Oxidative stress and alterations of antioxidant status in goats naturally infected with Mycoplasma agalactiae

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SUMMARY

The aim of this study was to determine some oxidative stress parameters and antioxidant vitamin concentrations in goats naturally infected with Mycoplasma agalactiae. Forty seven goats showing clinical signs of contagious agalactia and 20 healthy goats (control group) were used in the study. Blood samples were taken from the jugular vein in order to measure the concentrations of vitamin A, E, C, β-carotene, glutathione (GSH) and malondialdehyde (MDA) and activities of catalase (CAT) and glutathione peroxidases (GSHPx), whereas milk and mastitic secretions were taken from the sick animals for M. agalactiae identification by culture and PCR. Significant increase of MDA concentrations, significant reductions of GSH, vitamin C and E concentrations and of GSHPx activity compared to the controls were evidenced in M. agalactiae infected goats. The results clearly demonstrate the occurrence of an oxidative stress leading to deficiency of enzymatic and non-enzymatic antioxidant systems during this infection.

Keywords : Mycoplasma agalactiae, goat, antioxidant vitamins, malondialdehyde, glutathione peroxidases, catalase.

Introduction

Caprine Contagious Agalactiae (CCA) is one of the most serious disease affecting small ruminants and is characterised by mastitis, arthritis, keratoconjunctivitis, and occasionally abortion [27, 32]. The etiologic agent of the classical disease is Mycoplasma agalactiae. However, similar clinical and pathological features can be produced by other mycoplasma species in small ruminants included Mycoplasma mycoides subsp. mycoides, Mycoplasma capricolum subsp. capricolum and Mycoplasma mycoides subsp. capri [2, 24]. Goats seem to be more susceptible to the natural disease than sheep, but M. agalactiae is an important pathogen for both species [21]. The diagnosis of contagious agalactiae is established on the basis of epidemiological findings and the presence of clinical signs. The clinical diagnosis should be confirmed by laboratory examination, i.e. isolation and identification of the infectious agent. The most suitable samples include milk, ocular, nasal or vaginal swaps, articular exudates, blood and urine for isolation attempts [15, 21, 24].

Excessive formation of free radicals and concomitant damage at cellular and tissue levels are controlled by cellular antioxidant defence systems. The preventive defence systems can be accomplished by enzymatic or non-enzymatic mechanisms including reduced glutathione (GSH), vitamin C and vitamin E. The antioxidant enzymes such as glutathione peroxidase (GSHPx) and catalase (CAT) may also have important functions in alleviating the toxic effects of reactive oxygen species (ROS) [12, 30, 31].

The aim of this study was to investigate plasma concentrations of some oxidative stress parameters such as GSH, GSHPx, CAT and malondialdehyde (MDA), and antioxidant vitamins such as vitamin A, E, C and β-carotene in goats with agalactia caused by M. agalactiae.

RÉSUMÉ

Stress oxydatif et altérations du statut anti-oxydant chez des chèvres infectées naturellement par Mycoplasma agalactiae.

L’objectif de cette étude était de déterminer les paramètres biochimiques d’un stress oxydatif et les concentrations en vitamines anti-oxydantes chez des chèvres naturellement infectées par Mycoplasma agalactiae. Quarante-sept chèvres cliniquement atteintes d’agalaxie contagieuse et 20 chèvres saines ont été utilisées comme témoins. Des prélèvements sanguins ont été effectués à la veine jugulaire afin de mesurer les concentrations en vitamines A, E, C, en β-carotène, en glutathion (GSH) et en malondialdéhyde (MDA) ainsi que les activités de la catalase (CAT) et des glutathione peroxidases (GSHPx). L’identification de M. agalactiae a été réalisée par mise en culture et PCR à partir du lait et des sécrétions mammaires des animaux malades. Par rapport aux témoins une augmentation significative des concentrations plasmatiques de MDA, associée à une réduction significativement importante des concentrations en GSH, β-carotène, vitamine C et vitamine E et de l’activité GSHPx a été mise en évidence chez les chèvres infectées par M. agalactiae. Ces résultats montrent clairement l’existence d’un stress oxydatif conduisant à un déficit des capacités anti-oxydantes enzymatiques et non enzymatiques durant cette infection.

