Influence of high dietary nitrate intake and sulphur supplementation on oxidant / anti-oxidant balance and on some haematological parameters in Angora goats

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SUMMARY
This study was conducted in order to determine the potential effects of a high nitrate food intake, known to cause chronic poisoning, on the oxidant / anti-oxidant balance and on some haematological parameters in goats and the putative protecting effects of a dietary sulphur supplementation. Eighteen male adult angora goats were divided into three equal groups: in the first group, goats were fed with a nitrate enriched diet (1500 ppm per day) and in the second group, animals received diets enriched with nitrate (1500 ppm) and sodium sulphate (1.8%) for 60 days whereas controls were fed with a standard diet. At the end of the trial, haematological parameters (red blood cell and white blood cell counts, haemoglobinemia and haematocrit), plasma malondialdehyde (MDA), nitrate/nitrite, glutathione (GSH) concentrations, total antioxidant activity (AOA), glutathione peroxidase (GPx) activity and erythrocyte catalase (CAT) activity were determined in all animals. While haematological parameters were not altered among groups, significant increases in the MDA concentrations and GPx activities were coupled to marked decreases in the antioxidant systems (GSH, CAT and AOA) in goats chronically exposed to nitrate compared to controls and intermediate values except for GPx activity. The increases in the antioxidant systems (GSH, CAT and AOA) and the erythrocyte catalase activity were reported only in the last group of the experiment. While hematological parameters were not altered among groups, significant increases in the antioxidant systems (GSH, CAT and AOA) and the erythrocyte catalase activity were reported only in the last group of the experiment. These results show that an oxidative stress occurred in chronic nitrate poisoning that can be markedly alleviated with dietary sulphur supplementation in goats.

Keywords: Angora goat, high nitrate food intake, lipid peroxidation, antioxidant systems, glutathione, Sulphur supplementation.

RÉSUMÉ
Effets d’une alimentation enrichie en nitrate et d’une supplémentation en soufre sur l’équilibre oxydant / antioxydants et sur quelques paramètres hématologiques chez la chèvre Angora

Cette étude a été entreprise afin de déterminer les effets éventuels d’une teneur élevée en nitrate dans l’alimentation, connue pour induire une intoxication chronique, sur l’équilibre oxydants / antioxydants et sur quelques paramètres hématologiques ainsi que les possibles effets protecteurs d’une supplémentation en soufre chez la chèvre Angora. Pour cela, 18 chèvres adultes Angora, mâles ont été réparties en 3 groupes égaux : les animaux du 1er groupe ont été nourris avec une ration enrichie en nitrate (1500 ppm par jour), ceux du 2e groupe ont reçu une ration enrichie en nitrate (1500 ppm) et en sulfate de sodium (1,8 %) pendant 60 jours alors que les chèvres contrôles ont été nourries avec une ration standard. A la fin de l’expérimentation, les paramètres hématologiques en malondialdéhyde (MDA), nitrates / nitrites et en glutathion (GSH), l’activité plasmatique totale en antioxydants (AOA), celle de la glutathion peroxydase (GPx) ainsi que l’activité érythrocytaire de la catalase ont été déterminées chez tous les animaux. Tandis que les paramètres hématologiques n’ont significativement pas varié entre les groupes, les concentrations plasmatiques de MDA et les activités plasmatiques de GPx ont significativement augmenté chez les chèvres chroniquement intoxiquées au nitrate par rapport aux contrôles et ont été associées à de nettes diminutions des systèmes antioxydants (GSH, CAT et AOA). Des valeurs intermédiaires de ces marqueurs biochimiques ont été observées chez les ruminants chroniquement intoxiqués par le nitrate mais supplémentés en soufre, à l’exception de l’activité plasmatique de la GPx qui a aussi été fortement augmenté dans ce groupe. Ces résultats montrent qu’un stress oxydatif intervient au cours d’une intoxication chronique au nitrate dont l’intensité peut être fortement amoindrie par une supplémentation alimentaire en soufre chez la chèvre.

Mots clés : Agneau, acide malique, ration mélangée, concentrés, croissance pondérale, digestibilité, métabolites du rumen.

