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A survey of biochemical and acute phase proteins changes in sheep experimentally infected with *Anaplasma ovis*

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ABSTRACT

Objective: To determine the biochemical and acute phase proteins changes in sheep experimentally infected with *Anaplasma ovis* (*A. ovis*). **Methods:** One Iranian sheep naturally infected with *A. ovis* (parasitemia 0.02%) but with no other blood parasites based on blood smear and polymerase chain reaction methods was selected as donor, and it was splenectomized to induce high level of parasitemia. Then, three weeks after splenectomy when parasitemia was 6%, donor's blood was intravenously administered to each recipient animal. Five 5-6 months old Iranian male sheep without any blood parasites were selected as recipient animals. The percent of parasites, packed cell volume, serum biochemical parameters (urea, creatinine, bilirubin, aspartate aminotransferase activity, cholesterol, total protein, albumin, globulin, Fe), acute phase proteins (haptoglobin, total iron binding capacity, fibrinogen), were evaluated in sheep before and after being experimentally infected with *A. ovis* (until day 38). In addition, body weights of sheep were measured on days 0, 20 and 38. **Results:** In recipient sheep, microscopic examination of erythrocytes revealed a significant rise of parasitemia on days 12 and 15. The lowest level of packed cell volume in sheep was seen on day 15 post infection. A significant rise existed in mean urea and bilirubin (total, direct and indirect) on days 15 and 20. The increase of indirect bilirubin level was higher than direct bilirubin. Furthermore, serum Fe significantly increased on days 20 and 23. The mean total protein concentration significantly increased on day 38. A significant increase was found in the serum globulin concentration from days 20 and 27 to 38. Maximum values of haptoglobin were observed on days 27 and 30. Moreover, aspartate aminotransferase activity (from days 20-30) and cholesterol concentration (on day 20) significantly decreased. However, no significant changes were found in other parameters. **Conclusions:** Experimental ovine anaplasmosis caused by *A. ovis* could be associated with some changes in measured parameters, which presumably could be helpful for evaluation on staging of disease.

1. Introduction

Anaplasma ovis (*A. ovis*) is an obligate intraerythrocytic rickettsia pathogen which infects sheep and goats in the tropical and subtropical areas of the world[1,2]. The disease caused by *Anaplasma* spp. has been recognized over a century, and is still an important

issue worldwide. Although the disease appears to be widespread, the extent of the infection and the loss of livestock productivity remain poorly understood[3]. The life cycle of *A. ovis* involves vertebrates,

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ticks and biting flies[4,5]. Anaplasmosis is usually a subclinical or mild condition without clinical signs in sheep; but, moderate to severe clinical disease may occur with fever, anemia and icterus[6]. Khaki et al.[1] described a natural co-infection of *A. ovis* with *Theileria ovis*. Their observation showed that the effect of a mixed infection can be more severe than a single infection with *Anaplasma* or *Theileria*.

In small ruminants, natural infection of *A. ovis* was previously reported from different areas of Iran[7–11]. However, there is very limited information about laboratory studies, particularly biochemical and acute phase protein (APP) changes of *A. ovis* in sheep. This study was conducted to evaluate the serum biochemical parameters and APPs, percent of parasites and packed-cell volume (PCV) changes in sheep experimentally infected with Iranian strain of *A. ovis*.

2. Materials and methods

2.1. Experimental animals

All experimental procedures involving animals were approved by the ethical committee at the faculty of veterinary medicine, University of Tehran, Tehran, Iran (No: 7508017.6.11).

Five 5-6 months old Iranian male sheep were selected as recipient animals. All selected animals were microscopically tested negative for the infection of blood parasites (*Anaplasma* spp., *Theileria* spp. and *Babesia* spp.), which were confirmed by the polymerase chain reaction method. Before infection, hematological and biochemical assessment was conducted to check on any diseases affecting the experimental animals.

2.2. Source of experimental infection

One Iranian sheep (parasitemia 0.02%) naturally infected with *A. ovis* but with no other blood parasites (*Theileria* or *Babesia* spp.) based on blood smear and polymerase chain reaction methods was selected as donor animal, and then it was splenectomized to induce high level of parasitemia. One hundred mL blood was collected from donor three weeks after splenectomy (with parasitemia 6%) in a container with heparin, and an inoculum of 20 mL blood was intravenously administered to each recipient animal.

2.3. Sampling and body weight measurement

Blood samples were collected from jugular vein into test tubes with EDTA-K2 for parasitological and molecular analysis and without anticoagulant for serum separation and biochemical assessment. Samples of serum were separated and stored at -20 °C until being analyzed.

Samples on zero day were taken as controls before the infection was given and were collected thereafter on days 5, 9, 12, 15, 20, 23, 27, 30, 34, and 38 post inoculation. Additionally, body weights were measured on days 0, 20 and at end of the experiment (day 38).

