EVALUATION OF THE PROTECTIVE EFFECT OF ASCORBIC ACID AND/OR THYMOQUINONE ON NITRATE TOXICITY IN RABBITS

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ABSTRACT:
Background: Risk of nitrate toxicity among animal and human. The objective of this work is designed to study the capacity of thymoquinone and/or ascorbic acid for detoxification of drinking water nitrate in growing New Zealand White rabbits.

Methods: In this experiment, Hematological and serobiochemical parameters as well as histopathological examination of liver and kidney were estimated in 5 assigned groups as follows, the 1st group served as a control, the 2nd group was orally received sodium nitrate 1gm/L drinking water for 8 weeks. The 3rd group was orally received sodium nitrate 1gm/L drinking water and treated with ascorbic acid 300mg/L drinking water for 8 weeks. The 4th group was orally received sodium nitrate 1gm/L drinking water for 8 weeks and treated with thymoquinone(10mg/kg/day) via the intraperitoneal route for 7 successive days. The 5th group was orally received sodium nitrate 1gm/L drinking water and treated with ascorbic acid 200mg/L drinking water for 8 weeks along with thymoquinone(10mg/kg/day) via the intraperitoneal route for 7 days.

Results: The leukocyte count and red blood cells were decreased significantly (P<0.05) in the nitrate treated group compared to the control and other treated groups. Total protein and albumin concentrations were decreased significantly (P<0.05) in the nitrate treated group compared to the control and other treated groups. The activity of AST, ALT as well as Cholesterol and glucose concentrations were significantly increased (P<0.05) in the nitrate treatment group compared to the control and other treated groups. The creatinine and blood urea nitrogen (BUN) were significantly increased (P<0.05) in the nitrate treatment group compared to the control and other treated groups. The supplementation of thymoquinone at the rate of 10mg/kg/day and/or ascorbic acid at the rate of 300mg/Litre lead to an improvement of the blood and biochemical parameters, and also ameliorate hepatic and renal damage. Conclusion: The present study suggests the possible beneficial effects of thymoquinone either alone or with ascorbic acid against sodium nitrate toxicity via an antioxidant mechanism.

Key words: Ascorbic acid, thymoquinone, nitrate, histopathology. Liver, kidney.

INTRODUCTION
The most common causes of high nitrate levels in water include shallow wells contaminated with surface water, water containing animal wastes, and surface runoff from heavy rain after fertilization with ammonium nitrate [1]. Water containing more than 500 ppm NO3 is potentially toxic [2]. In the body, nitrate is reduced to nitrite and the absorption of nitrite leads to methaemoglobinemia which cannot transport oxygen to body tissues [3]. Exposure to nitrates has been reported to have an adverse effect on animals and human health [4]. Nitrate toxicity has been observed commonly in ruminants [5]. A high level of nitrate can lead to a change in blood constituents [6]. Vitamin C has potentials to scavenge free radicals and protect cells from oxidative damage [7]. Thymoquinone (TQ) is the major constituent of the volatile oil from Nigella sativa seeds. Its valuable effects are related to its anti-oxidant, anti-infective, anti-tumor, anti-inflammatory properties [8]. Previous studies have demonstrated that TQ has protective effect against oxidative injury induced bya variety of free radical generating agents [10,11,12]. As far as we know there are no documented reports on the protective effect of TQ on nitrate toxicity. Therefore, the present study is designed to investigate whether oral supplementation of thymoquinone and/or ascorbic acid could ameliorate or protect against nitrate toxicity.

MATERIALS AND METHODS
Animals: A total of 40 growing New Zealand White rabbits were obtained from the Animal Care Unit of College of Veterinary Medicine and Animal Resources, King Faisal University (KFU). The rabbits were assigned to five groups (8 rabbits/group) at 8 weeks of age. Animals were housed under good ventilation with free access to food and water.

Chemicals: Sodium nitrate (BDH)-Chemicals Ltd Poole England, L-Ascorbic acid- Techno-Pharmhem-Haryana-India, thymoquinone-99%-Sigma Aldrich Chemical Company, St. Louis, MO, 63103 USA, which dissolved in dimethylsulphoxide (DMSO) as 14 mg/ml and then diluted in PBS as required.

