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Determination of plasma fibrinogen and haptoglobin, hematological and blood biochemical changes in Bulgarian local goats with experimentally induced *Staphylococcus aureus* mastitis

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Abstract: The aim of the present study was to assess changes in acute phase proteins fibrinogen and haptoglobin, as well as hematological, blood biochemical, and microbiological alterations in local Bulgarian goats with experimentally induced *Staphylococcus aureus* mastitis. The experiments were conducted with 6 clinically healthy local Bulgarian goats, 2 to 6 years of age, weighing 46–57 kg. The experimental infection was done with a stationary phase culture of a field *Staphylococcus aureus* strain isolated from a cow with clinical mastitis. Blood haptoglobin in goats was considerably higher ($P < 0.05$) as early as 8 h after the pathogen's inoculation. The most significant differences from baseline values occurred 24 and 48 h ($P < 0.01$) after infection. Plasma fibrinogen increased significantly ($P < 0.05$) 8 h after infection and reached the highest mean concentration (9.12 g/L) by 72 h. Leukocytosis was established as early as 4 h after the experimental infection when leukocyte counts were significantly higher than baseline ($P < 0.001$). The analysis of results indicates that in experimentally induced *Staphylococcus aureus* mastitis, significantly higher ($P < 0.05$) levels of the leukocyte, fibrinogen, and haptoglobin are established as early as 8 h after the infection.

Key words: Acute phase proteins, goats, mastitis

1. Introduction

Acute phase proteins (APPs) are a group of blood proteins whose concentrations vary under external or internal influences such as inflammation, surgical trauma, or stress (1). Positive APPs (haptoglobin, C-reactive protein, serum amyloid A, ceruloplasmin, fibrinogen, and alpha-1-acid glycoprotein) are elevated in response to inflammation. In contrast, negative APPs reduce their levels in blood in response to inflammation; they include the proteins albumin and transferrin (2).

APPs are important early diagnostic markers of inflammation in animals (3). This thesis is supported by numerous recent studies on their concentrations in various experimental inflammation models (4–8).

González et al. (1) reported the blood levels of APP haptoglobin (Hp), serum amyloid A, acid soluble glycoprotein, fibrinogen, and albumin in goats with inflammation induced by subcutaneous turpentine oil application.

Blood APP analysis has been performed during the first postpartum days (9) in goats with experimentally induced pregnancy toxemia (10) as well in goats with viral arthritis encephalitis (11).

There is no information about the blood levels of fibrinogen and Hp in experimentally induced staphylococcal mastitis in local Bulgarian goats.

The aim of the present study was to assess changes in fibrinogen and Hp as well as the hematological, blood biochemical, and microbiological alterations in local Bulgarian goats with experimentally induced *Staphylococcus aureus* mastitis.

2. Materials and methods

2.1. Experimental animals

The experiment was approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine, Trakia University, Stara Zagora, in compliance with Ordinance

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No. 20 of the Ministry of Agriculture and Food from 1 November 2012 on the minimum requirements for the protection and welfare of experimental animals and the requirements for the utilization, rearing, and/or delivery facilities. The experiments were conducted with 6 clinically healthy local Bulgarian goats about 2 to 6 years old, weighing 46-57 kg. The animals were reared in the facilities of the Productive Animal Clinic of the Faculty of Veterinary Medicine, Stara Zagora. The goats were in the sixth lactation months, third to fifth lactation, and were milked manually twice a day (in the morning and in the evening).

