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

Hasan Özen a, *, Ufuk Kamber b, Musa Karaman a, Süleyman Gül c, Emine Atakişi d, Kadir Özan e, Onur Atakişi f

a Department of Pathology, Faculty of Veterinary Medicine, Kafkas University, Kars, Turkey
b Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Kafkas University, Kars, Turkey
c Department of Molecular Biology and Genetics, Faculty of Art and Science, Kafkas University, Kars, Turkey
d Department of Biochemistry, Faculty of Veterinary Medicine, Kafkas University, Kars, Turkey
e School of Health, University of Üşak, Üşak, Turkey
f Department of Biochemistry, Faculty of Art and Science, Kafkas University, Kars, Turkey

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ABSTRACT

The aim of this study was to investigate the effects of long term Sodium nitrite (NaNO₂) consumption. Swiss albino mice were given NaNO₂ (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, immuno-histochemical, biochemical and genotoxic investigations. Mild to moderate degenerative changes were observed in liver, kidney, intestine, lung and spleen of NaNO₂-given mice. Inducible nitric oxide synthase and nitrotyrosine activities increased in liver and kidney of NaNO₂-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 NaNO₂-given mice. The results showed that NaNO₂ 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.

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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
low level consumption of sodium nitrite (NaNO₂) 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 in mice 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 NaNO₂, respectively. NaNO₂ 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 immunohistochemically 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/H₂O₂ solution until color development. Finally, following rinses in distilled H₂O 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 deoxyguanosinucleotidyl 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 H₂O₂ 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/H₂O₂. Finally, the sections were rinsed with distilled H₂O, counterstained with 0.1% methyl green, orderly processed through several changes of distilled H₂O, 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 Yoshiko et al. (1979), respectively.

2.6. Genotoxic investigations

Four groups of mice, namely negative control, positive control, NaNO₂ (10 mg), and NaNO₂ (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 NaNO₂ (10 mg) and NaNO₂ (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.
Statistical analyses of data from genotoxic investigations were performed using GraphPad InStat version 3.05 for Windows 95 (GraphPad Software, San Diego, California, USA).

3. Results

In microscopic examination, mostly mild to moderate hydropic degeneration that was characterized by swelling of hepatocytes was seen especially at the pericentral regions of liver in mice given NaNO₂ (Fig. 1a). The degree of degeneration was more prominent in mice received the higher dose of NaNO₂ tested. Occasional necrotic cells, hyperemia and perivascular mononuclear cellular infiltration were also observed in some mice. Mild to moderate renal tubular degeneration in the proximal tubules was determined in kidneys of NaNO₂ given mice (Fig. 1b). As in liver, while mostly mild cellular degeneration was present in the lower dose group moderate cellular degeneration was noted in the higher dose group in kidney. Degenerative changes characterized by

![Representative microscopic tissue images of a mouse given 20 mg/kg/day NaNO₂ (H&E).](image)

(a) Liver: pericentral cellular degeneration (circled area), congestion (black arrow) and few mononuclear cells (white arrow). Inset figure shows cellular degeneration at higher magnification (arrows). (b) Kidney: moderate tubular degeneration (black arrows). (c) Small intestine: degeneration (black arrows) and sloughing off (white arrows) of some mucosal epithelia. (d) Spleen: many megakaryocytic cells (black arrows) and brown colored pigmentation (white arrows). (e) Lung: mononuclear cellular infiltration (black arrow) in the interalveolar septa. (For interpretation of the references to color in text, the reader is referred to the web version of the article.)
erosion of crypt epitheliums and sloughing off some epithelial cells were detected in small intestine of three mice received the higher dose (Fig. 1c). In spleen, many megakaryocytic cells in the red pulp and yellow-brown pigmentation were noted (Fig. 1d). In two mice of the higher dose group, a few foci of mononuclear cellular infiltrates were detected in lungs (Fig. 1e).