Experimental design: The 1st group (n=8) served as a control, the 2nd group (n=8) was orally received sodium nitrate 1gm/L drinking water for 8 weeks. The 3rd group (n=8) was orally received sodium nitrate 1gm/L drinking water and treated with ascorbic acid...
300mg/L drinking water for 8 weeks. The 4th group (n=8) was orally received sodium nitrate 1gm/L drinking water for 8 weeks and treated with thymoquinone (10mg/kg/day) via the intraperitoneal route for 7 successive days. The 5th group (n=8) was orally received sodium nitrate 1gm/L drinking water and treated with ascorbic acid 200mg/L drinking water for 8 weeks along with thymoquinone (10mg/kg/day) via the intraperitoneal route for 7 days.

Biochemistry and Hematology:
The rabbits were observed daily for clinical signs. Before necropsy, the rabbits were euthanized with sodium pentobarbital (300mg/kg, i.p.) according to Animal Welfare Advisory Committee (AWAC). Blood samples were collected via cardiac puncture in EDTA tubes for hematologic analysis (WBC, RBC, Hb, PCV, MCV, MCH and MCHC) using a Coulter counter electronic analyzer (Vet Scan 5 HM-ABAXIS-USA). Serum samples were collected in plain tubes, then separated and kept in -80°C until analyzed for total protein, albumin, cholesterol, glucose, ALT, AST, BUN and creatinine using a biochemical blood analyzer (ELLIPSE, E0217, Italy, 2003).

Gross pathology and histopathology:
Necropsy was performed at the end of an experiment and samples of liver, kidney and lung were collected at 10% neutral buffered formalin for histopathology. Samples fixed in formalin were embedded within paraffin in 48 hours and processed routinely for hematoxylin and eosin staining (HE) (ELLIPSE, E0217, Italy, 2003).

Statistical analysis:
Data were analyzed by the General Linear Model (GLM) procedure (SAS, Institute, Inc, 2002). The least Square Mean (LSM) ± standard errors for each group were calculated and tested for significance using the "t" test [14].

RESULTS
Clinical signs, blood and biochemical parameters:
No mortality was recorded during the whole experiment. At 4 weeks, lethargy and diarrhea were observed in the nitrate treated group which can persist until the end of the experiment. These findings were not observed in control and other treated groups. The leukocyte count and red blood cells were decreased significantly (P<0.05) in the nitrate treated group compared to control and other treated group (Table 1). Total protein and albumin concentrations were significantly decreased (P<0.05) in the nitrate treated group compared to control and other treated groups (Table 2). The activity of AST, ALT as well as cholesterol and glucose concentrations were significantly increased (P<0.05) in the nitrate treatment group compared to a control and other treated groups (Table 2). The creatinine and blood urea nitrogen (BUN) were significantly increased (P<0.05) in the nitrate treatment group compared to the control and other treated groups (Table 2).

Gross and histopathology findings:
Gross findings revealed enlarged, pale livers and mild gastroenteritis in the nitrate treated group, however, in other treated groups, these organs were more or less quite similar to the control group. The liver sections of rabbits intoxicated with the nitrite showed disturbed lobular architecture and severe degenerative changes characterized by swelling and vacular appearance of hepatocytes with pyknotic nuclei (Fig.1a). The vast majority of hepatocytes had significant scattered single necrotic cells (apoptotic cells). These necrotic cells were frequent in the peribital areas, but they occurred to a lesser extent in mid zonal areas and were absent in centrilobular areas. Severe degrees of inflammatory reaction manifested with minute foci of macrophages and lymphocytes was noticed everywhere. The livers intoxicated with nitrate and treated with ascorbic acid showed a mild degree of degenerative changes of some hepatocytes manifested with vacular cytoplasm (Fig.1b). However, The most hepatocytes appeared recovered and have an almost normal architecture. The livers intoxicated with nitrate and treated with thymoquinone showed almost recovered and healthy hepatocytes, except for occasional infiltration of mononuclear cells in the portal areas (Fig.1c). The livers intoxicated with nitrate and treated with ascorbic acid and/or thymoquinone revealed recovered and healthy hepatocytes, which have an almost normal architecture (Fig.1d).

