Introduction

The rumen microorganisms have the ability to convert non-protein nitrogen compounds into protein [4]. They hydrolyze urea into ammonium (NH₄⁺) and ammonia (NH₃) to synthesize their own protein. Usually, most of the urea is transformed to ammonium that is available to the rumen microbe. At pH 7.0, approximately 99% of the compound is ammonium and 1% is ammonia, but at more alkaline pH, the ammonia concentration gets greater [32]. Conversely, the small amount of ammonia produced, which is a lipid-soluble compound, is readily absorbed into the blood stream [6]. Normally, the liver can efficiently detoxify ammonia into urea, but at higher blood concentrations, the detoxification capacity of hepatocytes will be overwhelmed and ammonia concentrations increase into blood, cerebrospinal fluid and in tissues, resulting in ammonia poisoning [8, 32]. Animals usually received high crude protein enriched diets or adapted to urea have greater ability to detoxify ammonia in the liver [6]. While liver plays an important role in ammonia detoxification, dysfunction of the organ leads to higher susceptibility to ammonia poisoning [24]. Clinical signs due to ammonia

Keywords: Lamb, ammonium sulphate, intoxication, oxidative stress, nitric oxide, antioxidant depletion, organic acid, retinol.

SUMMARY

In this study, the effects of the ammonium sulphate intoxication on the blood antioxidant/oxidant status were investigated in Sakiz crossbred lambs. For that, circulating blood urea nitrogen (BUN), nitric oxide (NO), malondialdehyde (MDA), glutathione (GSH), β-carotene, retinol and ceruloplasmin concentrations were measured in 6 lambs accidentally poisoned with ammonium sulphate and in 6 healthy control lambs. Oral treatment with 10% glumatic acid (1g/kg), 2.5% acetic acid (2.5 mL/kg) and vitamin A (400 IU/kg) was daily administered to diseased animals for five days. Poisoned lambs exhibited neurological signs (sleepiness, ataxia, tonic and clonic spasms) coupled to a rumen atony and acceleration of heart and respiratory rates compared to healthy controls. Biochemically, the circulating MDA, NO and BUN concentrations were markedly increased and the GSH, β-carotene and vitamin A concentrations were significantly depressed compared to healthy controls whereas the ceruloplasmin concentrations were not significantly altered. After treatment, clinical and biochemical signs were significantly alleviated but, however 2 lambs died. For them, the histopathological examination revealed cell degeneration in liver, lungs and kidney associated to mononuclear cell infiltrates and proliferation of Kupffer cells. These results clearly showed the occurrence of an oxidative stress induced by ammonium sulphate poisoning leading to cell damage and proved the efficiency of a treatment based on organic acids and retinol supplementation.

Keywords: Lamb, ammonium sulphate, intoxication, oxidative stress, nitric oxide, antioxidant depletion, organic acid, retinol.

RÉSUMÉ

Effets d’une intoxication accidentelle par le sulfate d’ammonium sur le statut oxydant/antioxydant des agneaux

Au cours de cette étude, les effets d’une intoxication par le sulfate d’ammonium sur le statut sanguin antioxidant/oxidant des agneaux croisés de race Sakiz ont été explorés. Pour cela, les concentrations circulantes de BUN (Blood Urea Nitrogen), d’oxyde nitureux (NO), de malondialdéhyde (MDA), de glutathion (GSH), de β-carotène, de rétinol et de céruloplasmine ont été mesurées chez 6 agneaux accidentellement intoxiqués par du sulfate d’ammonium et chez 6 agneaux sains servant de contrôles. Un traitement par de l’acide glutamique 10 % (1g/kg), de l’acide acétique 2,5 % (2,5 mL/kg) et de la vitamine A (400 UI/kg) a été administré oralement pendant une semaine. Les animaux intoxiqués ont présenté des signes neurologiques (somnolence, ataxie, spasmes et tétanies) couplés à une atonie du rumen et une accélération des rythmes cardiaques et respiratoires par comparaison aux contrôles sains. Biochimiquement, les concentrations circulantes de MDA, NO et BUN ont été nettement augmentées et celles en GSH, β-carotène et vitamine A nettement diminuées alors que les concentrations en céruloplasmine n’ont pas été significativement affectées. Après le traitement, les signes cliniques et biochimiques ont été significativement atténués mais 2 animaux sont morts. Dans ces cas, un examen histopathologique du foie, des reins et des poumons après coloration des tissus à l’hémalun-éosine a permis de mettre en évidence des signes de dégénérescence cellulaire associés à une infiltration par des cellules mononucléées et à une prolifération des cellules de Kupffer. Ces résultats montrent clairement l’apparition d’un stress oxydatif durant une intoxication par le sulfate d’ammonium conduisant à des lésions cellulaires et prouvant aussi l’efficacité d’un traitement à base d’acides organiques et de vitamine A.