Introduction
Nitrogen is a very important nutrient element in agriculture. However, changes in patterns of agricultural practice, food processing and industrialization have impacted accumulation of nitrates/nitrites in the environment. Due to the intense use of synthetic nitrogen fertilizers and livestock manure in modern day agriculture, food and drinking water may contain higher concentrations of nitrate than in the past. Therefore, nitrate could be a major threat to environment in different agricultural situations and a potential health risk for humans and animals [22].

Ruminants are more susceptible to nitrate poisoning than non-ruminant species [5]. However, nitrite or nitrate in the rumen is reduced to ammonia by some ruminal bacteria and the reductive process detoxifies nitrite and provides N for microbial protein synthesis [16]. Nitrate poisoning occurs when
the nitrite concentration in the rumen exceeds the capacity of the bacteria to convert it to ammonia. During this reduction process, nitrite is absorbed through the rumen wall into the bloodstream where it reacts with haemoglobin to form methaemoglobin [5, 16].

Chronic nitrate toxicity is a form of nitrate poisoning where the clinical signs of disease are not observed but it commonly induces reduction in animal performance and a greater susceptibility to infections, resulting in financial losses to the producer. These production related problems or losses are not often recognized and will occur when nitrate concentrations are consumed at 800–2000 ppm in the diet [22]. Oxidative stress in chronic nitrate toxicity may also be a contributory factor to increased disease susceptibility in animals because oxidative stress occurs when the production of reactive oxygen species (ROS) exceeds the antioxidant defence mechanisms present in the body [8, 10]. In fact, a considerable body of literature exists documenting an increased production of indicators of oxidative stress in blood and tissue of laboratory rats in response to nitrate intake [7, 30, 37]. However, a limited number of high nitrate studies in ruminants have been conducted investigating high nitrate and oxidative stress.

Ruminants have a highly efficient anaerobic fermentor located at the beginning of their digestive tract. This allows them to digest fibrous feed and to use non-protein nitrogen (NPN) to synthesize microbial matter [16]. Sulphur (S) is well known as part of the amino acids methionine, cystine, and cystein, and occurs in animal tissues in many sulphate forms [16, 35]. Sulphur is used by the ruminal bacteria to make S-containing amino acids from ammonia [6] and thus, may promote the conversion of nitrate to ammonia and decrease nitrite accumulation in the rumen. TAKAHASHI et al. [35] found that the amount of nitrite formed by rumen microorganisms in vitro decreased with increasing concentration of S added. Therefore, S supplementation to diet in ruminants may be a potential way to compensate for sulphur loss and to decrease the formation of nitrite in the rumen.

Following digestion of the ruminal bacteria in the lower alimentary tract, S may become a part of body tissue. However, the role of S-containing amino acids has only been evaluated in terms of protein synthesis, but never in terms of their ability to contribute to metabolic and/or detoxification mechanisms throughout sulphur. For example, sulphation is a major pathway for both the sulphation of thyroglobulin (Tg), the thyroid hormone precursor in the thyroid gland, and detoxification of pharmacological agents by the liver [17, 20, 29]. Therefore, providing sulphur supplementation to the diet can reduce oxidative stress in the ruminant animals fed with high nitrate diet. However, bacteria in the rumen may produce sulphites, which are readily absorbed through the rumen wall into the bloodstream [4]. When sulphites absorbed, they may inhibit the functions of some enzymes in the cell such as catalases and peroxidases, adversely affecting oxidative metabolism [32].

Little is known about the effects of high dietary nitrate and sulphate supplementation on oxidative stress and antioxidants in ruminant animals. Therefore, this study was conducted in order to determine effects of sulphate supplementation to diet containing high nitrate on oxidant-antioxidant balance and some haematological parameters in goats.