In immunohistochemical staining, light to moderate immunoreactivity for iNOS was seen at cytoplasm of the sinusoidal endothelial cells in liver of the control group mice (Fig. 2a). In NaNO2 given mice, strong immunoreactivity on the same cells as well as some hepatocytes was determined (Fig. 2b). Immunostaining on hepatocytes was located in the cytoplasm, and the immunoreactive hepatocytes were mostly shown pericentral localization. Although there was no recognizable difference in terms of the immunostaining between the lower and the higher dose groups in general, few showed strong immunoreactivity in the higher dose group (Fig. 2c). In kidney of the control group mice, light immunostaining for iNOS was detected on tubule epitheliums and sinusoidal epitheliums (Fig. 3a). Immunostaining on the renal tubule and sinusoidal epitheliums as well as glomerula was generally more pronounced in the higher dose group (Fig. 3c) than the lower dose group (Fig. 3b). Immunostaining for nitrotyrosine both in liver and kidney tissues showed similar results to those of iNOS (data not shown). Immunohistochemical staining for PCNA revealed nuclear immunoreactivity, and there were only few immunostained hepatocytes in the liver of control mice under 20× objective in a given field (Fig. 4a). In NaNO2 given mice, many stained hepatocytes mostly located pericentrally were detected (Fig. 4b and c). Immunohistochemical staining for PCNA in kidney tissue did not show recognizable difference between the control and the NaNO2 given groups (data not shown).

In situ TUNEL staining revealed many apoptotic cells in liver of mice given NaNO2 (Fig. 5b) in contrast to no apoptotic cells in the control group (Fig. 5a). Distribution of apoptotic cells throughout the liver sections did not present any significant pattern. It seemed that the degree of apoptotic cell death was more pronounced in the higher dose group, although this was not tested by a statistical mean. There were no detectable apoptotic cells in the kidneys of both control and NaNO2 given groups.

Biochemical analysis for NO and MDA was performed to investigate the tissue response and damage in liver and kidney of NaNO2 given mice. The results of these investigations were summarized in Table 1. The level of NO in liver and kidney significantly decreased in NaNO2 given groups compared to those of control. In terms of the NO level, while there was no statistical difference between the lower and the higher dose groups in liver, it was more drastically reduced in kidney of the mice in the lower dose group. As a marker of lipid peroxidation, MDA increased in NaNO2 groups, and there was no significant difference between the lower and the higher dose groups. In kidney, MDA level did not show any significant change among the groups.

Genotoxic investigations on chromatid and chromosome breaks, chromotid association, polyploidy formation and mitotic index were performed on the treatment, positive and negative
Fig. 3. Immunohistochemical staining for iNOS in the kidneys. Light immunoreactivity in the renal tubule and sinusoidal epithelia in a control mouse (a); light to moderate immunoreactivity in renal tubule and sinusoidal epithelia as well as glomerulia in a mouse given 10 mg/kg/day NaNO₂ (b); and strong immunoreactivity in kidney of a mouse given 20 mg/kg/day NaNO₂ (c).

Table 1

<table>
<thead>
<tr>
<th>Groups</th>
<th>Liver NO</th>
<th>Liver MDA</th>
<th>Kidney NO</th>
<th>Kidney MDA</th>
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<td>Control</td>
<td>12.45 ± 0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.86 ± 0.42&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.25 ± 0.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.80 ± 0.94&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>NaNO₂ (10 mg)</td>
<td>5.22 ± 1.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.06 ± 0.87&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.33 ± 0.76&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10.25 ± 0.73&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>NaNO₂ (20 mg)</td>
<td>5.29 ± 0.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.65 ± 0.48&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.28 ± 0.68&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.56 ± 1.18&lt;sup&gt;a&lt;/sup&gt;</td>
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control groups (Table 2). It was found that NaNO₂ treatment would cause significant increase in the number of chromatid and chromosome breaks, and chromatid association (Fig. 6). Polyploidy formation, which was not observed in the control group, took place in the NaNO₂ treatment groups. On the other hand, mitotic index decreased significantly in the NaNO₂ treatment groups compared to the negative control.

