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Clinicobiochemical investigations of gangrenous mastitis in does: immunological responses and oxidative stress biomarkers*

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Abstract: A total of 50 does were used to determine selected hematological and biochemical parameters with special references to oxidative stress markers, acute phase protein profiles, and proinflammatory cytokines in healthy and gangrenous mastitis affected does. Animals were divided into two equal groups represented as clinically healthy (control) and diseased groups, respectively. The bacteriological examination of milk samples from diseased does revealed many types of bacterial infection. The isolated bacteria were *Staphylococcus aureus* (*N*=23/25), *Escherichia coli* (*N*=11/25), and *Clostridium perfringens* (*N*=4/25). There was a significant increase in the levels of β-hydroxybutyrate, non-esterified free fatty acids, triglyceride, low density lipoprotein cholesterol (LDL-C), aspartate aminotransferase, and alanine aminotransferase and a significant reduction in the levels of glucose, cholesterol, and high density lipoprotein cholesterol (HDL-C) in does with gangrenous mastitis compared to healthy does. Moreover, there was a significant increase in the levels of malondialdehyde and uric acid with a significant decrease in the levels of reduced glutathione, super oxide dismutase, and catalase in does with gangrenous mastitis compared to healthy does. In addition, there was a significant increase in the haptoglobin, serum amyloid A, fibrinogen, interleukin 6 (IL-6), IL-1β, and tumor necrosis factor-α (TNF-α) in does with gangrenous mastitis compared to healthy ones. Conclusively, oxidative stress biomarkers, acute phase proteins, and proinflammatory cytokines play an essential task as biomarkers for gangrenous mastitis in does. Mastitis may be considered as one of the ketotic stressors in does after parturition.

Key words: Haptoglobin, Serum amyloid A, Malondialdehyde, Interleukin 1β, Interleukin 6, Tumor necrosis factor-α
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1 Introduction

Gangrenous mastitis in goats is a severe clinical condition of the inflammatory process in mammary glands (Ribeiro *et al*., 2007). Fatal clinical course is characterized by worsening of corporal condition, pneumonia, septicemia, and/or toxemia (Cable *et al*., 2004). A variety of infectious diseases of farm animals, for instance pneumonia, enteritis, and mastitis, are coupled with oxidative stress (Sordillo, 2005; Spears and Weiss, 2008; Sordillo and Aitken, 2009). Antibacterial activity of neutrophils is interceded, in part, through reactive oxygen species (ROS) (Rinaldi *et al*., 2007). Although essential for the body, an excess of oxidative reactions from the antibacterial processes may cause tissue damage. A surplus of ROS and the absence of optimal amounts of antioxidants are leading to oxidative stress (Lykkesfeldt and Svendsen, 2007). Many cells are susceptible to this oxidative stress, which can cause necrosis or apoptosis. The acute phase response (APR) in small ruminants is poorly described. The different acute phase proteins (APPs) may play a similar role both in sheep and goat, but some differences have been reported. Haptoglobin (Hp) and serum amyloid A (SAA) are considered as major APPs and ceruloplasmin (Cp) as

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a minor APP in both ovine and caprine APRs (González *et al*., 2008). Nonetheless, fibrinogen (Fb) participates as a minor APP in sheep but as a moderate APP in goat. In both species, the concentration of albumin diminishes after an appropriate stimulus (González *et al*., 2008). Most of the studies performed on sheep were focused on the role of APPs after several inflammatory stimuli, but few studies were carried out in relation to specific bacterial, viral, or parasitic infections. Some studies are focused on the expression of APPs against lentiviral infections, with a local expression of SAA (Sack and Zink, 1992), but no serum enhancement of Hp or Fb concentrations (de la Concha-Bermejillo *et al*., 2000). Few studies have been focused on the changes of APPs in milk secretions of sheep. Whereas SAA levels in milk may be useful for the diagnosis of subclinical mastitis in individual ewes, further studies are needed to determine its usefulness from bulk milk (Winter *et al*., 2006). Interestingly, opposite to bovine, the changes in the concentration of SAA in ewes with mastitis experimentally induced by *Staphylococcus epidermidis* are observed earlier in serum than in milk (Winter *et al*., 2003). In goats, the studies are rather limited than in sheep. The measure of APPs has been shown to not imply any advantage against the traditional markers observed for the diagnosis of pregnancy toxemia (González *et al*., 2011). Moreover, an increase of several APPs has been observed in the Alpine ibex with sarcoptic mange (Rahman *et al*., 2010), which probably would act in the same way in domestic goats.

