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Research Article

Investigation of Antioxidant Enzymes and Some Biochemical Parameters in Ewes with Gangrenous Mastitis

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Abstract: This paper investigates the effects of gangrenous mastitis seen with cell necrosis and tissue damage on the activity of erythrocyte glutathione peroxidase (GSH-Px), level of plasma lipid peroxidation (MDA), and some biochemical parameters. To do that, 20 clinically gangrenous mastitis diagnosed and 20 healthy ewes, all from the same flock in a local ewes unit were used as material. Blood samples were kept in test tube containing the disodium salt of ethylenediamine tetraacetic acid (EDTA) as anticoagulant for plasma MDA and erythrocyte GSH-Px analysis and in vacutainer tube for biochemical analyses from the jugular vein. The following measurements were performed in blood serum: glucose, cholesterol, triglyceride, total protein, albumin, sodium (Na), potassium (K), chloride (Cl), calcium (Ca), total bilirubin, alanine transaminase (ALT), aspartate transaminase (AST), urea and creatinine in an autoanalyzer.

Erythrocyte GSH-Px activities and plasma MDA levels were found to be significantly higher in the ewes with gangrenous mastitis than healthy ewes. While there was an increase in creatinine and total bilirubin, Na, K, Ca, glucose and albumin were significantly decreased in the gangrenous mastitic group than the control group.

In conclusion, in ewes, the severe damage in the inflamed mammary gland tissue during gangrenous mastitis might be said to cause high oxidant stress, which leads to increases in the levels of erythrocyte GSH-Px and plasma MDA.

Key Words: Glutathione peroxidase, lipid peroxidation, biochemical parameters, gangrenous mastitis, ewes

Gangrenli Mastitisli Koyunlarda Antioksidan Enzimlerin ve Bazı Biyokimyasal Parametrelerin Araştırılması

Özet: Bu çalışmada amaç, koyunlarda doku harabiyeti ve hücre nekrozuna neden olan gangrenli mastitisin eritrosit glutation peroksidaz (GSH-Px) aktivitesi, plazma lipit peroksidasyon (MDA) düzeyi ve bazı biyokimyasal parametreler üzerindeki etkilerini araştırmaktır.

Bu amaçla bir koyun işletmesinde klinik olarak gangrenli mastitis teşhisi konulan 20 baş hasta ve aynı sürüdeki 20 baş sağlıklı koyun materyal olarak kullanıldı.

Kan örnekleri plazma MDA ve eritrosit GSH-Px ölçümleri için etilendiamin tetraasedik asit (EDTA)'li tüplere, diğer biyokimyasal parametre ölçümleri için ise vakumlu tüplere vena jugularisten alındı. Kan serumlarında glukoz, kolesterol, trigliserid, total protein, albumin, sodyum (Na), potasyum (K), klor (Cl), kalsiyum (Ca), total bilirubin, alanin transaminaz (ALT), aspartat transaminaz (AST), üre ve kreatinin ölçümleri otoanalizörde yapıldı.

Eritrosit GSH-Px aktiviteleri ve plasma MDA düzeyleri gangrenli mastitisli koyunlarda sağlıklı koyunlarla karşılaştırıldığında önemli derecede yüksek bulundu. Gangrenli mastitisli grupta kreatinin ve total bilirubin değerlerinde kontrol grubuna göre bir artış olurken Na, K, Ca, glukoz ve albumin değerlerinde önemli bir azalma gözlendi.

Sonuç olarak koyunlarda ganrenli mastitiste yangılı meme dokusundaki şiddetli hasarın yüksek oksidan strese neden olarak eritrosit GSH-Px ve plasma MDA düzeylerinde artışa yol açtığı söylenebilir.

Anahtar Sözcükler: Glutation peroksidaz, lipid peroksidasyon, biyokimyasal parametre, gangrenli mastitis, koyun

Introduction

Mastitis continues to be an economically vital disease all over the world. Due to its anatomic topography, the udder is exposed to environmental effects, leading to inflammatory and non-inflammatory diseases (1). The disease in small ruminants is very important because of its high mortality rate on acute and peracute forms even though it is seen relatively rarely (2).