Mots-clés : Mycoplasma agalactiae, chèvre, vitamines anti-oxydantes, malondialdéhyde, glutathion peroxidases, catalase.
Material and Methods

ANIMALS AND SAMPLES

The study was performed on 20 healthy hair goats (control group) and 47 naturally infected hair goats with \textit{M. agalactiae} in the province of Elazığ, Turkey. None of these animals had been vaccinated against \textit{M. agalactiae}. The clinical signs in the sick animals were mostly mastitis and arthritis, and there was no history of agalactia-related symptoms or signs in the control animals at the time of sampling. Ages of the animals varied between 2 and 3 years and mean body weights between 45 and 55 kg. All the animals were kept under the same care and feeding conditions. In both groups, the goats were fed with classic diet containing straw, barley and concentrates.

Blood samples were taken by veni puncture from the jugular vein into heparinized and nonheparinized vacutainer tubes. The nonheparinized blood samples were kept at room temperature for 2 h in order to obtain serum. Plasma and serum was separated by centrifugation (700 g, at +4°C) and stored at -20°C until analysis. Milk and mastitic secretions were collected aseptically of culture and identification by polymerase chain reaction (PCR) from the sick goats.

ANALYTICAL PROCEDURES

Identification \textit{M. agalactiae} (Culture and PCR)

Approximately 0.1 ml of milk samples were inoculated by Mycoplasma Broth Base (Oxoid) and by Mycoplasma Agar Base (Oxoid) containing Mycoplasma Supplement G (Oxoid) and incubated in a humid atmosphere containing 5% carbon dioxide at 37°C for 7 days. The broths were checked daily for growth and when they became turbid, loopfuls were inoculated into Mycoplasma Agar Base (Oxoid) and the plates were incubated under the same conditions. The plates were examined under a stereomicroscopic microscope (Olympus 209376, Tokyo, Japan) for 'fried egg' colonies, and viewed typical 'fried egg' appearance of \textit{Mycoplasma} colonies.

PCR was performed in a PCR Sprint Temperature Cycling System (Thermo Hybaid) in a reaction volume of 50 µL, consisting of 5 µL of a PCR buffer (750 mM of Tris HCl, pH 8.8, 200 mM of (NH4)2SO4, 0.1% Tween 20), 5 µL of MgCl2 (25 mM), 26 µL of sterile distilled water, 1 µL (total 4 µL) of each deoxynucleotide triphosphate (datp, dttp, dctp, dgtp) (25 mM), 5 µL (1.25 U) of Taq DNA Polymerase (MBI, Fermentas), 5 µL (total 50 pmol) of each primer (IDT) and 25 ng of template DNA. Primers ma-mp 1F (5'-AGCAGCAGCCTACCTGAGA-3) and ma-mp 1R (5'-AACACCTGGAATTGTTTGTGTT-3') were derived from the ma-mp81 gene of \textit{M. agalactiae} [7]. PCR products were cycled by 30 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min and extension at 72°C for 1 min, with a final extension step at 72°C for 10 min. The amplified products were detected by staining with ethidium bromide (0.5 µg/mL) after electrophoresis at 80V for 2 h in 1.5% agarose gels. PCR products with a molecular size of 176 bp were considered indicative for \textit{M. agalactiae}.

Oxidative stress parameters

The plasma CAT activity was measured as previously described by GOTH [9]. Briefly, 0.2 mL of plasma samples was incubated in 1.0 mL substrate (65 µmol of hydrogen peroxide per mL of a 50 mM phosphate buffer, pH 7.0) at 37°C for 60 seconds. The enzymatic reaction was terminated with 1.0 mL of a 32.4 mM ammonium molybdate solution. Hydrogen peroxide was measured at 405 nm against blank containing all the components except the enzyme on a spectrophotometer (Shimadzu UV-1208 UV-VIS, Japan). The catalase activity was expressed as kU/L.