To the best of our knowledge, the correlations between gangrenous mastitis, oxidative stress biomarkers, APPs, and proinflammatory cytokines are lacking in does. The aim of the present study is to throw the light on the oxidative stress biomarkers, APPs, and proinflammatory cytokines as possible biomarkers for gangrenous mastitis in does.

2 Materials and methods

2.1 Animals

Twenty-five does ((3.4±0.2) years old), native breeds, were referred to the Veterinary Teaching Hospital, King Faisal University, Saudi Arabia, presenting peracute clinical signs of mastitis, two to three weeks after the parturition. The animals were affected with gangrenous mastitis for 7–10 d. Treatment of affected does was initially attempted using amoxicillin (10 mg/kg, once a day, subcutaneously) and enrofloxacin (10 mg/kg, 24 h, subcutaneously), along with flunixin meglumin (1.1 mg/kg, 24 h, intravenously). In despite of initial therapy, the animal was submitted to unilateral mastectomy (Cable *et al*., 2004) 24 h after the clinical examination, due to worsening of general clinical signs and extension of the mammary lesions. In addition, other 25 healthy does of similar age and post-partum period were used as a control group. The diseased does were examined clinically. No verification of traumatic lesions or foreign body was pragmatic during the clinical examination of mammary glands of the affected does. All the animal procedures were performed according to the guidelines of the Animal Ethics Committee of College of Veterinary Medicine and Animal Resources, King Faisal University, Saudi Arabia.

2.2 Sampling

Two separate blood samples were collected from healthy does and affected does with gangrenous mastitis. One sample was taken in an Eppendorf tube, which was mixed with ethylenediaminetetraacetic acid (EDTA) as anticoagulant for hematological studies. The second blood sample was taken in plain tubes. Samples were centrifuged at 3000 r/min for 10 min and the clear serum was separated carefully and stored in Eppendorf tubes at −20 °C until estimation of serum biochemistry.

Milk samples were aseptically collected from diseased does and submitted to microbiological culture on 5% defibrinated sheep blood agar (0.05 g/ml), MacConkey agar, and Sabouraud agar, and incubated under aerobiosis, at 37 °C for 72 h. The same material was submitted to 5% sheep blood agar, and incubated under anaerobic conditions, at 37 °C for 120 h. The isolated microorganisms were identified using VITEK2 Compact, Biomeriux, France.

2.3 Determination of oxidative stress biomarkers

2.3.1 Malondialdehyde (MDA)

MDA is determined by colorimetric method by readymade kits provided by Bio-diagnostic, Egypt, according to the instruction of the enclosed pamphlet. Thiobarbituric acid (TBA) reacts with MDA in acidic

medium at 95 °C for 30 min to form TBA reactive product, and the absorbance of the resultant pink product can be measured at 534 nm.

2.3.2 Reduced glutathione (GSH)

GSH is determined by colorimetric method by readymade kits provided by Bio-diagnostic, Egypt, according to the instruction of the enclosed pamphlet. GSH determination is based on the reduction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) with GSH to produce a yellow compound. The reduced chromogen is directly proportional to GSH concentration and its absorbance can be measured at 405 nm.

2.3.3 Super oxide dismutase (SOD)

SOD is determined by colorimetric method by readymade kits provided by Bio-diagnostic, Egypt, according to the instruction of the enclosed pamphlet. SOD assay relies on the ability of the enzyme to inhibit the phenazine methosulfate-mediated reduction of nitroblue tetrazolium dye.

2.3.4 Catalase

Catalase is determined by colorimetric method by readymade kits provided by Bio-diagnostic, Egypt, according to the instruction of the enclosed pamphlet. Catalase reacts with a known quantity of H_2O_2. The reaction is stopped after exactly 1 min with catalase inhibitor. In the presence of horseradish peroxidase (HRP), remaining H_2O_2 reacts with 3,5-dichloro-2-hydroxybenzene sulfonic acid (DHBS) and 4-aminophenazone to form a chromophore with a color intensity inversely proportional to the amount of catalase in the original sample.