2.4. Microscopic examination and PCV assessment

Giemsa-stained smears were used to determine the infection rate of erythrocytes. Parasitemia ratio was assessed by counting the number of infected red blood cells on examination of at least 200 oil immersion fields. Then, the number of parasitized cells was expressed as a percentage[1].

The percentage of PCV was determined by the microhematocrite method[12].

2.5. Polymerase chain reaction (PCR) analysis

DNA was prepared from the blood samples by using the MBST kit (Tehran, Iran), according to the manufacturer's instructions. A PCR method was performed to detect *Anaplasma* spp. (*A. ovis* and *Anaplasma marginale*) using one pair of primers, based on the *MSP4* gene sequence of *Anaplasma* spp. Primers were forward strand primer 5'-TTGTTTACAGGGGGCCTGTC-3' and reverse strand primer 5'-GAACAGGAATCTTGCTCCAAG-3'. *A. ovis* and *Anaplasma marginale* were differentiated from each other by PCR-RFLP using *Hpa* II enzyme[9].

Theileria and *Babesia* infections were diagnosed by a PCR technique using forward strand primer FThBab 5'-GCATTTCGTATTTAACTGTCAGAGG-3' and reverse strand primer RThBab 5'-GATAAGGTTCCAAAACCTCCCTAG-3' which were specific for 18S rRNA gene sequence of *Theileria* and *Babesia* spp. PCR-RFLP was performed to differentiate *Theileria* and *Babesia* as well as various *Theileria* species infecting sheep (*Theileria ovis* and *Theileria lestoquardi*) using *Hind* II and *Vsp* I enzymes, respectively[13].

2.6. Biochemical and APP assessment

The concentrations of urea, creatinine, total, direct and indirect bilirubin, aspartate aminotransferase (AST) activity, cholesterol, total protein, albumin and iron (Fe) were measured by commercial kits of Pars Azmoon (Tehran, Iran) using an Elitech automated analyzer (SELECTRA Prom, France). In addition, serum globulin concentration was calculated by subtracting albumin from total protein concentration[12].

Furthermore, APPs such as haptoglobin (Hp) (Tridelta Development Ltd, Wicklow, Ireland), total iron binding capacity (TIBC) (Pars Azmoon kit, Tehran, Iran), and plasma fibrinogen (Fib) (heat precipitation and refractometry method) were measured[12].

2.7. Statistical analysis

Data were analyzed using analysis of variance (ANOVA) and Tukey's *post hoc* tests. All values were presented as mean and standard error (SE), and *P* value less than 0.05 was considered as significant difference.

3. Results

3.1. Parasitological, PCV, body weight and clinical findings

During the post infection days, maximum level of parasitemia was revealed on day 12 ($1.44 \pm 0.46\%$) by microscopic examination of blood smears (Table 1).

As shown in Table 1, the value of PCV gradually decreased from 5 days post inoculation and reached approximately the lowest level on day 15 [$16.25 \pm 3.19\%$] of post infection compared to day 0.

However, no significant difference was found in the mean body weight of sheep before and after being infected experimentally with *A. ovis* ($P > 0.05$) (Table 1).

In recipient sheep, body temperature was unremarkable during post infection days. However pale mucous membranes were observed in some infected animals on days 12 and 23, but anemic membranes were seen in all infected sheep on days 15 and 20. During mentioned days, relative lethargy and anorexia were also observed, but clinical jaundice was not seen.

3.2. Biochemical and APP findings

Table 2 showed the mean serum biochemical parameters. Significant rises were recorded for urea values on days 15 and 20 compared to day 0 and other days ($P < 0.05$). However, no significant changes were observed in serum creatinine concentrations.

Our results also revealed that serum total, direct and indirect bilirubin amounts significantly increased with the progress of infection and reached a peak on days 15 and 20 of experiment. Then, a gradual decline existed in their concentration up to day 38.

As for AST activity, a significant reduction in AST activity was observed on days 20 to 30, with the lowest level on day 30, when the concentration was (71.0 ± 1.2) U/L compared to (89.6 ± 4.7) U/L on day 0 and (90.8 ± 4.5) U/L on day 38. The concentration

Table 1

Parasitemia, PCV and weight body during experimental period (mean \pm SE).