2.2. Clinical examination of the udder and microbiological monitoring of infection

The clinical examination of the udder included inspection, palpation, and a California mastitis test of the milk. In this experiment, a stationary phase culture of a field *Staphylococcus aureus* strain obtained from the milk of a cow with clinical mastitis was used. The infection dose was standardized using the first tube of McFarland's standard at 1.5×10^8 cfu/mL. The staphylococcal strain was cultivated on blood agar (Trypticase-soy agar base, Difco, supplemented with 7% sheep erythrocytes) at 37 °C for 24 h and the stationary growth phase was used for preparation of bacterial culture for inoculation. One milliliter of standardized bacterial culture was administered intracisternally into one-half of udders of the 6 experimental goats. The other udder halves served as negative control; they were treated with 1 mL of saline solution. Microbiological monitoring of infection was performed 6, 24, 48, 72, 120, 144, and 168 h after infection. For bacteriological examination for the presence of staphylococci, milk samples were collected from infected and control udder halves of all goats.

2.3. Hematological assays and blood biochemistry

Blood was sampled from *v. jugularis externa* after immobilizing the animals, shaving their hair, and scrubbing their skin with 5% iodine in sterile vacutainers. Complete blood counts were determined with an automated hematological analyzer Exigo EOS Vet (Exigo, Sweden), and blood biochemical parameters were assessed by an automated biochemical analyzer BS 120 (Mindray, China). All samples were assayed in the Laboratory Diagnostic Center of the Faculty of Veterinary Medicine, Stara Zagora. Hematological reference ranges reported by Byers and Kramer (12) were used, whereas those described by Kaneko et al. (13) were used for blood biochemical parameters. The studied parameters were determined 0, 4, 8, 24, 48, 72, 96, and 168 h after infection.

2.4. Analysis of blood plasma fibrinogen and Hp

Plasma fibrinogen concentrations were determined by the nephelometric method of Podmore (14). To 0.25 mL of plasma was added 2.5 mL of 10.5% Na_2SO_4. A blank sample consisting of 0.25 mL of plasma and 2.5 mL of 0.9% sodium chloride was simultaneously run. The absorbance was measured after 3 min at 570 nm. The result was calculated using a standard curve prepared with different dilutions of plasma. Fibrinogen analysis was done in the biochemistry unit lab of the Faculty of Veterinary Medicine, Stara Zagora. Plasma Hp was assayed by sandwich ELISA in the lab of the Department of Veterinary Science and Public Health, University of Milan (Italy). APPs were measured 0, 4, 8, 24, 48, 72, 96, and 168 h after infection.

2.5. Treatment of experimental animals

After performing antibiogram testing of the used staphylococcal strain, the animals were treated with antibiotics from infection hour 96 onward using an intramammary injector (Mastijet fort, Intervet, Holland), applied intracisternally 3 times at 12-h intervals.

2.6. Statistical analysis

Data were processed using StatSoft (Statistica 7, Microsoft Corp. 1984–2000 Inc.) statistical software and presented as means and standard deviations (mean ± SD). Differences were considered statistically significant at $P \leq 0.05$.

3. Results

3.1. Clinical data

The clinical examination of udders of all goats showed that infected halves were swollen, warm, painful, and hyperemic. These alterations were most pronounced at 48 and 72 h postinfection. The collected milk samples showed coagula, first appearing at 48 h postinfection and more frequent at 72 h. The California mastitis test detected a strong positive reaction in all goats 48 h after pathogen inoculation.

3.2. Hematological and blood biochemistry results

The studied hematological parameters (mean ± SD) are presented in Table 1. The analysis showed leukocytosis ($15.9 \pm 1.53 \times 10^3/\mu L$) as early as 4 h after the inoculation and a statistically significant difference ($P < 0.001$) from the preinfection counts. Leukocyte counts were also considerably higher ($P < 0.01$) 8, 24, and 48 h after infection. The trend continued at 72 and 96 h ($P < 0.001$). On the third day after the beginning of the treatment (168 h), leukocyte counts were within the normal reference range. The other hematological parameters did not change substantially over the trial's duration as compared to hour zero.

Blood biochemical analysis included glucose, total protein, albumin, aspartate aminotransferase, alanine aminotransferase, urea, creatinine, cholesterol, and triglycerides (Table 2). Throughout the entire experimental period, these analytes were within the respective reference ranges.