2.3.5 Uric acid

Uric acid was determined by uricase-POD enzymatic colorimetric method using kits provided by Spinreact, Spain, according to Young and Friedman (2001).

2.4 Determination of biochemical parameters

The levels of glucose, triglyceride, cholesterol, high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), and blood urea nitrogen (BUN), as well as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were determined in serum samples on a Beckman CX-7 autoanalyser using commercial kits (Sigma Chemical Co. Ltd., Poole, Dorset, UK).

Serum concentration of β-hydroxybutyrate was determined by a kinetic enzymatic method using a commercially available kit (Ranbut D-3-hydroxybutyrate, Randox, Crumlin Co., Antrim, UK), according to the methods described by Bani Ismail *et al*. (2008). The assay is based on the reversible reaction between 3-hydroxybutyrate and NAD catalyzed by 3-hydroxybutyrate dehydrogenase, and the change in nicotinamide adenine dinucleotide (NADH) concentration is measured by changes in the absorbance at 340 nm.

Serum concentration of non-esterified free fatty acid (NEFA) was carried out using commercially available test kits supplied by Randox laboratories Ltd., Crumlin Co., Antrim, UK.

2.5 Determination of APPs and plasma cytokines

Serum Hp was determined using the haemoglobin binding assay described by Makimura and Suzuki (1982). SAA was measured by a solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) with a commercially available ELISA kit (Phase SAA kit, Tridelta Ltd., Ireland), according to the manufacturer's instructions. The analytical sensitivities in serum have been determined by the manufacturer as 0.3 μg/ml. Interleukin 6 (IL-6), IL-1β, and tumor necrosis factor-α (TNF-α) levels were determined from undiluted serum samples using commercially available ELISA kits (Biosource, Diagnostic Corporation, USA). The plates were read at 450 nm on a computerized automated microplate ELISA reader (Bio TEC, ELX800G, USA).

2.6 Statistical analysis

The statistical significance between means was compared using Student's *t*-test; $P \leq 0.05$ was considered significant. All data are presented as means± standard error of the means (SEM). All tests were performed using computer package of the statistical analysis system (SAS, 2002).

3 Results

The diseased does displayed fever, anorexia, and dyspnea. The mammary gland was enlarged,

blue-blackish, and cold, with marked line of the affected tissue and milk containing blood and pus secretion in very small amounts. The bacteriological examination of milk samples revealed many types of bacterial infection. The isolated bacteria were *Staphylococcus aureus* (*N*=23/25), *Escherichia coli* (*N*=11/25), and *Clostridium perfringens* (*N*=4/25). Concerning the biochemical investigation of diseased does, Table 1 showed that there was a significant ($P\leq0.05$) increase in the levels of β-hydroxybutyrate, NEFA, triglyceride, LDL-C, AST, and ALT ((1.60±0.13) mmol/L, (1.20±0.12) mmol/L, (71.32±0.21) mg/dl, (29.32±0.45) mg/dl, (62.10±0.52) U/L, and (32.23±0.32) U/L, respectively), with a significant ($P\leq0.05$) reduction in the levels of glucose, cholesterol, and HDL-C ((22.20±0.12) mg/dl, (58.32±0.54) mg/dl, and (31.80±0.32) mg/dl, respectively) in does with gangrenous mastitis compared to healthy does. Table 2 revealed a significant ($P\leq0.05$) increase in the levels of MDA and uric acid ((9.13±2.38) nmol/ml and (0.21±0.001) mg/dl, respectively) with a significant ($P\leq0.05$) reduction in the levels of GSH, SOD, and catalase ((0.57±0.13) mg/dl, (112.25±4.29) U/ml, and (183.03±5.13) U/L, respectively) in does with gangrenous mastitis compared to healthy does. Table 3 revealed that there was a significant ($P\leq0.05$) increase in the Hp, SAA, Fb, IL-6, IL-1β, and TNF-α ((1.11±0.11) mg/L, (30.40±4.42) mg/L, (3.84±0.21) g/L, (22.30±2.31) pg/ml, (20.83±2.40) pg/ml, and (19.36±1.75) pg/ml, respectively) in does with gangrenous mastitis compared to healthy ones ((0.09±0.001) mg/L, (3.36±1.20) mg/L, (2.42±0.24) g/L, (13.20±1.23) pg/ml, (12.74±3.43) pg/ml, and (5.54±1.23) pg/ml, respectively).

