope.

### 2.3. Immunohistochemical investigations

Sections of liver and kidney were immunochemically stained for inducible nitric oxide synthase (iNOS) and nitrotyrosine in order to show nitrosative tissue stress. In order to investigate cellular proliferative activity in these tissues, immunohistochemistry for proliferating cell nuclear antigen (PCNA) was also used. Immunohistochemical staining of the samples was performed on 4–5 μm thick formalin-fixed paraffin-embedded sections. Following deparaffinization in xylene and rehydration in degrading series of ethanol, the sections were washed with phosphate buffered saline (PBS, 0.1 M, pH 7.4). Antigen retrieval of the tissues was performed by microwave treatment in 0.1 M sodium citrate solution, pH 6.0 at 600 W for 10 min. The sections were then treated with 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. Non-specific antibody binding was blocked by incubating the sections with 1.5% non-immune goat serum for 30 min. Then, in a humidified chamber antibodies against iNOS (Lab Vision, Cat No: RB-9242-P0), nitrotyrosine (Millipore, Cat No: 06-284) or PCNA (Chemicon, Cat No: 50-174-619) were allowed to incubate with the sections for 1 h at room temperature at 1:100, 1:1000 and 1:2000 dilutions, respectively. Following the primary antibody incubation, the sections were washed trice with PBS and incubated with a biotinylated rabbit antibody for 30 min. Then, the sections were washed as previously described and incubated with streptavidin-biotin immunoperoxidase (Lab Vision, Fremont, CA, USA) for 30 min. Peroxidase activity was visualized by treating the sections with 3,3-diaminobenzidine/$H_2O_2$ solution until color development. Finally, following rinses in distilled $H_2O$ the sections were counterstained with hematoxylin, rinsed under running tap water, and coverslipped. Negative controls were provided by exchanging the primary antibodies with PBS.

### 2.4. In situ TUNEL assay

Apoptotic cell death in liver and kidney tissues was investigated by DeadEnd™ Colorimetric TUNEL System (Promega, Cat No: G7130). Briefly, the sections were deparaffinized with xylene and rehydrated with degrading series of ethanol. Following rinses in PBS, the sections were treated with Proteinase K solution for 30 min. The enzyme solution was washed away with PBS rinses and then the sections were incubated with an equilibration buffer containing 200 mM potassium cacodylate, 25 mM Tris–HCl, 0.2 mM dithiothreitol, 2.5 mM cobalt chloride, and 0.25 mg/ml bovine serum albumin for 10 min. In a humidified chamber, a reaction buffer containing biotinylated nucleotide mix and terminal deoxynucleotidyl transferase was applied onto the sections at 37 °C for 1 h. Then, sodium citrate solution was applied for 15 min and the sections were rinsed with PBS. Endogenous peroxidase activity was blocked by $H_2O_2$ for 5 min. Following incubation with a streptavidin horseradish peroxidase solution for 30 min, peroxidase activity was assessed by color development *via* a solution of 3,3-diaminobenzidine/$H_2O_2$. Finally, the sections were rinsed with distilled $H_2O$, counterstained with 0.1% methyl green, orderly processed through several changes of distilled $H_2O$, butanol, and xylene and then coverslipped for microscopic examination. Negative control was provided by exchanging the reaction buffer containing biotinylated nucleotide mix with PBS.

### 2.5. Biochemical investigations

Liver and kidney tissues of treatment and control groups were rinsed with ice-cold 0.9% NaCl. Then, 1 g of tissue samples were homogenized in four fold of phosphate buffer in 0.1 M KCl, pH 7.4, in an ice bath. The homogenates were centrifuged in 5000 × *g* at 4 °C for 15 min. The tissue nitric oxide (NO) and malondialdehyde (MDA) contents were colorimetrically measured by the methods of Miranda et al. (2001) and Yoshoiko et al. (1979), respectively.

### 2.6. Genotoxic investigations

Four groups of mice, namely negative control, positive control, $NaNO_2$ (10 mg), and $NaNO_2$ (20 mg), each containing 5 animals were set. The negative control mice were given 0.9% NaCl, intraperitoneally (ip) while the positive control mice were treated with a single dose of 2 μg/g mitomycine-C. Groups of $NaNO_2$ (10 mg) and $NaNO_2$ (20 mg) mice were fed with a diet at 10 mg/kg/day and 20 mg/kg/day doses, respectively for 8 months as previously described. All mice were injected ip with 4 μg/g colchicine 3 h prior to sacrifice. Both femurs were dissected out and cleaned of any adhering muscle. Bone marrow cells were collected by flushing in 8 ml KCl (0.075 M) solution and incubated at 37 °C for 25 min. The cell suspension was centrifuged at 1000 × *g* for 10 min, fixed in ice-chilled acetic acid:methanol (1:3, v/v). Centrifugation and fixation were repeated thrice. The material was resuspended in a small volume of the fixative, dropped onto chilled slides, flame-dried, and stained the following day with 5% buffered Giemsa (pH 6.8). Finally, one hundred good metaphases containing 40 chromosomes were examined per animal to score different types of aberrations (chromosome and chromatid breaks, chromatid association). Well-spread chromosome arms that can be clearly distinguished and not crossed with others at the metaphase were accepted as good metaphases. Mitotic index and polyploidy were also determined in 1000 metaphases per animal.

### 2.7. Statistical analysis

Statistical analyses of biochemical data were done using SPSS software (Windows version 10.0). The data were expressed as median ($X$) ± standard deviation (SD). One-way analysis of variance followed by Student's $t$ test was to compare the values among the groups. A $P$ value of less than 0.05 was considered significant.