Table 1. Hematological parameters (mean ± SD) in goats during the course of the experiment (n = 6).

Parameter	Hours after the infection							
	0 h	4 h	8 h	24 h	48 h	72 h	96 h	168 h
Hemoglobin, g/L	83.5 ± 8.26	81.0 ± 7.95	87.5 ± 8.8	88.5 ± 4.46	88.5 ± 7.66	92.0 ± 8.41	84.4 ± 8.32	89.0 ± 4.38
Hematocrit, %	24.05 ± 2.99	23.73 ± 3.76	25.83 ± 3.69	26.05 ± 2.51	25.18 ± 3.49	25.9 ± 3.22	24.7 ± 3.32	26.03 ± 3.0
Erythrocytes, $\times 10^6$ cells/μL	15.58 ± 1.72	14.94 ± 1.23	14.88 ± 1.67	15.79 ± 0.92	15.43 ± 1.4	15.39 ± 1.31	15.62 ± 1.2	14.03 ± 0.7
Leukocytes, $\times 10^3$ cells/μL	11.13 ± 1.46	15.9 ± 1.53 ***	18.11 ± 4.86 **	18.17 ± 5.53 **	19.58 ± 6.5 **	15.8 ± 1.66 ***	15.62 ± 1.84 ***	8.78 ± 2.44
MCV, fL	17.57 ± 1.27	16.83 ± 1.0	17.37 ± 1.03	17.87 ± 1.06	17.05 ± 0.93	17.02 ± 0.89	18.02 ± 0.96	17.27 ± 0.92
MCH, pg	5.97 ± 0.5	5.77 ± 0.38	5.98 ± 0.43	5.83 ± 0.6	5.75 ± 0.43	5.87 ± 0.48	5.93 ± 0.52	5.95 ± 0.66
MCHC, g/L	345.3 ± 16.1	335.8 ± 18.6	362.8 ± 24.6	356.7 ± 13.3	346.3 ± 13.9	347.8 ± 14.9	346.4 ± 13.6	345.4 ± 19.34

Differences compared to hour 0 (before infection) are statistically significantly different at ** $P < 0.01$; *** $P < 0.001$.

Table 2. Blood biochemical parameters (mean ± SD) in goats during the course of the experiment (n = 6).

Parameter	Hours after the infection							
	0 h	4 h	8 h	24 h	48 h	72 h	96 h	168 h
Glucose, mmol/L	3.44 ± 0.6	3.04 ± 0.4	3.32 ± 0.4	3.56 ± 0.4	3.57 ± 0.34	3.83 ± 0.7	3.64 ± 0.4	3.08 ± 0.28
Total protein, g/L	76.4 ± 5.9	75.06 ± 3.47	76.15 ± 4.1	77.5 ± 3.01	76.42 ± 3.84	74.85 ± 4.74	76.08 ± 4.6	75.63 ± 3.89
Albumin, g/L	37.6 ± 1.8	38.35 ± 1.88	38.67 ± 1.7	38.77 ± 1.43	37.98 ± 1.81	37.6 ± 2.29	38.74 ± 1.58	37.93 ± 2.0
AST, U/L	92.0 ± 22.08	71.83 ± 16.31	75.67 ± 18.06	71.83 ± 15.06	70.0 ± 11.4	69.33 ± 12.4	72.82 ± 11.4	73.83 ± 5.56
ALT, U/L	24.83 ± 2.93	23.5 ± 3.83	22.5 ± 2.07	25.33 ± 3.38	25.67 ± 2.5	26.5 ± 2.34	25.7 ± 2.5	26.67 ± 2.34
Urea, mmol/L	7.68 ± 0.66	8.05 ± 1.99	7.63 ± 0.98	6.02 ± 0.93	6.22 ± 1.9	7.68 ± 1.29	7.82 ± 1.32	7.28 ± 1.09
Creatinine, μmol/L	70.17 ± 4.83	68.0 ± 4.19	62.67 ± 5.05	64.67 ± 5.43	64.67 ± 4.84	58.17 ± 4.4	65.27 ± 4.8	68.0 ± 7.04
Cholesterol, mmol/L	1.71 ± 0.27	1.59 ± 0.15	1.58 ± 0.14	1.65 ± 0.21	1.59 ± 0.11	1.53 ± 0.11	1.62 ± 0.12	1.24 ± 0.2
Triglycerides, mmol/L	0.19 ± 0.09	0.17 ± 0.06	0.09 ± 0.03	0.15 ± 0.1	0.13 ± 0.05	0.12 ± 0.07	0.15 ± 0.06	0.11 ± 0.04