Table 1 Mean levels of selected biochemical parameters in clinically healthy does and those with gangrenous mastitis

Group	Glucose (mg/dl)	β-Hydroxybutyrate (mmol/L)	NEFA (mmol/L)	Triglyceride (mg/dl)	Cholesterol (mg/dl)
Control healthy does (*N*=25)	61.40±0.29	0.92±0.12	0.54±0.12	55.90±0.15	78.20±0.66
Does with gangrenous mastitis (*N*=25)	22.20±0.12*	1.60±0.13*	1.20±0.12*	71.32±0.21*	58.32±0.54*
Group	LDL-C (mg/dl)	HDL-C (mg/dl)	AST (U/L)	ALT (U/L)	BUN (mg/dl)
Control healthy does (*N*=25)	27.37±0.17	49.60±0.22	47.50±0.63	17.13±0.23	25.50±0.54
Does with gangrenous mastitis (*N*=25)	29.32±0.45*	31.80±0.32*	62.10±0.52*	32.23±0.32*	24.70±0.43

* Means are significantly different at the level ($P\leq0.05$)

Table 2 Mean levels of oxidative stress markers in control healthy does and those with gangrenous mastitis

Group	MDA (nmol/ml)	SOD (U/ml)	GSH (mg/dl)	Catalase (U/L)	Uric acid (mg/dl)
Control healthy does (*N*=25)	5.19±0.30	161.48±4.06	1.26±0.12	330.60±6.67	0.11±0.004
Does with gangrenous mastitis (*N*=25)	9.13±2.38*	112.25±4.29*	0.57±0.13*	183.03±5.13*	0.21±0.001*

* Means are significantly different at the level ($P\leq0.05$)

Table 3 Mean levels of acute phase proteins and proinflammatory cytokines in clinically healthy does and those with gangrenous mastitis

Group	Hp (mg/L)	SAA (mg/L)	Fb (g/L)	IL-6 (pg/ml)	IL-1β (pg/ml)	TNF-α (pg/ml)
Control healthy does (*N*=25)	0.09±0.001	3.36±1.20	2.42±0.24	13.20±1.23	12.74±3.43	5.54±1.23
Does with gangrenous mastitis (*N*=25)	1.11±0.11*	30.40±4.42*	3.84±0.21*	22.30±2.31*	20.83±2.40*	19.36±1.75*

* Means are significantly different at the level ($P\leq0.05$)

