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Hematological and Serum Biochemical Analyses in Experimental Caprine Besnoitiosis

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Abstract: This study was undertaken to investigate the hematological and biochemical changes in experimentally infected goats with *Besnoitia caprae* from the time of infection till 360 days post-infection (PI). Six male goats were inoculated subcutaneously with 13×10^7 bradyzoites of *B. caprae*, and blood samples were collected from the jugular vein. The total erythrocyte and total leukocyte counts, hematocrit value, and differential leukocyte counts were determined. Serum biochemical analysis, including the total protein, albumin, total globulin, cholesterol, triglyceride, chloride, testosterone, calcium (Ca^{2+}), inorganic phosphorus, sodium (Na^+), potassium (K^+), iron (Fe^{2+}), glucose, serum amyloid A (SAA), haptoglobin (Hp), fibrinogen, ceruloplasmin, aspartate aminotransferase, alanine aminotransferase, creatine kinase, lactate dehydrogenase, and alkaline phosphatase, was undertaken. Skin biopsy from the limbs were collected at weekly intervals and histologically examined for *Besnoitia* cysts. Cysts were present in the skin biopsies of the leg of the infected goats from day 28 PI. There were variations in hematological analyses, but no significant difference was seen. From day 30 to 360 PI, results showed that SAA, Hp, fibrinogen, and ceruloplasmin concentrations increased, whereas testosterone concentrations decreased. Infected goats exhibited decrease of albumin and increase of serum total protein and globulin concentrations. By contrast, there were no significant differences in the remained analyses concentrations.

Key words: *Besnoitia caprae*, hematological parameter, serum biochemical analysis, goat

INTRODUCTION

Besnoitiosis is a disease of both domestic and wild animals with a worldwide distribution [1-4]. Parasites of the genus *Besnoitia* are classified in the subfamily Toxoplasmatinae of the phylum Apicomplexa [5]. Species of the coccidian genus *Besnoitia* parasitize cattle, goats, equids, reindeer, caribou, opossums, rabbits, rodents, and lizards [3,6]. Clinical besnoitiosis in goats have been reported in wild and domestic goats in Iran [1,7] and Kenya [2,8]. Its extensive skin damage criteria, together with condemnation of infected carcass organs and its adverse effects on growth and weight gain in association with reduction in male fertility, make this disease of great economic concern to the mutton and leather industries and animal breeders [5,7-10]. Besnoitiosis causes severe metabolic disturbances in various organs of the intermediate hosts and may affect enzymatic activities of lactate dehydrogenase (LDH), alkaline phosphatase (ALP), alanine aminotransferase (ALT), creatine kinase (CK), aspartate aminotransferase (AST) as well as immunoglobulin concentrations and serum minerals Na^+, K^+, Ca^{++}, and phosphorus concentration.

Although the changes in biochemical and hematological analyses in the naturally infected domestic goats by *Besnoitia caprae* were studied previously [11,12], no report is yet available on comparison of these changes at different time intervals after infection. Therefore, the present experimental study was conducted to provide information on the hematological and biochemical changes in experimentally infected goats with bradyzoites of *B. caprae* from early infection to 1 year post-infection (PI).

MATERIALS AND METHODS

Isolation of bradyzoites

Goats with naturally occurring chronic besnoitiosis were identified by observing *Besnoitia* cysts in their conjunctiva and

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subsequent confirmation on histopathological sections of the skin covering the tarsal or carpal area (Fig. 1) [7]. Two naturally infected goats, exhibiting clinical features of caprine besnoitiosis were culled and then painlessly euthanized according to the Iranian Veterinary Organization rules. The skin and subcutaneous tissues from these animals were collected and stored at 4℃, and transported to the laboratory. To remove surface contaminations, tissue pieces were separately washed in a petri dish containing Dulbecco's modified eagle medium (DMEM) supplemented with 2% fetal calf serum (FCS), 1% antibiotic solution (10,000 IU penicillin and 10,000 mg streptomycin/ml solution) and 1% amphotericin B (250 mg/ml). Subsequently, the tissue sections were cut into 1-2 mm^3 pieces with scalpel. The liberated bradyzoites was collected by spinning the DMEM at 770 *g* for 15 min at 4℃. The pellets were resuspended in DMEM and the *B. caprae* bradyzoites were counted using a Neubauer chamber. The bradyzoites were cryopreserved by resuspending in FCS containing 10% DMSO, and stored in liquid nitrogen.