3.3. Blood plasma fibrinogen and Hp concentrations

Preinfection fibrinogen concentration was 3.2 ± 0.69 g/L. By 4 h after infection, the levels were higher, although not significantly different compared to the baseline; 8 h after infection, fibrinogen was substantially elevated ($P < 0.05$). The highest level of statistically significant differences were established at infection hours 24 and 48 (Table 3).

Hp concentrations also differed considerably ($P < 0.05$) from the baseline by 8 h, and the difference became more consistent during the subsequent sampling intervals ($P < 0.01$).

3.4. Microbiological findings

Before the start of the experiment (hour 0), bacteriological examination of milk samples from both udder halves of experimental goats was performed and no bacterial cells were isolated. During the microbiological monitoring, the bacterial pathogen was reisolated 6, 24, 48, and 72 h after the intramammary application of the bacterial culture from respective udder halves. Reisolation of *Staphylococcus aureus* (1.2×10^6 cfu/mL) was achieved as early as infection hour 6, and this bacterial load was maintained by 24 and 72 h (Table 4). All control udder halves were bacteriologically negative throughout the entire period of the study. After initiation of therapy at infection hour 96, bacterial findings from both udder halves were negative.

4. Discussion

The occurrence of clinical and subclinical mastitis in goats is the main factor that contributes to decreased milk production. The prevailing mastitis pathogens in goats

Table 3. Blood concentrations of studied acute phase proteins (mean ± SD) in goats during the course of the experiment (n = 6).

Acute phase protein	Hours after the inoculation							
	0 h	4 h	8 h	24 h	48 h	72 h	96 h	168 h
Fibrinogen, g/L	3.2 ± 0.69	5.17 ± 2.81	7.37 ± 4.15 *	8.87 ± 4.87 **	8.33 ± 4.26 **	9.12 ±5.34 *	7.04 ± 4.48 *	3.51 ± 0.45
Haptoglobin, g/L	3.05 ± 1.08	3.55 ± 1.08	4.48 ± 0.97 *	4.73 ± 0.83 **	4.75 ± 0.51 **	4.27 ± 1.23 *	4.32 ± 1.05 *	3.43 ± 0.59

Differences between hour 0 and the other sampling times are statistically significantly different at * $P < 0.05$; ** $P < 0.01$.

Table 4. Results from bacteriological cultures of milk samples collected from the infected udder halves of the experimental goats.

Goat no.	Hours after *Staphylococcus aureus* inoculation							
	0 h	6 h	24 h	48 h	72 h	120 h	144 h	168 h
1	-	+++	+++	+++	++	-	-	-
2	-	+++	+++	+++	++	-	-	-
3	-	+++	+++	+++	+	-	-	-
4	-	+++	+++	+++	++	-	-	-
5	-	+++	+++	+++	++	-	-	-
6	-	+++	+++	+++	+++	-	-	-

Legend: (-) Negative bacteriological finding.
(+) 1.0×10^2 cfu/mL.
(++) 1.0×10^4 cfu/mL.
(+++) 1.2×10^6 cfu/mL.

are staphylococci. The main microbial agent involved in clinical forms is *Staphylococcus aureus*, while in subclinical mastitis it is coagulase-negative staphylococci (15).