4 Discussion

Mastitis is considered one of the most important diseases of domestic animals, caused by several etiological agents. Transmission of the microorganisms primarily occurs via teats canal by ascendance, usually involving agents from animals and environmental origin and from the milking process (Anderson *et al*., 2005). The presented study revealed a significant ($P\leq0.05$) reduction in glucose levels in does with gangrenous mastitis in comparison with healthy does. Moreover, there was a significant ($P\leq0.05$) elevation in the levels of β-hydroxybutyrate and NEFA in comparison with healthy does. The decreased levels of glucose and increased levels of β-hydroxybutyrate and NEFA could be attributed to the loss of appetite in diseased does. Interestingly, these data may present a ketotic state in does with gangrenous mastitis. These results of reduced glucose levels are consistent with those obtained by Çetin *et al*. (2005) in ewes and disagree with Fuguay *et al*. (1975) in cows with clinical mastitis. Furthermore, there was a significant ($P\leq0.05$) increase in the total lipids in diseased does compared to healthy ones. It has been reported previously that serum total lipid concentrations increased in ketotic state (Marteniuk and Herdt, 1988; Kaneko *et al*., 1997), which was confirmed in this study. Serum cholesterol levels decreased significantly ($P\leq0.05$) in hepatic insufficiency (Kaneko *et al*., 1997). In the present findings, serum concentrations of cholesterol in the diseased does were lower than that in healthy does ($P\leq0.05$). Furthermore, it was found that the serum levels of HDL-C in the diseased does were also significantly lower ($P\leq0.05$), while LDL-C was significantly ($P\leq0.05$) higher compared to the control group. AST and ALT activities increased in mononucleosis, liver damage, and kidney infection (Kaneko *et al*., 1997). In the present study, AST and ALT activities differed significantly ($P\leq0.05$) between diseased and healthy does. Higher activities of AST and ALT in diseased animals are associated with liver dysfunction. The mechanisms by which inflammation cause damage to mammary gland tissue during mastitis are still not fully understood. It is well known that inflammatory reactions, in which vascular permeability increases and leukocyte migration occurs, involve free radicals, such as $O_2\cdot$, $H_2O_2\cdot$, and $OH\cdot$ (Cuzzocrea *et al*., 1998; Poch *et al*., 1999). In the present study, there was a significant decrease in GSH in diseased does compared to healthy ones. Moreover, there was a significant decrease in the levels of SOD and catalase in diseased does compared to healthy ones. These changes might be due to the great demand of high GSH, SOD, and catalase activities for elevated level oxidant damage occurring because of inflammatory reactions in the mammary gland tissue or insufficient nutrition of the does with gangrenous mastitis. Eventually, defense activity of the animal increased against oxidants. Increased lipid peroxidation as a result of changed intracellular ratio between the free radicals and antioxidant system has been suggested to be correlated with mastitis (Ndiweni *et al*., 1991; Atroshi *et al*., 1996; Çetin *et al*., 2005). These findings are in concurrence with those obtained by Atroshi *et al*. (1996) in cows and Çetin *et al*. (2005) in ewes. In the present study, plasma MDA levels in does with gangrenous mastitis were significantly ($P\leq0.05$) higher than that in healthy ones. Moreover, the decrease in food intake may be considered as a further cause of increased lipid peroxidation in ruminants (Gaál *et al*., 1993). These findings agree with those obtained by Atroshi *et al*. (1996) who determined the levels of MDA in cows with mastitis to be 38%–44% higher than those in healthy cows, and are also in agreement with those obtained by Çetin *et al*. (2005) in ewes. The present results indicate that increasing levels of oxidative stress markers in does with gangrenous mastitis might have an essential role in the process of inflammation and tissue damage. Uric acid is the major low molecular weight intracellular antioxidant in upper respiratory fluids (Kassim *et al*., 2002). Similarly, our results showed a significant increase in the level of uric acid in diseased does compared to the healthy ones.

In the present study, there were significant ($P\leq0.05$) increases in the levels of IL-6, TNF-α, and IL-1β in does with gangrenous mastitis compared to healthy does. Moreover, APR revealed significantly ($P\leq0.05$) higher levels of Hp, SAA, and Fb in diseased does compared to healthy ones. The elevated levels of APPs could be attributed to the release of proinflammatory cytokines like TNF-α as a result of inflammation (Gabay and Kushner, 1999). The elevated levels of APPs are in agreement with those obtained by Winter *et al*. (2003) in ewes with mastitis induced experimentally with *Staphylococcus*

epidermidis, Eckersall *et al*. (2001) in dairy cows with clinical mastitis, and Gerardi *et al*. (2009) in dairy cows with subclinical mastitis. In the current study, chemotactic factors released by infectious bacteria and other components of the immune system are the signals for neutrophil recruitment to sites of infection. However, this influx of neutrophils is a double-edged sword. This may cause not only an inflammatory reaction that results in the elimination of infection, but also tissue damage that leads to fibrosis and impaired mammary function (Capuco *et al*., 1986). Neutrophils promote tissue injury and disturb mammary function via reactive oxygen metabolite generation (the respiratory burst) and granular enzyme release (degranulation) (Miller *et al*., 1993; Kehrli and Shuster, 1994). Release of the cytokine TNF in the present study is due to the binding of the lipopolysaccharide (LPS)/LPS-binding protein (LBP) complex to the cluster of differentiation 14 (CD14) molecule (Schumann *et al*., 1990). TNF is a potent activator of leukocytes and enhances the phagocytosis and killing of mastitis pathogens by bovine neutrophils (Kabbur and Jain, 1995). Under defined conditions, the CD14 receptor can also mediate phagocytosis of LBP-coated Gram-negative bacteria (Wright *et al*., 1989).

5 Conclusions

From this study it could be concluded that oxidative stress biomarkers, APPs, and proinflammatory cytokines play an essential task as biomarkers for gangrenous mastitis in does. Moreover, gangrenous mastitis may be considered as one of the ketotic stressors in does after parturition.

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