Infection of goats

Six male goats approximately 12-16 months old, from a non-infected area, carefully examined to be free of *Besnoitia* cysts in the scleral conjunctiva and in the biopsy from the carpal skin, were used in this study. No *Besnoitia* cysts were reported from at least 120 km surrounding the area in which the male goats were collected. Each goat was inoculated subcutaneously (SC) with approximately 13×10^7 bradyzoites of *B. caprae*. The goats were located in separate boxes, each in a different room, and the animals were observed daily for development of clinical besnoitiosis. Skin biopsies from the distal parts of the limbs were collected at weekly intervals and histologically examined for *Besnoitia* cysts. Five healthy goats were bou–ght from the herd and were used as controls.

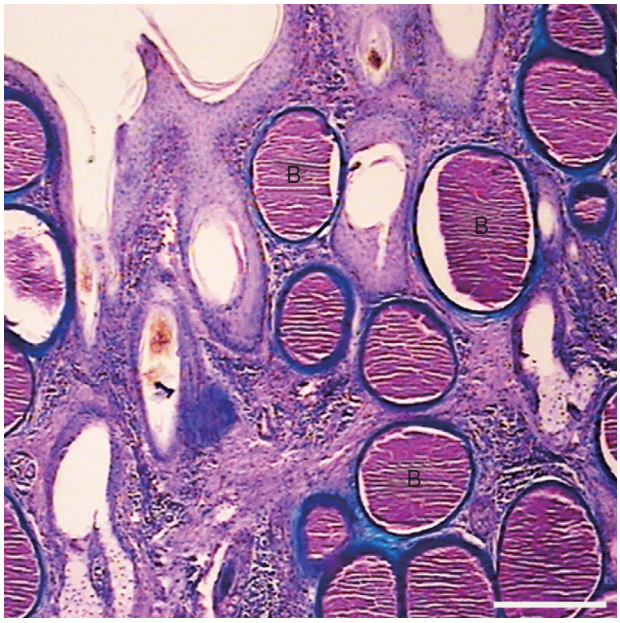

Fig. 1. *Besnoitia caprae* tissue cysts (B) in the skin of distal parts of the leg in an infected goat. Alcian blue/PAS. ×180.

Hematological and biochemical analyses

Blood samples were collected at weekly intervals from the jugular vein of the goats. For hematological analyses, blood samples were collected in vacutainers containing EDTA as an anticoagulant. For the serum biochemical analysis, blood samples were collected into vacutainers without anticoagulant, and serum was separated by centrifugation at 750 *g* for 15 min and stored in a freezer at -20℃ until use. Hematological analyses, including the total erythrocyte count, total leukocyte count, and hematocrit value, was determined using standard routine techniques. For differential leukocyte counts, blood smears were prepared and stained with Giemsa [13].

The total protein was measured by the Biuret method, albumin by the bromcresol green method, the total globulin by the difference of total protein and albumin, and cholesterol by a modified Abell-Kendall/Levey-Brodie (A-K) method. Triglyceride was measured by the enzymatic procedure of McGowan et al. [14], chloride by the mercury chloride colorimetric method, testosterone by a solid phase ELISA (DRG Instruments GmbH, Division of DRG International Inc, Marburg, Germany), and total calcium by the orthocresolphthalein method, inorganic phosphorus by the ammonium molybdate method. Sodium and potassium were measured by the flame photometric method (Flame Photometer - CL 26D - ELICO, UK), iron by atomic absorption spectrophotometer (Shimadzo AA-670, Kyoto, Japan), glucose by the glucose oxidase method, AST and ALT activities by the colorimetric method of Reitman and Frankel, CK by the Sigma colorimetric (modified Hughes) method, LDH by the Sigma colorimetric (Cabaud–Wroblewski) method, and ALP by the modified method of Bowers and McComb.

All the enzyme activities were measured at 37℃ and the results were presented in units per liter [15]. Biochemical analyses were measured using a standard autoanalyser with veterinary software (Cobas-Mira, ABX-Diagnostics, Tokyo, Japan).