Mots clés : Agneau, sulfate d’ammonium, intoxication, stress oxydatif, oxyde nitureux, déficit en antioxydants, acide organique, rétinol.

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poisoning have been reviewed and described by several authors, but they also exhibit some variability [6, 30, 33]. The onset of clinical signs may vary in a matter of a few minutes to hours after consumption of NPN (Non Protein Nitrogen) and they are usually acute and drastic leading to death in most of the cases [8, 23].

The activation of NMDA (N Methyl D Aspartate) receptors leads to increased intracellular Ca2+ which in turn activates neuronal nitric oxide synthase, increasing the formation of nitric oxide, which contributes to the toxic effects of ammoniac. This is supported by the fact that nitroarginine, an inhibitor of nitric oxide synthase, also prevents ammoniac-induced death and depletion of ATP [14, 16]. This indicates that ammoniac intoxication increases brain nitric oxide, which in turn plays a role in the mediation of ammoniac-induced depletion of ATP and death of animals [14]. Indeed, following activation of the NMDA receptors and secondary activation of the nitric oxide synthase, nitric oxide accumulates and inhibits some antioxidant enzymes. Nitric oxide would also contribute to increased oxidative damage by depleting glutathione (GSH) and by forming peroxynitrite [15].

In this study, the effects of ammonium sulphate intoxication on circulating nitrite, blood urea nitrogen (BUN), glutathione (GSH), malondialdehyde (MDA), β-carotene, retinol and ceruloplasmin concentrations and on histology of liver, lung and kidney tissues, were investigated in Sakiz crossbred lambs, which had accidentally ingested some ammonium sulphate fertilizer that is widely used in agriculture and animal husbandry.

Materials and Methods

ANIMALS

In this study, 6 Sakiz crossbred lambs, 4-8 months old, were accidentally poisoned with ammonium sulphate (20.5% ammoniac nitrogen), six healthy lambs were used as controls and 4 lambs after treatment were included in the study. Before and after the treatment, clinic and biochemical examinations were performed in all lambs. For five days treatment, glutamic acid 10% (1g/kg, orally), weak acid vinegar 2.5% (25 mL/kg, orally) and vitamin A (400 IU/kg) were used every day.

Blood samples were collected from the V. jugularis puncture into sterile heparinised tubes and into sterile polystyrene tubes without anticoagulant. Heparinised whole blood samples were washed three times with phosphate-buffered saline, pH 7.4 and centrifugation (1 000g, 10 minutes, 4°C) and the MDA and GSH concentrations were determined on the day of admission. Blood samples without anticoagulant were allowed to clot for 1 hour at 10°C, were centrifuged at 1 000g for 10 minutes at 4°C and thereafter sera were carefully harvested and stored at -70°C until analysis.

In addition, necropsy and histological analyses were conducted in one than two lambs died in spite of treatment. Analyses were carried out in the laboratories of Biochemistry Department of Faculty, Afyon Kocatepe University.

BIOCHEMICAL ANALYSIS

The plasma concentrations of MDA, an important indicator of oxidative stress, were measured according to the method of JAIN et al. [11]. The principle of the method is based on the spectrophotometric measurement of the colour that occurs during the reaction of thiobarbituric acid with MDA. Concentrations of thiobarbituric acid reactive substances (TBARS) were calculated by the absorbance coefficient of malondialdehyde–thiobarbituric acid complex and expressed in μmol/L.

The GSH concentrations were also determined by a spectrophotometric method [2]. After lysing whole blood red cells and removal of precipitate, disodium hydrogen phosphate and DTNB [5,5-dithiobis-(2-nitrobenzoic acid)] solutions were added and the absorbance of the formed complex was read at 412 nm. The results were expressed in mg/L.