### Material and Methods

#### ANIMALS AND EXPERIMENTAL DESIGN

In this study, 18 male angora goats, 1-1.5 year old, provided by the Department of Anatolian Agricultural Expertise, were used and after physical examination, randomly divided into three equal groups (n = 6) as control (basal diet, Table I), nitrate (1500 ppm nitrate added to the diet), and nitrate + sulphur (1500 ppm nitrate and 1.8% sodium sulphate added to the diet) groups. The experimental protocol was approved by the Ethic Committee of the Faculty of Veterinary Medicine (University of Afyon Kocatepe, Afyonkarahisar, Turkey). At the beginning of the study, all goats were treated against internal and external parasites with an injection of Ivermectin (Vilmectin®, Vilsan Co Ltd., Ankara, Turkey) and received an intramuscular injection of Vitamins A, D₃ and E (ADEMIN®, 1 mL: Vitamin A, 500 000 IU; Vitamin D₃, 75 000 IU; Vitamin E, 50 mg; Dogu İlac Firma, Istanbul, Turkey).

<table>
<thead>
<tr>
<th>Concentrate composition (%)</th>
<th>Group control</th>
<th>Group N</th>
<th>Group N + S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>30.76</td>
<td>30.76</td>
<td>30.76</td>
</tr>
<tr>
<td>Barley</td>
<td>15.00</td>
<td>15.00</td>
<td>15.00</td>
</tr>
<tr>
<td>Sunflower meal</td>
<td>18.89</td>
<td>18.74</td>
<td>16.94</td>
</tr>
<tr>
<td>Wheat-scab</td>
<td>25.00</td>
<td>25.00</td>
<td>25.00</td>
</tr>
<tr>
<td>Molasses</td>
<td>7.18</td>
<td>7.18</td>
<td>7.18</td>
</tr>
<tr>
<td>Limestone</td>
<td>2.23</td>
<td>2.23</td>
<td>2.23</td>
</tr>
<tr>
<td>Salt (NaCl)</td>
<td>0.84</td>
<td>0.84</td>
<td>0.84</td>
</tr>
<tr>
<td>VMM</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Sodium nitrate</td>
<td>0.00</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>Sodium sulphate</td>
<td>0.00</td>
<td>0.00</td>
<td>1.8</td>
</tr>
</tbody>
</table>

VMM: vitamin mineral mixture (Rovimix 302 (Roche)) provided per kg of diet vitamin A 15 000 000 IU; vitamin D₃ 3 000 000 IU; Vitamin E, 30 000 mg; Mn 50 000 mg; Fe 50 000 mg; Zn 50 000 mg; Cu 10 000 mg; iod 800 mg; Co 150 mg; Se 150 mg.

Table I: Composition (%) of the concentrates in the diet of the control and experimental groups (group N: 1500 ppm nitrate were added to the diet, group N + S: 1500 ppm nitrate and 1.8% sodium sulphate were added to the diet).
During the fifteen-day adaptation period and two-month trial period they were kept in the farm of Afyon Kocatepe University Breeding Research Center under the same keeping and feeding conditions. During the experimental period, the animals were given dry clover at the rate of 1% of live weight and 0.57 kg/day concentrated feed as stated in the diets of angora goats. Clean drinking water was provided ad libitum. The mean weights in the control group and in the N and N + S groups were 29.5, 31.2, 30.3 kg respectively, on day 0 (at the beginning of the 2 month trial period) and 32.2, 34.4, 34.3 kg on day 60, respectively.

**BIOCHEMICAL ANALYSES**

Blood samples were collected prior to the morning feeding on day 60, from the jugular vein puncture into sterilized tubes containing heparin as anticoagulant. Red blood cells (RBC), white blood cell (WBC), haemoglobin (Hb) and haematocrit were determined by the methods as described in YILMAZ [39] from whole blood samples. After centrifugation (1500 g, 15 minutes, 4°C) blood samples were separated into plasma and erythrocytes.

The red cells were washed three times with PBS (phosphate buffer solution, pH: 7.2) 0.9% NaCl solution and the packed cells were haemolysed by adding an equal volume of cold distilled water. Erythrocytes were prepared according to WITTERBOURN et al. [38]. Catalase (CAT) activity in erythrocytes was measured spectrophotometrically as described by LUCK [27].