Table 1. Hematological values (mean ± SD) in goats

Parameter	Days post-infection						
	Control	30	60	120	180	270	360
RBC ($\times 10^{12}$/l)	11.17 ± 1.35	10.70 ± 1.32	6.12 ± 0.37	8.93 ± 1.96	9.09 ± 1.68	7.52 ± 1.13	8.27 ± 1.49
PCV (l/l)	0.28 ± 0.02	0.28 ± 0.01	0.19 ± 0.01	0.22 ± 0.02	0.23 ± 0.01	0.24 ± 0.01	0.24 ± 0.02
WBC ($\times 10^9$/l)	9.72 ± 2.94	7.71 ± 1.72	12.74 ± 1.88	8.69 ± 0.85	9.04 ± 3.76	9.37 ± 4.98	11.80 ± 4.98
Segmented ($\times 10^9$/l)	3.24 ± 1.29	4.77 ± 1.64	7.47 ± 0.84	4.79 ± 0.61	4.89 ± 0.43	5.57 ± 2.62	7.50 ± 1.25
Lymphocyte ($\times 10^9$/l)	5.31 ± 1.16	2.61 ± 0.99	4.69 ± 0.73	3.32 ± 0.25	3.88 ± 0.92	3.38 ± 1.53	4.04 ± 0.80
Monocyte ($\times 10^9$/l)	0.63 ± 0.48	0.19 ± 0.08	0.24 ± 0.01	0.11 ± 0.03	0.11 ± 0.04	0.11 ± 0.05	0.12 ± 0.06
Eosinophil ($\times 10^9$/l)	0.43 ± 0.28	0.00 ± 0.00	0.29 ± 0.08	0.39 ± 0.15	0.05 ± 0.10	0.22 ± 0.13	0.15 ± 0.17
Band ($\times 10^9$/l)	0.11 ± 0.06	0.14 ± 0.13	0.05 ± 0.02	0.08 ± 0.02	0.11 ± 0.14	0.09 ± 0.05	0.11 ± 0.10