The serum concentrations of nitric oxide (nitrate and nitrite) and BUN were detected by the method of MIRANDA et al. [20]. After mixing sera with an ethanol/hexane solution at the ratio of 1:1:3, the serum concentrations of β-carotene and vitamin A (retinol) were determined at 425 nm and 325 nm respectively [31]. Finally, the serum ceruloplasmin concentrations were measured according to the spectrophotometric method of SCHOSINSKY et al. [28].

All absorbances were read with a Jenway 6305 UV/VIS spectrophotometer.

HISTOPATHOLOGICAL ANALYSIS

Necropsy of one died lamb was carried out in the Pathology Department of Veterinary Faculty, Afyon Kocatepe University. Tissue samples were systemically fixed in 10% neutral formalin and then embedded with paraffin after routine tissue preparation. Sections (5 mm in thickness) were stained with haematoxylin-eosin and examined with light microscope (Olympus CX41 attached Kameram® Digital Image Analyze System).

STATISTICAL ANALYSIS

Data were analysed with the SPSS 15.0 statistical package program (SPSS Inc, Chicago, Illinois USA). Differences between the groups were analysed using one-way Analysis of Variance (ANOVA) and when F value was significant Duncan’s multiple range test was performed. Statistical significance was considered to be P < 0.05. The results are expressed as means ± standard deviations.

Results

CLINICAL FINDINGS

Poisoned lambs exhibited marked increased respiratory and heart rates and significantly depressed rumen motility.
compared to healthy controls \((P < 0.05)\) (Table I) coupled to dyspnoea, convulsion, salivation, sleepiness, ataxia, tonic and clonic spasms before treatment. Tympany was observed in 2 lambs and regurgitation in two other lambs.

One week after the treatment, four lambs were clinically improved: heart and respiratory rates as well as the rumen movement frequency were found statistically significant before and after treatment \((P < 0.05)\) (Table I). Nevertheless, 2 lambs died. Lung oedema and hyperaemia, petechial haemorrhage of heart muscle and hyperaemia in small intestinal mucosa were observed in these animals.

**BIOCHEMICAL FINDINGS**

As shown in Table II, circulating BUN, nitric oxide and MDA concentrations were markedly increased \((P < 0.05)\) in the poisoned lambs compared to healthy controls whereas GSH, retinol and \(\beta\)-carotene concentrations were significantly decreased \((P < 0.05)\). No significant difference in ceruloplasmin concentrations was found between the controls and the intoxicated animals. On the other hand, the concentrations of biochemical and antioxidant / oxidant markers were significantly modified after treatment \((P < 0.05)\) and became closely related to values observed in the healthy controls.

**HISTOPATHOLOGICAL FINDINGS**

Peribronchiolitis, thickening of the interalveolar septum tissue, and mononuclear cell infiltration, were observed in lung tissue (figure 1A). Glomerulonephritis coupled to degeneration of tubule epithelial cells in kidney tissue (figure 1B) and vacuolar degeneration of hepatocytes leading to disruption of cord structures (figure 1C) were also noticed. In addition to the signs of cell degeneration, mononuclear cell infiltrates associated to the proliferation of the Kupffer cells in the liver (figure 1D) were evidenced.

**Discussion**

Acute ammonium poisoning is frequently observed in ruminants because they consume with appetite ammonium nitrate-containing fertilizers [9]. Accidental access to the powder or liquid form of the compound can cause severe mortalities [33]. Acute intoxication with large doses of ammoniac as used in the present work leads to high ammoniac concentrations in blood and to the rapid (half-life: 30 min) death of the animals [16]. Ammoniac intoxication increases brain nitric oxide, which in turn plays a role in the mediation of ammoniac-induced depletion of ATP and death of animals [14].

**Clinical parameters**

<table>
<thead>
<tr>
<th></th>
<th>Healthy lambs ((n = 6))</th>
<th>Poisoned lambs before treat. ((n = 6))</th>
<th>Poisoned lambs after treat. ((n = 4))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body temperature (^\circ\text{C})</td>
<td>39.27 ± 0.16</td>
<td>39.10 ± 0.22</td>
<td>39.03 ± 0.10</td>
</tr>
<tr>
<td>Heart rate ((\text{beats / min.}))</td>
<td>81.17 ± 5.46(^a)</td>
<td>134.67 ± 7.92(^b)</td>
<td>79.50 ± 2.12(^a)</td>
</tr>
<tr>
<td>Respiratory rate ((\text{cycles / min.}))</td>
<td>21.0 ± 0.89(^a)</td>
<td>30.00 ± 1.41(^b)</td>
<td>22.5 ± 0.71(^c)</td>
</tr>
<tr>
<td>Rumen movement ((\text{number / 5 min.}))</td>
<td>8.50 ± 0.55(^a)</td>
<td>2.33 ± 0.82(^b)</td>
<td>8.0 ± 0.45(^a)</td>
</tr>
</tbody>
</table>