Table 2

<table>
<thead>
<tr>
<th>Test Substance</th>
<th>Treatment</th>
<th>Structural aberrations</th>
<th>Numerical aberration</th>
<th>Mitotic index ± SD (%)</th>
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<tr>
<td></td>
<td></td>
<td>ctb</td>
<td>csb</td>
<td>cta</td>
</tr>
<tr>
<td>NC</td>
<td>0.9% NaCl</td>
<td>0.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PC</td>
<td>2 μg/g (24 h)</td>
<td>21</td>
<td>2.2</td>
<td>3.6</td>
</tr>
<tr>
<td>NaNO₂ 10 mg/kg</td>
<td>10 mg/kg</td>
<td>5.4&lt;sup&gt;*&lt;/sup&gt;</td>
<td>1&lt;sup&gt;*&lt;/sup&gt;</td>
<td>1.2&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>NaNO₂ 20 mg/kg</td>
<td></td>
<td>7&lt;sup&gt;*&lt;/sup&gt;</td>
<td>1.4&lt;sup&gt;*&lt;/sup&gt;</td>
<td>2.8&lt;sup&gt;*&lt;/sup&gt;</td>
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4. Discussion

Nitrite is a well-known toxic compound when its concentration is high in the organism. There are ample studies investigating the toxic effects of nitrate and nitrite compounds, however almost all of these previous studies conducted as a high dose-short time consumption experiment. Direct toxic effects characterized by changes...
Fig. 4. Immunohistochemical staining for PCNA in the livers. Few hepatocytes showing immunostaining in a control mouse (a); many immunoreactive cells in mice given 10 mg/kg/day NaNO2 (b) and 20 mg/kg/day NaNO2 (c).

in blood parameters and immune response due to high dose administration of these substances were well studied (Grant and Butler, 1989; Šireli et al., 1995; Abuharfeil et al., 2001; Miasodeova and Nazarov, 2004; El-Shiekh and Khalil, 2011). However, nitrate and nitrite are almost always used at low dosages especially as color and flavor preservatives in foodstuffs in daily life. Long time consumption of low dose nitrate and nitrite processed foodstuffs are therefore more likely scenario in reality. In the present study, intake of low dose NaNO2 for 8 months and the resulted histopathologic, biochemical and genotoxic effects were studied in mice.

Histopathological studies investigating nitrite and nitrate toxicity in rodents are quite few. It was reported that sodium nitrate given in drinking water at 1–3 g/L during pregnancy and lactation to Long–Evans rats decreased erythropoietic development, caused growth retardation, and increased mortality (Roth et al., 1987). Similar results were also reported in a later study (Mowafy et al., 2001). Some histopathological changes such as cytoplasmic vacuolization of centrilobular hepatocytes and decreased hematopoiesis in bone marrow and spleen were also recorded in these rats. In another study, mononuclear cellular infiltration in liver, kidney as well as lung tissues were reported together with hyperplasia in thymus, lymph nodes and Payer’s patches in mice consuming 0.4 g/L nitrate and nitrite containing water for 26 weeks (Kahraman et al., 1988). In an earlier study, co-administration of sodium nitrite and dimethylamine was shown to cause centrilobular necrosis in liver and congestion in portal veins in mice (Asahina et al., 1971). Similarly, we have detected hydropic cellular degeneration in liver and tubular degeneration in kidney especially in those animals received 20 mg/kg/day NaNO2. In addition, erosion of crypt epitheliums and sloughing off some epithelial cells in intestine, increased number of megakaryocytes and yellow-brown pigmentation in spleen, and mononuclear cellular infiltration in lungs was detected in some mice received the higher dose of nitrite tested. It is a well known fact that many of the above-mentioned histopathological changes are mediated by oxidative stress produced by the action of the metabolic pathways generated against the toxic compounds.

Reactive oxygen species are important mediators of cellular degeneration. The role of these molecules in oxidative stress and the following tissue degeneration has been shown in many kinds of toxicities as well as infectious conditions. In the process of nitrite toxicity, nitrite taken into the body is converted to nitrosonium ions which in turn react with amines and amides to form nitrosamines and nitrosamides, respectively. Diethylnitrosamine was shown to induce free radicals production in rat liver tissue in vivo (Yamada et al., 2006). N-nitrosamines have the ability to induce rapid oxidative stress (Ahotupa et al., 1987). Lipid peroxidation is the main mechanism in these processes. Lipid peroxidation is the prime factor especially in the breakdown of the lipid bilayers in biological systems, and therefore it causes interruptions in the cellular homoeostasis. Cellular health is known to be strictly regulated by the balance between the oxidative and anti-oxidative systems, and increased oxidative stress and/or decreased anti-oxidative mechanisms result with cellular degeneration and death.