Table 2. Serum biochemical values (mean ± SD) in goats

Parameter	Days post-infection						
	Control	30	60	120	180	270	360
Serum amyloid A[a] (mg/ml)	5.64 ± 1.67	5.93 ± 1.48	7.51 ± 2.93	8.54 ± 3.81	9.51 ± 4.48	11.26 ± 7.51	12.03 ± 8.22
Haptoglobin[a] (g/l)	0.10 ± 0.05	0.09 ± 0.01	0.09 ± 0.02	0.10 ± 0.02	0.11 ± 0.04	0.14 ± 0.09	0.13 ± 0.08
Fibrinogen[a] (g/l)	1.99 ± 0.07	2.01 ± 0.05	2.21 ± 0.24	2.22 ± 0.26	2.35 ± 0.29	2.47 ± 0.68	2.62 ± 0.82
Ceruloplasmin[a] (g/l)	0.06 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.08 ± 0.02	0.08 ± 0.02
Total protein[a] (g/l)	63.4 ± 3.3	60.5 ± 2.5	63.9 ± 1.7	64.0 ± 1.3	64.2 ± 3.4	66.7 ± 7.4	66.7 ± 7.2
Albumin[a] (g/l)	38.6 ± 2.4	36.5 ± 1.2	35.3 ± 1.2	34.2 ± 2.2	34.0 ± 3.1	34.4 ± 4.7	33.5 ± 4.8
Globulin[a] (g/l)	24.8 ± 3.1	24.0 ± 3.3	28.6 ± 3.0	28.6 ± 5.2	28.7 ± 5.4	32.3 ± 12.1	33.1 ± 12.1
Albumin/Globulin (A/G)	1.55 ± 0.18	1.54 ± 0.27	1.22 ± 0.15	1.16 ± 0.25	1.12 ± 0.27	0.97 ± 0.42	0.89 ± 0.40
Testosterone[a] (ng/ml)	3.11 ± 0.31	3.04 ± 0.34	2.92 ± 0.23	2.35 ± 0.73	2.27 ± 0.98	2.12 ± 0.85	2.16 ± 0.81
AST (U/l)	143.64 ± 21.25	131.7 ± 50.7	141.4 ± 29.3	141.0 ± 23.7	140.8 ± 20.1	142.1 ± 19.4	136.9 ± 13.4
ALT (U/l)	78.27 ± 4.19	76.7 ± 8.5	77.0 ± 3.11	73.8 ± 3.35	73.7 ± 3.90	71.02 ± 1.60	73.5 ± 3.10
ALP (U/l)	188.67 ± 39.17	190.7 ± 78.2	181.3 ± 29.8	176.0 ± 21.8	175.0 ± 22.8	146.0 ± 26.2	183.5 ± 34.7
CK (U/l)	129.43 ± 25.26	94.7 ± 76.6	127.1 ± 39.7	122.8 ± 28.4	123.1 ± 27.7	110.4 ± 15.6	166.9 ± 30.2
LDH (U/l)	381.59 ± 59.34	377.7 ± 96.8	367.0 ± 87.9	376.1 ± 51.1	384.0 ± 41.2	375.8 ± 32.9	370.9 ± 23.2
Glucose (mmol/l)	3.41 ± 0.13	3.24 ± 0.96	3.40 ± 0.23	3.45 ± 0.11	3.50 ± 0.04	3.11 ± 0.06	3.42 ± 0.22
Triglyceride (mmol/l)	0.33 ± 0.03	0.26 ± 0.04	0.32 ± 0.01	0.32 ± 0.01	0.32 ± 0.02	0.32 ± 0.03	0.33 ± 0.02
Cholesterol (mmol/l)	2.06 ± 0.22	2.02 ± 0.55	1.95 ± 0.01	1.95 ± 0.02	2.05 ± 0.14	1.98 ± 0.26	2.09 ± 0.13
Na^+ (mmol/l)	147.0 ± 3.8	146.2 ± 3.4	142.7 ± 3.8	144.0 ± 2.6	145.2 ± 2.2	145.4 ± 1.2	145.2 ± 1.4
K^+ (mmol/l)	4.89 ± 0.21	5.02 ± 0.66	4.99 ± 0.09	4.96 ± 0.14	4.96 ± 0.21	4.95 ± 0.20	5.08 ± 0.10
Ca^{2+} (mmol/l)	2.91 ± 0.09	2.75 ± 0.33	2.90 ± 0.03	2.81 ± 0.04	2.83 ± 0.05	2.81 ± 0.06	2.78 ± 0.10
P (mmol/l)	1.94 ± 0.08	1.82 ± 0.25	1.85 ± 0.05	1.88 ± 0.07	1.92 ± 0.03	1.92 ± 0.05	1.95 ± 0.03
Cl (mmol/l)	104.0 ± 4.60	100 ± 6.4	101.1 ± 4.8	101.3 ± 2.9	102 ± 3.4	103.6 ± 3.4	113.5 ± 22.8
Fe (μmol/l)	32.87 ± 3.11	29.66 ± 3.09	32.93 ± 3.18	32.62 ± 2.24	32.97 ± 2.14	32.38 ± 0.85	32.13 ± 2.09

AST, aspartate aminotransferase; CK, creatine kinase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase.
[a]Statistically significant difference ($P < 0.05$).

Hp was measured with a commercial colorimetric kit, and SAA by a solid phase sandwich ELISA (Tridelta Development Plc, Wicklow, Ireland). The analytical sensitivities of these tests in serum have been determined as 0.3 μg/ml for SAA and 0.0156 g/L for Hp by the manufacturer. Ceruloplasmin was measured using the Sunderman and Nomoto method [16] and fibrinogen by precipitation- refractory method as described by Thrall et al. [17].

Statistical analysis

The data were presented as mean ± standard deviation (SD), and the level of significance was set at $P < 0.05$. The data were analyzed statistically by repeated measure method using statistical analysis system (SAS) software.

RESULTS

Cysts were present in the skin biopsies of distal parts of the

leg of infected goats from day 28 PI. The mean ± SD of hematological and serum biochemical values from different samples of each goat at weakly intervals are presented in Tables 1 and 2, respectively. However, there were variations in hematological analyses; the differences were not statistically significant with those of the normal values. From day 30 to day 360 PI, statistically results showed that the acute phase proteins concentrations, including SAA, Hp, fibrinogen, and ceruloplasmin, increased, whereas testosterone concentrations decreased. Serum total protein concentrations increased. Infected goats exhibited decrease of albumin and increase of globulin concentrations. These parameters were statistically significant ($P < 0.05$) in infected animals. By contrast, there were no significant differences in AST, ALT, ALP, CK, LDH, Na^+, K^+, Ca^{2+}, Fe^{2+}, Cl, P, cholesterol, triglyceride, and glucose concentrations in the sera of infected goats.