Main pathogen bacteria causing mastitis in ewes are *Staphylococcus aureus* and *Pasteurella haemolytica* (1). *S. aureus*-caused mastitis beginning with color changes in the skin produces gangrenous mastitis with severe general and local conditions. Systemic symptoms are high fever (40.5-42 ºC), anorexia, and difficult breathing within 1-2 days. Lameness due to swollen udder is a very essential symptom to clinically observe the sick animals (3). If not treated the disease kills 50-85% of the flock within 1-5 days as a result of septicemia and toxemia (4).

Inflammatory reaction accompanying mastitis causes damages to the mammary secretory epithelium and, consequently, reduces milk production (5,6). However, the biochemical mechanisms underlying mammary cell damages are unclear. Mastitis is characterized by an accumulation of neutrophils in the mammary gland (7). More recently, active bovine blood neutrophils were shown to be cytotoxic for mammary epithelial cells in vitro (8). However, damage-causing agents are still unidentified.

Reactive oxygen species (ROS), such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$), are released by neutrophils and have been shown to play an important role in inflammation and cell injury (9-11). Cytotoxic effects of oxidants include protein oxidation, lipid peroxidation, DNA damage, and the inhibition of cellular metabolic pathways. Catalase, superoxide dismutase and glutathione peroxidase (GSH-Px) are part of intracellular defense systems against oxidation. Both have been shown to prevent, to a certain extent tissue damage in experimental pancreatitis (12). Thus, these antioxidants might reduce mammary cell damage (13).

The present study investigated the effects of gangrenous mastitis causing tissue damage and cell necrosis in ewes, on erythrocyte GSH-Px activity, level of malondialdehyde (MDA) in the plasma as a marker of lipid peroxidation or free radical activation and some biochemical parameters.

Materials and Methods

In the present study, 20 clinically gangrenous mastitis diagnosed and 20 healthy ewes, all from the same flock in a local ewes unit were used as subjects. Milk samples from sick udder lobes were obtained in laboratory conditions for bacteriological examination. The samples were stained by Gram method, and were observed under light microscope. Hence, samples were streaked on 7% sheep blood agar (Oxoid), the plates were incubated at 37 ºC for 24-48 h. Macroscopic and microscopic morphologies of the developed colonies were identified by commonly known biochemical methods. The following features of the bacteria isolated in this study were analyzed: oxidase, catalase, coagulase, oxidation/ fermentation-glucose (O/F), hemolysis, DNAase, pigment production, urease, mannitol fermentation (mannitol salt agar), maltose fermentation (purple agar+1% maltose), aesculine hydrolysis, and sensitivity to polymyxin B, novobiocin, bacitracin and furazolidone.

The end-product of polyunsaturated fatty acid peroxidation, MDA, reacting with thiobarbituric acid in serum samples was determined by the modification of Satoh (14) and Yagi (15). The values of MDA reactive material were expressed in terms of MDA (nmol / ml plasma).

Erythrocyte GSH-Px activity was measured by the method of Beutler (16) in which cumene hydroperoxide was used as substrate. Oxide glutathione (GSSG) produced by the action of erythrocyte GSH-Px and cumene hydroperoxide, was reduced by glutathione reductase (GSH-az) and NADPH. The decrease in the concentration of NADPH was measured at 340 nm. The enzyme activity was expressed as units per g of Hb (U/ g Hb).

Additionally, blood was taken from the jugular vein for blood serum, was centrifuged at 3000 g for 30 min, and serum was acquired and kept at –20 ºC until analyzed. Levels of the following were examined in the blood serum: glucose, cholesterol, triglyceride, total protein, albumin, sodium (Na), potassium (K), chloride (Cl), calcium (Ca), total bilirubin, alanine transaminase (ALT), aspartate transaminase (AST), urea and creatinine in an autoanalyzer (Beckman Coultur Ix20).

Student's t test was used to determine differences between groups by Minitab for Windows (17).

Results

In clinical examination, the mastitic udders in the ewes were swollen, hot, hard and highly painful, milk production was sharply decreased and breathing and body heat were increased. The affected udder lobes were gangrened entirely, were bluish or black, and the skin came off in some.

In incubation, bright, hemolytic colonies gold-yellow in color and 3-4 in diameter were seen in 7% sheep blood agar, and Gram-positive cocci were observed in the samples. The microorganism was identified by known biochemical tests to be *S. aureus*.

Mean values of investigated parameters and differences between with gangrenous mastitic and healthy ewes are presented in tables 1,2. In the blood of the gangrenous mastitic animals, erythrocyte GSH-Px activities and plasma MDA levels were significantly ($P < 0.01$ and $P < 0.001$) higher than in the healthy animals.