It has been stated that nitrate and nitrite are ready sources of NO (Chow and Hong, 2002). Production of NO from nitrate and nitrite was shown to be mediated by both commensal bacteria
Fig. 5. In situ TUNEL assay for apoptosis in the livers. No apoptotic cell death in a control mouse (a); few versus many apoptotic cells (black arrows) in mice given 10 mg/kg/day NaNO2 (b) and 20 mg/kg/day NaNO2 (c), respectively.

(Lundberg et al., 2004) and bacteria present in the urogenital tract (Lundberg et al., 1997). NO plays a key role in vascular vasodilatation, and therefore it is important in cardiovascular diseases (Machha and Schechter, 2011). However, high concentrations of NO may cause tissue degeneration. Rapid interaction of NO with superoxide forms highly reactive peroxynitrite which causes lipid peroxidation (Rubbo et al., 1994).

There are numbers of molecules used as an indicator of lipid peroxidation. Serum and/or tissue levels of these molecules have been practically in use to estimate oxidative stress. However, since there are several pathways in the process of lipid peroxidation and several different end-products, the results might change depending on which parameters are being used (Ahotupa et al., 1987). Elevated levels of serum MDA and NO, arginase activity, decreased glutathione (GSH) concentration, increased glutathione S-transferase (GST) and decreased catalase (CAT) activities were previously shown in rats given NaNO2 (El-Sheikh and Khalil, 2011). Similarly, decreased GSH and CAT activity in NaNO2-intoxicated rats was reported earlier (Hassan and Yousef, 2010). Decreased NO levels in NaNO2-given mice in the present study seems to contradict with the previous investigations. However, the decrease in tissue NO level might be explained by the fast and/or efficient removal of this molecule from liver and kidney. NO in tissue eventually cause an increase in serum, because of the metabolic removal from the tissue. Therefore, increased NO level in serum actually does not contradict with the decreased NO level in tissue. While increased MDA level in liver is in accordance with the earlier studies we did not observe any change in MDA level in kidney of NaNO2–given mice at both of the two tested dosages. Possibly different cellular scavenging activities in chronic toxicity observed in the current study and the acute toxicities in earlier investigations might be attributed to the resulting differences. Moreover, no change in MDA level in kidney tissue contrary to liver might be explained by that low amount of NaNO2 could show enough destructive effect in liver unlike in kidney. It has been shown that total protein and

Fig. 6. Chromatid break (black arrows), chromatid association (white arrows) and chromosome break (arrowhead) in a mouse given 20 mg/kg/day NaNO2, Giemsa 1000×.
albumin levels in serum was significantly reduced in NaNO₂-given rats indicating that liver might become more vulnerable against the destructive substances and could not able to perform sufficient protection (El-Sheikh and Khalil, 2011). It seems that such an effect is less prominent in kidney especially in chronic toxicities, and may be due to presence of enough time to implement necessary anti-oxidant systems.

Increased iNOS activity has been shown by us and others in indicating oxidative stress in variety of occasions. iNOS mediates the catalytic formation of NO from L-arginine (Moncada and Higgs, 1993). Interaction of NO with some oxygen free radicals such as superoxide yields peroxynitrite, which may further interacts with tyrosine residues in biological molecules forming nitrotyrosine (Beckman and Koppenol, 1996; Quijano et al., 2005). Peroxynitrite was also suggested to be parted to form NO₂ and NO₃, which might involve in DNA damage (Ichiropoulos et al., 1992; Nordberg and Arner, 2001). In the preset investigation, increased expressions of iNOS and also nitrotyrosine were shown to take place in both liver and kidney tissues of mice received NaNO₂. Strong immunochmical reactivity against both antibodies clearly has shown that NaNO₂ consumption in long term also causes oxidative stress in these organs. Mild to moderate tissue degeneration observed in both organs also correlates these findings. Strong immunohistochemical staining for nitrotyrosine was also previously shown in stomach epithelial cells in rats given NaNO₂ and catechol together (Ishi et al., 2006).