Plasma malondialdehyde (MDA) concentrations were estimated according to the method of DRAPER and HARDLEY [11] which is based on coupling MDA with thiobarbituric acid. Blood reduced glutathione (GSH) concentrations were assayed by colorimetric method of BEUTLER et al. [3]. Plasma GPx (Glutathione peroxidase) activities were determined by commercially available kits (Bioxytech GPx, No: 21014, OxisResearch, CA, USA) with ELISA reader (THERMOLABSYSTEMS Multiskan spectrum, No: 1500, Finland). Plasma antioxidant activity (AOA) was measured according to KORACEVIC et al. [24]. The assay measured the capacity of the samples to inhibit the production of thiobarbituric acid reactive substances (TBARS) from sodium benzoate under the influence of the free oxygen radicals derived from the Fenton’s reaction. This reaction can be measured spectrophotometrically and the inhibition of colour development defined the AOA. A solution of 1 mmol uric acid was used as standard. Nicrit oxide was assayed by the colorimetric method of Griess [in 28] in the plasma.

**Statistical Analysis**

Statistical analysis was performed with SPSS statistical software (SPSS for Windows; Standard Version 10.0). Comparisons between different groups were analysed by one-way ANOVA by Duncan’s multiple range tests. Data were expressed as the mean ± standard error. Differences of values with a confidence level of \( P < 0.05 \) were considered to be significant.

**Results**

None of the goats in the treated groups showed neither an abnormal growth compared to the controls, nor behaviour and signs of illness during the study period.

In the same way, the haematological parameters (red blood cell (RBC) and white blood cell (WBC) counts, haemoglobinemia (Hb) and haematocrit (Ht)) were not significantly affected by the dietary addition of nitrate alone or coupled to sulphate (Table II).

The lipid peroxidation intensity and the antioxidant status were presented in Table III. Compared to the controls, the plasma MDA concentrations were significantly increased in goats chronically exposed to the high nitrate dietary content (group N) for 60 days \( (P < 0.01) \) whereas the total plasma antioxidant activity \( (P < 0.05) \), the erythrocyte catalase activity \( (P < 0.01) \) and the blood GSH concentrations \( (P < 0.01) \) were markedly depressed in this group. For all these parameters, intermediate values (which have not significantly differed with the control values and with values observed in the group N) were observed in ruminants receiving both nitrate and sulphate. By contrast, the plasma GPx activity was significantly increased compared to the activity in controls in animals treated by nitrate alone or coupled to the sodium sulphate (groups N and N + S) \( (P < 0.01) \).

**Discussion**

ROS interact with other molecules within cells and cause oxidative damage to proteins, membranes and genes. Similarly, reactive nitrogen species such as NO, peroxynitrite and nitrite are known to have numerous biological actions [37]. The reactive free radicals can oxidize biomolecules and may cause extensive lipid peroxidation (LPO) in biological membranes [31], which leads to cell death and tissue injury. LPO is a complicated radical chain reaction leading to the formation of various products.

<table>
<thead>
<tr>
<th>Haematological parameters</th>
<th>Group control</th>
<th>Group N</th>
<th>Group N + S</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (10^12/L)</td>
<td>1.29 ± 0.06</td>
<td>1.30 ± 0.03</td>
<td>1.30 ± 0.03</td>
</tr>
<tr>
<td>WBC (10^9/L)</td>
<td>72.3 ± 3.5</td>
<td>83.7 ± 8.5</td>
<td>71.3 ± 5.1</td>
</tr>
<tr>
<td>Haemoglobinemia (g/L)</td>
<td>98.9 ± 0.3</td>
<td>95.9 ± 0.5</td>
<td>115.4 ± 9.1</td>
</tr>
<tr>
<td>Haematocrit (L/L)</td>
<td>0.267 ± 0.009</td>
<td>0.263 ± 0.007</td>
<td>0.272 ± 0.014</td>
</tr>
</tbody>
</table>