The levels of Na, K, Ca, glucose and albumin were significantly ($P < 0.05$, $P < 0.001$) lower in the ewes with gangrenous mastitis. However, serum levels of creatinine and total bilirubin were significantly ($P < 0.01$ and $P < 0.05$) increased in gangrenous mastitic animals than healthy controls.

Other parameters considered within this trial (ALT, AST, Cl, cholesterol, triglyceride, total protein, urea) did not show statistically valuable differences between the groups.

Discussion

To our knowledge, there are no studies related directly to mastitis, gangrenous mastitis and antioxidant enzymes in ewes. Therefore, due to the lack of references about gangrenous mastitis and antioxidant metabolism in ewes, we will discuss the results of our study in the light of mastitis in cows.

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, and $OH^{\cdot}$ (10,12). GSH-Px is one of intracellular defense system against free radicals.

Atroshi et al. (18,19) reported GSH-Px activity in cows with mastitis to be very low. Ndiweni et al. (13) also showed that there was a negative correlation between the incidence of mastitis and GSH-Px activity in the cows in England, that is, the lower the GSH-Px activity the higher the incidence of mastitis. In cows, there is generally no decrease in appetite and gangrene is not seen in clinic mastitis, except in the peracute ones. The disease in ewes, however, causes severe general and systemic damage, ceases appetite, and the incidence of gangrenous mastitis by staphylococci is higher (4). Insufficient nutrition due to low appetite leads to a complex and serious effect in the free radical defense mechanism (20). One of the most important effects of hungriness is the decrease in GSH levels in the liver and other tissues (21). As a result, although it has been shown (21) that lower level of substrate (reduced glutation) might decrease the GSH-Px activity, Gaal et al. (22) have indicated that hungriness in ewes increases the erythrocyte GSH-Px activity.

In this research, erythrocyte GSH-Px activities of the ewes with gangrenous mastitis and healthy ones were 95.0 ± 5.10 U/gHb and 62.0 ± 6.00 U/gHb, respectively, revealing a statistically significant difference ($P < 0.01$) (Table 1). This might be due to the need of high GSH-Px activity for high level oxidant damage occurring because of inflammatory reactions in the mammary gland tissue or insufficient nutrition of the ewes with gangrenous mastitis. Eventually, defense activity of the animal is 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 related with mastitis (13,18). In our study, plasma MDA levels (2.81 ± 0.16 nmol/ml) in the ewes with gangrenous mastitis were significantly higher than those (1.62±0.17 nmol/ml) in healthy ones ($P < 0.001$) (Table 1). One

Table 1. The results of plasma MDA and erythrocyte GSH-Px levels, from both gangrenous mastitic and healthy ewes.

	Gangrenous mastitic ewes n: 20	Healthy ewes n: 20	P
GSH-Px (U/gHb)	95.0 ± 5.10	62.0 ± 6.00	**
MDA (nmol/ml)	2.81 ± 0.16	1.62 ± 0.17	***

**: $P < 0.01$ ***: $P < 0.001$

cause of this might be the decrease in food consumption. As a matter of fact, Gaal et al. (23) have reported that hungriness or insufficient nutrition in ruminants has an important influence in the level of the blood lipid peroxidation and lack of energy increases the blood plasma levels of MDA in cows.

Atroshi et al. (18) determined the levels of MDA in cows with mastitis to be 38-44% higher than those in healthy cows. The present results suggest that increasing levels of oxidant stress in gangrenous mastitis might have an essential role in the process of the inflammation and tissue damage.

Studies in cows with clinic mastitis (24,25) have shown the levels of glucose to be numerically high but in normal levels. On the other hand, clinic mastitis in ewes is the cause of high fever and decrease in food consumption (3), thus leading to hypoglycemia (26). In this study, glucose levels (15.81 ± 1.50 mg/dl) in the animals with gangrenous mastitis were statistically lower than those (58.75 ± 2.30 mg/dl) in the healthy ones ($P < 0.001$) (Table 2). This is because of the hypoglycemia following hungriness, as reported in the literature (3,26).

Some researchers (27-29) said that levels of Na and K in cows with mastitis were not significantly different compared to those in healthy ones despite tending to decrease. Some (18,30,31) also indicate no difference between those. In contrast, Risvanli et al. (25) reported the Na and K levels in cows with clinic mastitis to be significantly low. The levels of Na and K in the ewes with mastitis in this research were also significantly low. This result on Na is in parallel to the findings of Risvanli et al. (25).