Cellular degenerations mostly end up with necrosis if the degenerative cause is strong enough. Hepatic necrosis was shown to take place following acute toxicity with sodium nitrite and secondary amines in mice (Asahina et al., 1971). In certain occasions, apoptotic cell death may develop as a result of various degenerating agents. Intrinsic or extrinsic pathways are involved in the process of apoptosis (Portt et al., 2011; Ryter and Choi, 2013). Oxidative stress and the free radicals produced might be involved in these pathways (Sinha et al., 2013). In the current study, apoptotic cell death was detected in liver of mice received 20 mg/kg/day NaNO₂. While few apoptotic cells were seen at the lower dose group none was observed in kidney at both low and high doses. These results seem to correlate with the unchanged level of MDA in kidney but not in liver. Moreover, the finding of increased PCNA immunoreactivity, especially only in the higher dose group, in liver with no reactivity in kidney is in accordance with the results of biochemical investigations. Therefore, overall these findings might indicate that NaNO₂ needs to be at certain dosage to cause degenerative effects, and the degree of degenerative changes might show differences among organs.

N-nitroso compounds produced from excess nitrate and nitrite consumption have been suggested to cause cancer (Sander et al., 1975; Bruning-Fann and Kaneene, 1993; Eichholzer and Gutzwiller, 1998). Many studies have solely focused on the carcinogenic effects of these substances. Esophageal, gastric and nasopharyngeal cancers have been associated with N-nitrosamines (Lownfels et al., 1978; Tricker and Preussmann, 1991; Ishii et al., 2006). It has been stated that P450-dependent hydroxylation and following formation of electrophilic alkyldiazonium ion plays role in carcinogenesis of N-nitrosamines (Bartsch et al., 1989; Tricker and Preussmann, 1991). In the present study, no carcinogenic changes were detected in any of the organs examined. Absence of any detectable cancerous activity might be explained by either low amounts of nitrite used in the experiment to cause such an impact or minute quantities of cancer cells that could not be detectable solely by microscopic investigation. No cancer risk for nitrite consumption was also previously evaluated for humans and suggested that colon cancer due to nitrite consumption via public water supplies might only occur among susceptible people (De Roos et al., 2003). Although no cancerous growth was detected in the current study some degenerating changes characterized by erosion of crypt epitheliums and sloughing off some cells were noted in the intestines of some mice received the higher dose of nitrite.

Even though no cancer cells were detected in histological observations, we have tested genotoxic potential of sodium nitrite in mice. In nitrite given mice at both doses tested significantly increased chromosome and chromatid breaks, chromatid association, and polyplody as well as decreased mitotic index were detected in cells collected from bone marrow as compared to the negative control animals. It is known that many different types of abnormalities would take place during the course of cancer development (Albertson et al., 2003). The use of nitrite in mice was therefore shown to cause abnormalities at chromatid and chromosomal levels.

Polyplody may occur due to abnormal cell division (Comai, 2005). In most neoplastic processes, mitotic index increases especially in those that are malignant. However, with the decreased mitotic index, presence of polyplody in the current study indicates that abnormal mitotic activity, but not only increased mitotic activity, might play role in the formation of polyplody. Above-mentioned changes in chromosome and chromatid structure may develop as a result of errors in DNA metabolism or repair. Many agents that are able to induce oxidative stress may also cause DNA damages which may further result with cancer if the impact of the agent is strong and/or long enough. It was previously shown that peroxidase-generated reactive nitrogen species could induce DNA damage (Zhang et al., 2012).

In the present study, decreased mitotic index may seem to contradict with the finding of increased PCNA immunoreactivity in liver. However, absence of any cancerous growth does not contradict with the decreased mitotic index. PCNA expression is generally accepted to increase in cancers. However, higher expression levels of PCNA can also be seen in a variety of different cellular events (Maga and Hubscher, 2003). One of the well known functions of PCNA beside DNA replication in cancer cells is on the DNA repair (Essers et al., 2005). Therefore, increased expression of PCNA might be explained by the repair event as a result of the chromosomal defects observed.

In conclusion, the results of the present study show that low level of long term NaNO₂ consumption causes important histopathological changes in liver and kidney of mice. Nitrosative tissue degeneration and lipid peroxidation take place in both organs though more prominently in liver than kidney, both was shown by immunohistochemical and biochemical means. Significant apoptotic cell death also occurs in liver of NaNO₂ given animals, though most prominently in the higher dose group. In addition, nitrite causes significant chromosomal changes which may indicate genotoxic potential of NaNO₂ in long term consumption. Based on the knowledge that nitrite metabolism in mice and human resembles to each other; the results of the present study suggest that NaNO₂ consumption should be limited even in long term use.

Conflict of interest

The authors declare that there are no conflicts of interest.

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