DISCUSSION

Despite the presence of variations in hematological values, there was no significant difference between hematological analyses in experimentally infected and control goats. Nazifi et al. [11] studied the changes of hematological analyses of the naturally infected domestic goats with *B. caprae*. They observed macrocytic-normochromic anemia, leukocytosis, neutrophilia, lymphopenia, basophilia, eosinophilia, and monocytosis in naturally infected goats. Naturally infected animals probably had mixed infections with other protozoa that might have affected the hematological analyses.

Lack of alterations in enzymes ALP, ALT, LDH and CK activities is due to their short half life (< 10 hr). They quickly decrease and reach a steady state within 1 to 2 days in case of acute infections. Their variations could not be detected during chronic diseases. Therefore, as CK and LDH are mainly of muscular origin [18], only cysts located in muscles would induce release of these 2 enzymes into the blood flow, whereas *Besnoitia* cysts were rarely found in subcutaneous skeletal muscles, and presence of *Besnoitia* cysts in deeper striated muscles has not been reported.

Analyses on total protein concentrations and protein fractions are important in various disease states [19]. In the present study, infected goats exhibited decrease of albumin and showed marked increases of serum globulin and total protein concentrations from 30 days after inoculation to day 360 PI. Globulin as a chronic phase protein increases in chronic diseases [17,18,20]. Albumin is considered as a negative acute-phase protein (APP), and its concentration tends to decrease mostly during chronic inflammation. Consequently, increase of serum total protein concentrations results from significant elevations of the globulin concentration and from a relative decrease of albumin [17,18,21].

The serum Hp, SAA, fibrinogen, and ceruloplasmin concentrations of the infected animals were significantly higher than those of the normal animals, and the level increased according to increase of PI days. The APPs are a group of blood proteins, and their concentrations in the blood of animals change subjected to external or internal challenges, such as infection, inflammation, surgical trauma, or stress [22-24]. They are mainly synthesized in the liver, mediated by proinflammatory cytokines, and their concentration can increase (positive APPs) or decrease (negative APPs) as a consequence of inflammatory stimuli. It has been suggested that APPs may be useful in the assessment of animal welfare [23,25,26]. On the other hand, it is suggested that various infections and inflammatory processes may be associated with different APP pattern details, which may lead to APP analyses being used for diagnostic purposes [24]. APPs and their changes due to various inflammatory and non-inflammatory conditions have been studied intensively in many animal species [19,23-26]. The specific type of APPs and the time course for alterations in these proteins vary in different species on the basis of the initiating disorder or underlying inflammatory processes [27]. SAA and Hp have been proposed to be markers of stress in cattle and other species [28-32].

Hp is a major APP in numerous species of livestock and domesticated animals. Elevated Hp concentrations occur not only with inflammation, but also with some conditions not generally associated with inflammation or tissue damage [23]. Statistically significant increase in Hp has been observed between clinically healthy and diseased cows in similar researches with same inflammatory diseases [33-35].

SAA proteins comprise a superfamily of apolipoproteins and are considered one of the major acute phase reactants in vertebrates. SAA proteins are highly conserved across evolutionarily distinct vertebrate species with respect to both their sequence and inductive capacity. It is, therefore, generally assumed that they have a crucial, yet ill-defined, protective role during inflammation [36]. Elevated SAA levels are found following inflammation and also under conditions unrelated to inflammation, such as physical stress or at parturition [23].

Glass et al. [37] reported that following experimental infection with *Theileria annulata* in cattle, SAA appeared first, followed by a rise in alpha-1-acid glycoprotein in all animals, whereas Hp only appeared in a low level in some of the animals.

Plasma fibrinogen in all species is synthesized in the liver, and hyperfibrinogenemia, which is characteristic of a broad range of bacterial infections and other inflammatory conditions, has been reported in a wide range of vertebrates [38].

Localization of the *Besnoitia* cysts in the parenchyma of the testis and epididymis and its adverse effect on spermatogenesis and goat production in male animals is previously reported [10,39,40]. The presence of *Besnoitia* cysts in the parenchyma between the seminiferous tubules of the testis and ducts of the epididymis of these infected animals caused severe tubular degeneration. The seminiferous tubules of the testis showed no spermatogenic activity. It can be considered as the cause of decreased testosterone concentrations observed in the present study which the severity of it increased from day 30 to day 360 PI.

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