The analysis of results from goats with experimentally induced *Staphylococcus aureus* mastitis demonstrated statistically significant changes in leukocyte counts, fibrinogen, and Hp, and reisolation of *Staphylococcus aureus* between 6 and 72 h after inoculation.

All animals included in this study showed clinical signs of acute mammary gland inflammation. According to the literature, one of the main factors contributing to the development of experimental clinical mastitis is the dose of the bacterial strain. The dose used in this study was 1.5×10^8 cfu/mL, whereas Lievaart-Peterson and Chousalkar (16) induced subclinical *Staphylococcus aureus* mastitis in goats with inoculation of a dose of approximately 58 cfu/mL. Lasagno et al. (17) applied a dose comparable to ours (1.7×10^8 cfu/mL) of *Streptococcus uberis*, and Jing et al. (18) induced acute colimastitis in goats with 3×10^3 cfu/mL *Escherichia coli*. In our opinion, our successful experimentally induced mastitis was mainly due to the high dose used for inoculation. The lack of risk factors leading to the occurrence of mastitis in goats used in the experiment was the main motive to use this high dose for infection.

In experimental goats, leukocytosis was detected as early as 4 h after infection. The leukocyte counts were significantly ($P < 0.001$) higher compared to the levels at hour 0. Unlike us, Lievaart-Peterson and Chousalkar (16) did not observe differences in white blood cell counts between control goats and goats experimentally infected with low doses of *Staphylococcus aureus*. According to Paape and Capuco (19), the first line of udder defense consists of neutrophil leukocytes, which migrate rapidly into the udder in response to inflammation. Considerably elevated leukocyte counts compared to the baseline ones ($P < 0.001$) were observed until 96 h after the reproduction of experimental mastitis.

The quantitation of APPs in blood plasma or serum is of exceptionally high diagnostic value for the detection, prediction, and monitoring of inflammations in many animal species (4). The production of APPs in the bovine udder in response to bacterial mastitis provides evidence for the potential of their quantitative determination in detection of economically relevant diseases in large ruminants, especially in automated milking production systems (20).

The analysis of blood Hp concentrations in goats revealed substantially ($P < 0.05$) increased values as early as 8 h after the intracisternal application of the inoculum. At

hours 24 and 48 of infection, the differences from baseline concentrations were the most pronounced ($P < 0.01$). According to González et al. (1), there are no reference values or standards for APPs in goats, and hence the analytical precision is evaluated indirectly from linearity assays. Of the positive APPs, Hp and fibrinogen belong to the group of moderate APPs, whose concentrations increase 2 to 10 times during the response to inflammation (4).

In contrast to our data, Eckersall et al. (21) have reported lower values of blood and milk Hp in cows with subclinical experimental *Staphylococcus aureus* mastitis. In our opinion, this could be attributed to the inoculation dose (0.5×10^4 cfu/mL), which caused a subclinical form of inflammation.

González et al. (1) observed the highest average values of Hp on the third day of inflammation induced by subcutaneous application of turpentine oil in goats, which also differ significantly from our results.

According to Kaneko et al. (13), blood fibrinogen in goats ranges between 1.0 and 4.0 g/L. In our study, significantly ($P < 0.05$) higher fibrinogen concentrations were measured 8 h after infection of the goats, while the highest mean value (9.12 g/L) of this acute phase protein was registered at 72 h. Comparable average fibrinogen levels were reported by González et al. (1) in goats (9.6 g/L). El-Deeb (22) reported significantly lower levels of fibrinogen (3.84 ± 0.21 g/L) in goats with gangrenous mastitis.

In conclusion, the analysis of results indicates that in experimentally induced *Staphylococcus aureus* mastitis significantly higher ($P < 0.05$) levels of leukocytes, fibrinogen, and Hp are established as early as 8 h after the infection. Their determination has an important diagnostic value as an early marker of acute inflammation of the mammary gland in goats caused by *Staphylococcus aureus*.

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