Studies (28,32) have observed a decrease in serum total Ca level in cows with mastitis while Wegner and Stull (29) found an increase in that. Rişvanlı et al. (24), however, showed no difference. The results of the present study were parallel to the literature (28,32), in which the total Ca level (8.62±0.29 mg/dl) in the ewes with mastitis was significantly lower than that (10.70±0.26 mg/dl) of healthy ones ($P < 0.001$) (Table 2). Similarly, serum albumin level in the ewes with mastitis showed a decreased tendency, in agreement with the literature (28,33). Insufficient nutrition is reported to possibly lower the albumin synthesis (26). In this research, similar results were obtained on the serum albumin levels, which were 0.98±0.02 g/dl in the ewes with mastitis and 1.23±0.03 g/dl in the healthy ones ($P < 0.001$) (Table 2).

Table 2. Some biochemical parameters from gangrenous mastitic and healthy ewes.

	Gangrenous mastitic ewes n: 20	Healthy ewes n: 20	P	Physiological levels (+)
Na (mmol/l)	145.10 ± 0.91	148.88 ± 1.0	*	141.6-159.6
K (mmol/l)	5.21 ± 0.15	5.83 ± 0.19	*	4.3-6.3
Cl (mmol/l)	103.20 ± 0.94	104.75 ± 1.20		100.8-113.0
Ca (mg/dl)	8.62 ± 0.29	10.70 ± 0.26	***	9.3-11.7
Glucose (mg/dl)	15.81 ± 1.50	58.75 ± 2.30	***	44.0-81.2
Cholesterol (mg/dl)	62.30 ± 5.50	52.90 ± 4.40		44.1-90.1
Triglyceride (mg/dl)	12.16 ± 2.20	11.00 ± 1.70		
T.Protein (g/dl)	7.96 ± 0.24	8.23 ± 0.23		5.9-7.8
Albumin (g/dl)	0.98 ± 0.02	1.23 ± 0.03	***	2.7-3.7
Urea (mg/dl)	56.9 ± 8.2	59.6 ± 5.1		
Creatinine (mg/dl)	1.14 ± 0.14	0.65 ± 0.02	**	0.9-2.0
T.Bilirubin (g/dl)	0.37 ± 0.04	0.25 ± 0.02	*	0.0-0.5
ALT (IU/l)	14.33 ± 0.90	17.33 ± 1.40		14.8-43.8
AST (IU/l)	194.00 ± 25.0	132.90 ± 22.0		49.0-123.3

(+): Physiological levels were kindly obtained from Merck Manual (34).
*: $P < 0.05$ **: $P < 0.01$ ***: $P < 0.001$

The level of serum Ca is said to lessen as the level of serum albumin is low (26). The present findings appear to support this fact clearly.

Total bilirubin in cows with mastitis has been determined to increase in general (24,25,33). Likely, the results in this study showed that the level of total serum bilirubin (0.37±0.04 g/dl) in the ewes with mastitis was significantly higher than that (0.25±0.02 g/dl) of healthy animals ($P < 0.05$) (Table 2).

Rişvanlı et al. (25) found the level of serum creatinine in cows with mastitis not to change while Katholm et al. (28) reported that it increased in the dead cows with mastitis. Similarly, our results revealed that it was significantly higher in the sick animals than healthy ones ($P < 0.01$). This increase is thought to be due to severe necrosis in the udder tissue, which possesses very rich smooth muscle (26).

Additionally, the fact that there were, in biochemical analyses, no differences between the sick and healthy animals in terms of the levels of Cl, triglyceride, total protein, urea, ALT, and AST is similar to the literature (18,25,28). Although cholesterol levels in cows with mastitis have been reported to decrease (24,25), this study done the ewes with gangrenous mastitis revealed no difference on the cholesterol level compared to that in healthy animals.

In summary, even though the levels of glucose, Ca and albumin were low in the ewes with mastitis this decline is thought to have no use in diagnosing the disease because it is due to insufficient nutrition during the disease process. Some biochemical changes in the gangrenous mastitis in ewes, though, might be because of the severe damage in the udder tissue, and this might be one of the vital factors in deteriorating the antioxidant defense system (GSH-Px, MDA) on disease pathogenesis.

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