The plasma GSHPx activity was determined according to the method of LAWRANCE [16]. The reaction mixture contained of 50 mM of a potassium phosphate buffer (pH 7.0), 1 mM of EDTA, 1 mM of sodium azide (Na3), 0.2 mM of reduced nicotinamide adenine dinucleotide phosphate (NADPH), 1 U/ml of oxidized glutathione (GS(GSSG))-reductase, 1 mM of GSH, and 0.25 mM of H2O2. Enzyme source (0.1 mL) was added to 0.8 mL of the above mixture and incubated for 5 min at 25°C before the initiation of the reaction by the addition of 0.1 mL of peroxide solution. The absorbance at 340 nm was recorded for 5 min on a spectrophotometer. The activity was calculated from the slope of the lines as micromoles of NADPH oxidized per minute. The blank value (the enzyme was replaced with distilled water) was subtracted from each value. The protein concentration was also measured by the method of LOWRY [20]. The results were expressed as U/g of proteins.

The plasma lipid peroxidation level was measured according to the concentration of thiobarbituric acid reactive species [26]. The amount of produced MDA was used as an index of lipid peroxidation. Briefly, one volume of the test sample and two volume of stock reagent (15%, w/v trichloroacetic acid in 0.25 N HCl and 0.375%, w/v thiobarbituric acid in 0.25 N HCl) were mixed in a centrifuge tube. The solution was heated for 15 min in boiling water. After cooling, the precipitate was removed by centrifugation at 500 g 10 min and then absorbance of the supernatant was measured at 532 nm against a blank containing all reagents except test sample on a spectrophotometer. The lipid peroxidation level was expressed as µmol/L.

The plasma GSH levels were measured spectrophotometrically using Ellman’s reagent [28] and results were expressed as mmol/L.

Antioxidant vitamins analysis

Vitamin A and β-caroten values were spectrophotometrically determined according to the method described by SUZIKI and CATOH [29], vitamin C values were colorimetrically determined using a phosphotungstic acid method described by KYAW [13] and vitamin E values were determined spectrophotometrically according to Martinke’s method [22]. All vitamin analyses were performed in the serum.
Changes in the plasma oxidative stress parameters and serum antioxidant vitamin concentrations are presented in Table I. Plasma MDA concentrations were markedly increased in naturally infected goats compared to controls (p<0.05). Plasma GSH concentrations and GSHPx activities were also significantly depressed in the infected group (p<0.05). By contrast, CAT activities did not significantly differ from control values, although this enzyme activity was lower in the infected group. In parallel, significant reductions of concentrations of vitamin E (p<0.05), C (p<0.01) and β-carotene (p<0.05) were also recorded in diseased goats compared to controls. No significant difference for vitamin A concentrations was evidenced between the 2 groups.

A negative correlation was determined between MDA and GSH concentrations in the infected goats (r = -0.611, p<0.05).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Infected goats</th>
<th>Healthy goats</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (µmol/L)</td>
<td>1.78 ± 0.35</td>
<td>1.43 ± 0.48</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>GSHPx (U/g protein)</td>
<td>0.86 ± 0.21</td>
<td>1.01 ± 0.15</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>GSH (mmol/L)</td>
<td>0.224 ± 0.015</td>
<td>0.244 ± 0.028</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>CAT (kU/L)</td>
<td>25.54 ± 8.20</td>
<td>28.88 ± 14.02</td>
<td>NS*</td>
</tr>
<tr>
<td>Vitamin E (mg/L)</td>
<td>0.91 ± 0.12</td>
<td>1.04 ± 0.11</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Vitamin C (mg/L)</td>
<td>3.22 ± 0.50</td>
<td>4.23 ± 0.40</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>β-carotene (µg/L)</td>
<td>146.5 ± 28.6</td>
<td>171.9 ± 21.6</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Vitamin A (mg/L)</td>
<td>1.89 ± 0.26</td>
<td>1.97 ± 0.24</td>
<td>NS*</td>
</tr>
</tbody>
</table>

*NS: no significant.

Table 1: Plasma oxidative stress parameters and antioxidant vitamin concentrations in goats naturally infected by *Mycoplasma agalactiae* (n = 47) and in healthy goats (n = 20). Results are expressed as mean ± standard deviation.
Discussion

A differential diagnosis is necessary for distinguishing mastitis and keratoconjunctivitis of mycoplasmal origin from the diseases caused by other microorganisms [21]. Recently, PCR has been accepted as a valuable method for diagnosis of mycoplasma infections. The PCR has the advantage of an easy use, a rapid availability of results and is more suitable for processing large number of specimens [7]. In the present study M. agalactiae was isolated by culture and identified by PCR.