*Treat.: treatment of ammonium sulphate poisoning. Different superscripts \(^a\), \(^b\), \(^c\) indicate significant differences \((P < 0.05)\) between groups.*

**Biochemical parameters**

<table>
<thead>
<tr>
<th></th>
<th>Healthy lambs ((n = 6))</th>
<th>Poisoned lambs before treat. ((n = 6))</th>
<th>Poisoned lambs after treat. ((n = 4))</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUN (mg/L)</td>
<td>75.3 ± 7.0(^a)</td>
<td>321.1 ± 30.1(^b)</td>
<td>82.7 ± 5.4(^a)</td>
</tr>
<tr>
<td>Nitric oxide ((\mu\text{mol/L}))</td>
<td>0.54 ± 0.16(^a)</td>
<td>0.83 ± 0.11(^b)</td>
<td>0.47 ± 0.08(^a)</td>
</tr>
<tr>
<td>GSH (mg/L)</td>
<td>357.7 ± 33.9(^a)</td>
<td>293.4 ± 22.3(^b)</td>
<td>372.3 ± 5.2(^a)</td>
</tr>
<tr>
<td>MDA ((\mu\text{mol/L}))</td>
<td>1.01 ± 0.12(^a)</td>
<td>1.26 ± 0.11(^b)</td>
<td>0.93 ± 0.08(^a)</td>
</tr>
<tr>
<td>Retinol ((\mu\text{g/L}))</td>
<td>367.2 ± 9.0(^a)</td>
<td>298.3 ± 13.8(^b)</td>
<td>369.0 ± 4.5(^a)</td>
</tr>
<tr>
<td>(\beta)-carotene ((\mu\text{g/L}))</td>
<td>169.1 ± 19.5(^a)</td>
<td>121.0 ± 7.4(^b)</td>
<td>160.5 ± 3.3(^a)</td>
</tr>
<tr>
<td>Ceruloplasmin ((\text{mg/L}))</td>
<td>367.5 ± 12.6</td>
<td>373.0 ± 5.4</td>
<td>377.9 ± 3.2</td>
</tr>
</tbody>
</table>

*Treat.: treatment of ammonium sulphate poisoning. Different superscripts \(^a\), \(^b\) indicate significant differences \((P < 0.05)\) between groups.*
AMMONIUM SULPHATE POISONING IN LAMBS

Clinical signs most often observed in ammoniac poisoning are restlessness, dullness, weakness, muscle tremors, salivation, rumen atony, dyspnoea, ataxia, lateral recumbency, regurgitation, bloating, incoordination, vocalization, lung oedema, tonic and clonic convulsion, and finally death by heart failure [4, 13, 25, 27]. These clinical findings were also observed in this study.

Acute ammonium intoxication leads to excessive activation of the NMDA receptors, leading to activation of neuronal nitric oxide synthase, increasing the formation of nitric oxide, which contributes to the toxic effects of ammoniac. Nitric oxide can reduce the activity of some antioxidant enzymes [3, 5, 21]. EDJTEHADI et al. [4] observed marked increases of plasma ammoniac and urea concentrations in sheep during acute urea intoxication. ISSI et al. [10] have also found high serum nitrite concentrations in goats suffering from acute nitrate intoxication. This increase in nitric oxide would be responsible for the rapid reduction of antioxidant enzyme activities. Nitric oxide can decrease these activities both directly and indirectly [3, 5, 21]. LUPERCHIO et al. [19] have also reported that nitric oxide in excess oxidized the reduced form of glutathione (GSH) into glutathione disulfide (GSSG) and mixed glutathione disulfides (GSSR), resulting in depletion of GSH and increased free radicals. KOSSENKO et al. [15] have observed that acute intoxication with large doses of ammoniac reduced the activity of antioxidant enzymes in liver and brain mitochondria and resulted in increased formation of the superoxide radical. They have also demonstrated that acute ammoniac intoxication reduced the total content of glutathione both in cytosol (by 68%) and in mitochondria (by 53%) and particularly the content of the reduced GSH (by 73% in cytosol and by 52% in mitochondria) [17]. Acute ammoniac intoxication also increased lipid peroxidation as measured by the determination of malondialdehyde (MDA) concentrations. Ammoniac increased MDA content by 27% in cytosol and by 36% in mitochondria [17]. ISSI et al. [9] have stated that plasma MDA values were high during the acute phase of poisoning but markedly decreased one week later. Oxidative stress and increase in free radical formation are expected events in acute nitrate poisonings. O’CONNOR and COSTELL [22] have reported