Table II: Haematological parameters (Red Blood Cell (RBC) and White Blood Cell (WBC) counts, haemoglobinemia and haematocrit) in the control goats (n = 6) and in goats orally exposed to nitrate (1500 ppm/day) for 60 days alone (group N, n = 6) or associated to sulphate (sodium sulphate 1.8%, group N + S, n = 6). Results are expressed as means ± standard errors.
including lipid hydroperoxides, conjugated dienes and MDA [10, 18]. Since membrane phospholipids are major targets of oxidative damage, LPO is often the first parameter analyzed for proving the involvement of free radical damage [10, 15]. Thus, the presence of MDA is considered as an indicator of free-radical damage through membrane lipid peroxidation [10]. In the present study, high dietary nitrate (1500 ppm) content, which was already known to cause chronic intoxication in animals [2, 22], increased MDA concentration in goats, which indicates an increased oxidative stress in agreement with the previous studies [1, 8, 19, 37]. This result suggests that lipid peroxidation could be an important factor in the pathogenesis of high nitrate toxicity in ruminants.

Sulphur amino acids contribute substantially to the maintenance and integrity of the cellular systems by influencing cellular redox state and the capacity to detoxify toxic compounds, free radicals and ROS [36]. If diets of ruminant animals contain NPN such as urea and nitrate, S may be needed for bacterial protein synthesis [16]. The rate of sodium sulphate added to the diet (1.8% in the present study) was reported as the highest rate of non-protein nitrogen supply in the diets including urea [14]. The rate of sodium sulphate added to the diet (1.8% in the present study) was reported as the highest rate of non-protein nitrogen supply in the diets including urea [14].

Carneiro et al. [6] found that sulphur supplementation increased cystein concentration in the bacterial proteins. This may indicate that the supplementation of sulphur to high nitrate diet may increase absorbed cystein concentration in goats. The availability of cystein appears to be the rate limiting factor for synthesis of GSH, which is a unique cellular tripeptide and plays a vital role in maintaining the oxidant/antioxidant balance in the tissue [36]. In the present study, the decreased lipid peroxidation (although not statistically significant) in goats fed with the nitrate and sulphur supplemented diet versus those fed with the nitrate supplemented diet, may indicate a relative improvement in efficiency of N from nitrate due to the sulphur utilisation. This result suggests that providing sulphur supplements to the diet can reduce oxidative stress in ruminants fed with diet containing high nitrate and improve the protection from oxidative damage caused by high dietary nitrate.

Cells have developed antioxidant defence systems to prevent free radical formation and to limit their damaging effects. The antioxidant systems protect the cellular biomolecules against damage caused by free radicals. They involve enzyme systems such as superoxide dismutase, glutathione peroxidase and catalase, and non enzyme systems such as glutathione and vitamins [12]. Changes in circulating levels of the antioxidants confirm the occurrence of an oxidative stress. Therefore, an imbalance between intracellular production of free radicals and the cellular defence mechanisms leads to oxidative stress. The total AOA of body fluids expresses a cooperative interaction between various antioxidants and has an important role on the maximum suppression of a free radical reaction in extracellular compartments [9]. In the present study, feeding goats with high dietary nitrate decreased the plasma AOA, GSH, a cystein-containing tripeptide, provides major protection in oxidative injury by participating in the cellular system of defence because it is the major non enzyme regulator of intracellular redox homeostasis [13, 15, 25]. Consequently, cells depleted in GSH may be more susceptible to nitrate toxicity. In the present study, feeding with high dietary nitrate in goats decreased GSH concentration in the blood and catalase activity in the erythrocytes, while it increased glutathione peroxidase activity in the plasma. This result is consistent with the results reported by SHUGALEI et al. [33] in rats and may indicate that the GSH depletion may depend upon the oxidative damage induced by high dietary nitrate content since decreased GSH concentrations reflect depletion of the antioxidant reserve [26]. In accordance with that, the elevated plasma GPx activity observed in goats chronically exposed to nitrate in the present study evidenced an increase in the hydroperoxide conversion into alcohols [34] that aggravated the cellular GSH consumption and an insufficiency in antioxidant defence mechanisms due to high nitrate intake may increase the lipid peroxidation in cells. In the present study, the supplementation of sulphur to diet containing high nitrate content has caused a numerically increase in both total AOA, GSH concentration and erythrocyte catalase activity compared to goats receiving dietary nitrate alone and has also sustained the plasma GPx activity, indicating that in this group, the ROS detoxification throughout GPx activity was not coupled to the intracellular GSH depletion. These results show that sulphur supplementation in ruminants may have a prophylactic effect in reducing oxidative stress caused by high dietary nitrate intake.