DISCUSSION
Nitrate is a health hazard because of its conversion to nitrite. Once ingested, the conversion of nitrateto nitrite takes place in the saliva and in the gastrointestinal tract of animals and human. The present study indicates that excess nitrate in drinking water leads to gastrointestinal disturbance. These signs might be due to the irritant effects of nitrite on the mucous membranes of the gastrointestinal tract [15]. The leukocyte count decreased significantly in the nitrate treated group compared to control and other treated groups. Furthermore, the number of red blood cells decreased significantly in the nitrate treated group compared to control and other treated groups. This finding might be...
due to the effect of nitrite on the Ca, Mg and ATPs activity of the cell membrane of RBC3. Dehydration due to diarrhea might be another factor for this decrease in RBC. Hemoglobin decreases significantly in the nitrate treated group compared to control and other treated groups. These results might be due to the decrease number of RBC or due to the conversion of nitrate to nitrite ions which can convert ferrous ions of hemoglobin into ferric form (methaemoglobin) resulting in tissue anoxia, which may be responsible for renal and hepatic damage[16]. Total protein and albumin concentrations were significantly decreased in the nitrate treated group. The decrease of total protein and albumin concentrations could be attributed to impair liver and kidney functions[17,18] or might be due to the formation of nitric oxide or peroxynitrite, which oxidizes proteins and lipoproteins[19]. The activity of AST and ALT were significantly increased in the nitrate treatment group. These results indicate hepatic damage [20]. Cholesterol concentrations were significantly increased in the nitrate treatment group. These findings might be due to the endothelial damage of blood vessels by nitric oxide and superoxide [19]. The creatinine and blood urea nitrogen (BUN) were significantly increased in the nitrate treatment group. These findings could be attributed to the renal damage. The addition of ascorbic acid to nitrate amended tissues changes, blood and biochemical parameters as well. These results indicated that ascorbic acid can reduce methaemoglobin [21] or may augment the function of endogenous free radical scavengers and decreases the adverse effect of nitrates on body cells [7]. The addition of thymoquinone either alone or along with ascorbic acid to nitrate also ameliorate tissues and blood changes. It has been shown that thymoquinone (TQ) works as a scavenger of various reactive oxygen species, including superoxide radical anion and hydroxyl radicals [10,22].

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Table 1: Blood parameters in control group, nitrate group, nitrate and ascorbic acid group, nitrate and TQ group, and nitrate, ascorbic acid and TQ group.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control group</th>
<th>Nitrate treated group</th>
<th>Nitrate and ascorbic acid group</th>
<th>Nitrate and TQ group</th>
<th>Nitrate, ascorbic acid and TQ group</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC 10^3/ml</td>
<td>6.20±0.15 a</td>
<td>3.00±0.26 b</td>
<td>5.56±0.14 c</td>
<td>5.15±0.12 d</td>
<td>5.75±0.06 e</td>
</tr>
<tr>
<td>LYM %</td>
<td>52.95±2.10 a</td>
<td>43.23±0.27 b</td>
<td>50.10±0.16 c</td>
<td>49.74±0.40 d</td>
<td>50.37±0.47 e</td>
</tr>
<tr>
<td>MON %</td>
<td>26.22±0.21 a</td>
<td>20.15±0.11 b</td>
<td>24.88±0.26 c</td>
<td>24.70±0.33 d</td>
<td>25.12±0.13 e</td>
</tr>
<tr>
<td>NEU %</td>
<td>19.24±0.07 a</td>
<td>13.32±0.13 b</td>
<td>17.32±0.15 c</td>
<td>17.98±0.08 d</td>
<td>18.06±0.09 e</td>
</tr>
<tr>
<td>RBC 10^9/ml</td>
<td>6.91±0.08 a</td>
<td>3.47±0.19 b</td>
<td>5.97±0.07 c</td>
<td>6.06±0.13 d</td>
<td>6.13±0.12 e</td>
</tr>
<tr>
<td>Hb g/dl</td>
<td>12.70±0.09 a</td>
<td>8.75±0.45 b</td>
<td>10.32±0.39 c</td>
<td>10.70±0.26 d</td>
<td>11.18±0.17 e</td>
</tr>
<tr>
<td>PCV %</td>
<td>39.51±0.24 a</td>
<td>30.27±0.41 b</td>
<td>37.17±0.23 c</td>
<td>38.04±0.09 d</td>
<td>38.12±0.07 e</td>
</tr>
<tr>
<td>MCV fl</td>
<td>68.22±0.06 a</td>
<td>58.02±0.27 b</td>
<td>66.22±0.12 c</td>
<td>66.82±0.09 d</td>
<td>67.10±0.14 e</td>
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<tr>
<td>MCH pg</td>
<td>22.14±0.04 a</td>
<td>15.74±0.22 b</td>
<td>20.86±0.18 c</td>
<td>20.93±0.10 d</td>
<td>21.03±0.11 e</td>
</tr>
<tr>
<td>MCHC g/dl</td>
<td>32.25±0.27 a</td>
<td>23.90±0.17 b</td>
<td>30.87±0.11 c</td>
<td>31.01±0.11 d</td>
<td>31.14±0.08 e</td>
</tr>
</tbody>
</table>