**FIGURE 1:** A. Lung tissue; peribronchiolitis (arrowheads), interalveolar septum thickening (bold arrows) and mononuclear cell infiltration (thin arrows) in a dead lamb after ammonium sulfhate poisoning, haematoxylin-eosin X100. B. Kidney tissue; glomerulonephritis (bold arrows) and focal mononuclear cell infiltrates in interstitial area (thin arrows) in a dead lamb after ammonium sulfhate poisoning, haematoxylin-eosin X100. C. Liver tissue; vacuolar degenerations in the hepatocytes (arrows) and deterioration in the cord structure (arrowheads), haematoxylin-eosin X200. D. Liver tissue; numerical increase of Kupffer cells (arrowheads) and mononuclear cell infiltration (arrows), haematoxylin-eosin X200.
that acute and chronic hyperammonemia in mice lead to increased lipid peroxidation in liver and brain, reflecting an oxidative stress situation. In the present study, the circulating BUN, nitrite (i.e. nitric oxide) and MDA concentrations were markedly and significantly increased in the acute ammonium poisoned lambs whereas blood GSH concentrations were significantly depleted. These findings are concordant with previous studies on ammonium poisoning which have reported increases in BUN, nitrite and MDA concentrations [4, 9, 10, 15, 17, 19, 22] and decrease in GSH concentrations [15, 17, 19]. ISSI et al. [9] have investigated acute nitrate poisoning in two cattle and reported that the β-carotene and vitamin A concentrations were notably depressed during and after treatment. Vitamin A deficiency has also been reported in cattle receiving nitrate enriched food or poisoned with fertilizers [8, 12, 26]. The depletion in provitamin A and in vitamin A would probably result from a direct effect of nitrates, which were reported to reduce the vitamin A store in liver [8, 12]. Significant decreases in β-carotene and retinol concentrations were evidenced in ammonium sulphate poisoned lambs in the current study.

As ammoniac increases the production of superoxide radicals, it is possible that the sporadic muscle damage occurring in acute ammonium poisoning in ruminants [6] would result from the induced oxidative stress. In addition, the hepatocellular and neuronal oxidative damage would be involved in the animal death [15]. SINGER and Mc CARTY [29] stated that sheep dead from ammoniac poisoning showed massive myocardial and skeletal muscle haemorrhage, whereas in liver no macroscopic lesion was detected and at the histological level, only a mild hepatocyte swelling was evidenced. Although it is well recognized that hyperammoneniama causes biochemical disarray in cellular metabolism and intense cellular damage to brain and lungs, only few studies [25] investigate induced hepatic and muscle lesions despite the large amounts of ammoniac accumulated in these tissues during poisoning. The clinical entity with brownish lungs and haemorrhage in gastrointestinal tract of two lambs were similar with results of YONG et al. [34] on cattle that died from nitrate poisoning in one hand, and of ISSI et al. [10] on acute nitrate poisoning in goats in the other hand. In the present study, necropsy and histopathological findings are compatible with the literature [6, 10, 14, 25, 29].

KULKARNI and KULKARNI [18] have reported that ruminal pH become more alkaline because of the hydrolysis of urea into ammoniac. Based on the exhibited symptoms the acute urea intoxicated buffalio heifers were treated with weak acetic acid, by a first oral administration of 2.5L of 5% acetic acid vinegar following by a second administration of 1L of vinegar after half an hour. Heifers completely recovered and reached values recorded in the healthy lambs in agreement with previous reports [7, 9, 10, 18].

In conclusion, biochemical and histopathological findings demonstrated the occurrence of an oxidative stress in intoxication with ammonium sulphate, widely used in agriculture and animal husbandry, leading to the degeneration of the epithelial cells. Moreover, 5% acetic acid (vinegar), glutamic acid and vitamin A could be successfully used in the treatment of the fatal intoxication.

References