Nitric oxide (NO), which is synthesized in vivo from L-arginine and has been shown to be involved in numerous physiological

**Table III: Oxidants / antioxidants balance in the control goats (n = 6) and in goats orally exposed to nitrate (1500 ppm/day) for 60 days alone (group N, n = 6) or associated to sulphate (sodium sulphate 1.8%, group N + S, n = 6). Results are expressed as means ± standard errors.**

<table>
<thead>
<tr>
<th>Biochemical parameters</th>
<th>Group control</th>
<th>Group N</th>
<th>Group N + S</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (mmol/L)</td>
<td>5.48 ± 0.10a</td>
<td>6.43 ± 0.18b</td>
<td>5.98 ± 0.24ab</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>NOx (µmol/L)</td>
<td>17.39 ± 2.78</td>
<td>19.92 ± 3.24</td>
<td>13.94 ± 1.04</td>
<td>NS</td>
</tr>
<tr>
<td>AOA (mmol/L)</td>
<td>2.25 ± 0.14a</td>
<td>1.53 ± 0.11b</td>
<td>1.90 ± 0.23ab</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>GSH (mg/L)</td>
<td>152.8 ± 8.8a</td>
<td>108.7 ± 6.1b</td>
<td>131.9 ± 11.9ab</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>GPx (U/L)</td>
<td>84.47 ± 3.98a</td>
<td>104.58 ± 8.46b</td>
<td>120.18 ± 4.29b</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>CAT (katal/gHb)</td>
<td>43.06 ± 3.41a</td>
<td>27.69 ± 3.34b</td>
<td>34.93 ± 1.33ab</td>
<td>&lt; 0.01</td>
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</tr>
</tbody>
</table>

MDA: Malondialdehyde; NOx: nitrate plus nitrite; AOA: Antioxidant activity; GSH: Glutathione; GPx: Glutathione peroxidase; CAT: catalase; Hb: haemoglobin; NS: not significant.

Different superscripts a,b in the same row indicate significant difference between groups (P < 0.05 or more).
and pathophysiological processes, can be oxidized to nitrite or nitrate in the blood. Thus, nitrate and nitrite have been considered as stable inactive end products of NO in the plasma [31] and the measurement of their plasma concentrations provide one of the most useful methods to quantify systemic NO production [21]. However, the reaction between superoxide produced by the cell and nitric oxide (NO) generates peroxy-nitrite, a potent oxidizing agent, and can cause biological oxidative stress [37]. In the present study, although sulphur supplementation has numerically decreased the NOX concentration in the blood, there was no difference in NOX concentrations among treatments. This result may suggest that feeding with high dietary nitrate could reduce the endogenous production of NO because nitrate and nitrite are known as both a NO oxidization product and a ready NOX source [7]. KELES-TIMUR et al. [23] reported that acute nitrate or nitrite poisoning in ruminants increased RBC, WBC, Hb and HT in the blood due to probably hypoxia. However, these haematological parameters in the present study remained included in the usual values reported for goats [39] and were not significantly affected by nitrate at 1500 ppm in the diet.

As a conclusion, although no clinical signs and haematological anomalies were not detected in goats fed with a nitrate enriched diet for 60 days, blood biochemical analyses have revealed marked alterations in the oxidant/antioxidant equilibrium, with significant increases in the circulating MDA concentrations and marked reductions of the antioxidant systems (total AOA, GSH concentrations and erythrocyte catalase activity). This may be an important factor in the pathogenesis of nitrate toxicity. On the other hand, the results have shown that sulphur supplementation in goats fed with diet containing high nitrate content decreased the oxidative stress intensity by increasing antioxidant activity, which demonstrates that adequate sulphur supplementation to diet containing high NPN in ruminants is crucial for the maintenance of GSH homeostasis. Moreover, these results provide a plausible explanation on the role of dietary sulphur supplementation in ruminants in protecting against the adverse effects of nitrates and nitrites.

References