Although laboratory changes are well examined in many bacterial and viral diseases in goats, especially oxidative stress parameters and antioxidant vitamins concentrations caused by mycoplasma are poorly studied. Furthermore, changes of oxidative status in the goats with mastitis caused by M. agalactiae are not explored. In the present study, significant increases of plasma MDA concentrations associated with decreases of antioxidant systems (GSH and GSHPx, β-carotene, vitamins E and C) were evidenced suggesting the oxidative stress occurrence during M. agalactiae infection.

Many potentially toxic reactive oxygen species are generated through normal oxidative metabolism, and the body has adapted by developing a complex system of protective antioxidants [10]. Oxidative stress may be defined as an alteration in the steady-state balance between oxidant and antioxidant agents in the cells. When intracellular sources of ROS are increased, several physiological processes may be disturbed [6, 19]. Under more stressful conditions such as mastitis, hydroxy radicals released by infiltrated neutrophils caused mammary cell injury [17]. This peroxidation-associated mechanism was demonstrated in an in vitro model where antioxidants were proved useful to prevent mammary tissue damage during bovine mastitis [3]. Mastitis is one of the most principal clinical symptoms in agalactia, hydroxy radicals were released by infiltrated neutrophils, and abnormal radical production leads to an oxidative stress, which caused damage of some macromolecules including proteins, lipids and nucleic acids. Furthermore, the other tissue damages observed during this disease such as arthritis and keratoconjunctivitis, could contribute to intensify oxidative damage.

Antioxidant enzymes and the determination of malondialdehyde (MDA) concentrations are among the most widely used methods for determination of oxidative stress. Increased plasma MDA concentration is considered as a marker of lipid peroxidation [10, 14, 23]. In this study, plasma MDA concentrations were found to be increased in the goats with M. agalactiae compared to the control group, while decreases of GSH concentrations and GSHPx activities were observed. As GSH and GSHPx are involved in the conversion of radicals into less effective metabolites, these changes coupled to the increase of MDA concentrations confirmed the occurrence of an oxidative stress during disease caused by M. agalactiae. A negative correlation was only obtained between MDA and GSH concentrations in the infected goats, whereas no correlation with the other parameters in the infected group was evidenced. These findings clearly indicate that GSH concentrations depressed when lipid peroxidation was increased, leading to an exacerbation of the oxidative stress.

Non-enzymatic antioxidants such as β-carotene attenuate lipid peroxidation intensity in the plasma of rats [8] and mouse [11]. The β-carotene is a quencher of singlet oxygen and has the ability to react directly with the peroxo radicals involved in lipid peroxidation [4]. Vitamin E (in the form of α-tocopherol) is the major lipid-soluble antioxidant of lipoproteins and biomembranes [14, 18, 31]. Vitamin C (ascorbic acid) functions as a potent water soluble chain-breaking antioxidant in the biological fluids, but it can not scavenge the radicals within the lipid region of the membranes [5]. The synergism between ascorbic acid and α-tocopherol in the inhibition of lipid peroxidation is well known. Vitamin C enhances the antioxidant activity of vitamin E and the depletion of the α-tocopherol is markedly reduced [1, 18, 25]. In this study, the concentrations of serum vitamin E, vitamin C and β-carotene, which are responsible for protecting the cells from damage caused by lipid peroxidation, were found to be significantly lower in the infected group than in the control group, while there was no significant change in the concentration of vitamin A. The increased consumption of these vitamins due to the disease-related oxidative stress may account for the reduction of serum vitamin E, vitamin C and β-carotene.

In conclusion, this study has highlighted the occurrence of an oxidative stress with important differences in antioxidant status as reflected by assessment of some enzymatic and non-enzymatic antioxidants between the goats infected by M. agalactiae and healthy controls. Hence, it can be useful to add the antioxidant vitamins to the classical treatment procedures for get ride of the disease.

References

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