a-e different letters between treatments are significant (p<0.05).

Table 2: Biochemical biomarkers in control group, nitrate group, nitrate and ascorbic acid group, nitrate and TQ group, and nitrate, ascorbic acid and TQ group.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control group</th>
<th>Nitrate treated group</th>
<th>Nitrate and ascorbic acid group</th>
<th>Nitrate and TQ group</th>
<th>Nitrate, ascorbic acid and TQ group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin (g/dL)</td>
<td>5.56±0.21 a</td>
<td>2.10±0.07 b</td>
<td>4.85±0.10 c</td>
<td>4.96±0.08 d</td>
<td>5.09±0.14 e</td>
</tr>
<tr>
<td>T protein (g/dL)</td>
<td>7.61±0.06 a</td>
<td>3.15±0.13 c</td>
<td>6.38±0.21 c</td>
<td>6.30±0.15 c</td>
<td>6.40±0.16 c</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>30.18±0.07 a</td>
<td>40.41±0.30 b</td>
<td>31.35±0.23 c</td>
<td>31.94±0.12 d</td>
<td>32.12±0.12 e</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>50.17±0.08 a</td>
<td>63.52±0.38 b</td>
<td>51.37±0.33 c</td>
<td>51.95±0.21 d</td>
<td>52.15±0.11 e</td>
</tr>
<tr>
<td>Cholesterol mg/dL)</td>
<td>40.35±0.34 a</td>
<td>74.07±0.18 b</td>
<td>41.90±0.18 c</td>
<td>42.52±0.32 d</td>
<td>41.04±0.52 e</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.81±0.02 a</td>
<td>3.33±0.27 b</td>
<td>0.98±0.04 c</td>
<td>0.99±0.04 d</td>
<td>0.88±0.04 e</td>
</tr>
<tr>
<td>BUN</td>
<td>25.19±0.08 a</td>
<td>49.68±0.13 b</td>
<td>26.59±0.34 c</td>
<td>26.33±0.19 d</td>
<td>25.99±0.10 e</td>
</tr>
<tr>
<td>Glucose</td>
<td>137.2±0.04 a</td>
<td>256±0.66 b</td>
<td>139.3±0.49 c</td>
<td>139.1±0.26 d</td>
<td>138.2±0.81 e</td>
</tr>
</tbody>
</table>

a-e different letters between treatments are significant (p<0.05).
Fig. 1a: Liver of the nitrate group showing sever vacuolar degeneration with pyknotic nuclei (arrow). HE bar 20 μm. Fig. 1b: Liver of the nitrate group treated with ascorbic acid showing vacuolar hepatocytes (arrow). HE bar 20 μm. Fig. 1c: Liver of the nitrate group treated with thymoquinone showing slight mononuclear infiltration in portal area (arrow). HE bar 20 μm. Fig. 1d: Liver of the nitrate group treated with ascorbic acid and/or thymoquinone showing recovered and healthy hepatocytes (arrow). HE bar 20 μm.

Fig. 2a: Kidney of the nitrate group showing multiple foci of mononuclear cells between glomeruli (arrow). HE bar 20 μm. Fig. 2b: Kidney of the nitrate group treated with ascorbic acid showing cellular casts in some tubules (arrow). HE bar 20 μm. Fig. 2c: Kidney of the nitrate group treated with thymoquinone showing a mild degree of vacuolar degeneration of some tubules (arrow).
REFERENCES