Indexes	0 d	5 d	9 d	12 d	15 d	20 d	23 d	27 d	30 d	34 d	38 d
Parasitemia (%)	0.00 \pm 0.00	0.28 \pm 0.04	0.85 \pm 0.26	1.44 \pm 0.46*	1.32 \pm 0.33*	1.08 \pm 0.20*	0.73 \pm 0.08	0.47 \pm 0.08	0.37 \pm 0.09	0.23 \pm 0.04	0.18 \pm 0.03
PCV (%)	32.75 \pm 0.94	28.62 \pm 1.21	23.75 \pm 1.31*	19.25 \pm 2.86*	16.25 \pm 3.19*	17.75 \pm 1.93*	20.50 \pm 1.19*	21.25 \pm 0.47*	22.00 \pm 0.90*	25.00 \pm 0.70	25.25 \pm 0.75
Weight body (kg)	28.60 \pm 1.91	–	–	–	–	30.50 \pm 2.21	–	–	–	–	31.40 \pm 2.17

*– Represents a significant difference between parameter's concentrations on different days with day 0 as control ($P < 0.05$).

Table 2

Serum biochemical parameters in sheep before and after being infected experimentally with *A. ovis* (mean \pm SE).

Days post infection	0	5	9	12	15	20	23	27	30	34	38
Urea (mg/dL)	49.0 \pm 6.1	40.8 \pm 4.4	47.0 \pm 7.7	40.5 \pm 2.4	64.6 \pm 3.9*	64.6 \pm 5.6*	45.8 \pm 3.5	51.2 \pm 3.1	49.6 \pm 2.4	52.8 \pm 2.3	36.8 \pm 4.5
Creatinine (mg/dL)	0.92 \pm 0.04	0.92 \pm 0.02	0.94 \pm 0.05	0.85 \pm 0.02	0.90 \pm 0.06	0.92 \pm 0.07	0.86 \pm 0.04	0.92 \pm 0.09	1.00 \pm 0.12	0.86 \pm 0.02	0.88 \pm 0.03
Total bilirubin (mg/dL)	0.10 \pm 0.00	0.10 \pm 0.00	0.14 \pm 0.02	0.12 \pm 0.02	0.34 \pm 0.12*	0.40 \pm 0.16*	0.12 \pm 0.02	0.22 \pm 0.08	0.18 \pm 0.05	0.18 \pm 0.05	0.12 \pm 0.02
Direct bilirubin (mg/dL)	0.04 \pm 0.00	0.05 \pm 0.00	0.09 \pm 0.01	0.06 \pm 0.02	0.15 \pm 0.04*	0.19 \pm 0.04*	0.08 \pm 0.01	0.13 \pm 0.05	0.10 \pm 0.02	0.11 \pm 0.04	0.06 \pm 0.00
Indirect bilirubin (mg/dL)	0.05 \pm 0.00	0.04 \pm 0.00	0.05 \pm 0.01	0.05 \pm 0.00	0.19 \pm 0.09*	0.21 \pm 0.12*	0.04 \pm 0.00	0.08 \pm 0.02	0.07 \pm 0.03	0.06 \pm 0.01	0.03 \pm 0.00
AST (U/L)	89.6 \pm 4.7	79.0 \pm 2.8	83.8 \pm 3.4	78.0 \pm 7.3	86.6 \pm 5.2	73.6 \pm 5.9*	74.2 \pm 3.6*	71.2 \pm 1.6*	71.0 \pm 1.2*	80.6 \pm 5.6	90.8 \pm 4.5
Cholesterol (mg/dL)	51.80 \pm 3.73	44.80 \pm 2.85	45.60 \pm 2.33	43.00 \pm 4.08	49.00 \pm 2.00	42.00 \pm 2.60*	45.20 \pm 1.74	51.00 \pm 3.01	54.80 \pm 2.03	46.80 \pm 2.03	51.00 \pm 2.88
Total protein (g/dL)	6.34 \pm 0.21	6.34 \pm 0.12	6.36 \pm 0.12	6.35 \pm 0.16	6.46 \pm 0.16	6.74 \pm 0.13	6.58 \pm 0.10	6.70 \pm 0.15	6.70 \pm 0.17	6.76 \pm 0.16	7.04 \pm 0.12*
Albumin (g/dL)	3.40 \pm 0.10	3.40 \pm 0.03	3.26 \pm 0.10	3.35 \pm 0.06	3.26 \pm 0.07	3.40 \pm 0.10	3.42 \pm 0.08	3.38 \pm 0.12	3.36 \pm 0.12	3.36 \pm 0.12	3.62 \pm 0.18
Globulin (g/dL)	2.94 \pm 0.14	2.94 \pm 0.14	3.10 \pm 0.13	3.00 \pm 0.14	3.20 \pm 0.10	3.34 \pm 0.07*	3.16 \pm 0.10	3.32 \pm 0.11*	3.34 \pm 0.12*	3.40 \pm 0.08*	3.50 \pm 0.70*
Fe (μ g/dL)	146 \pm 14	177 \pm 18	162 \pm 14	165 \pm 25	189 \pm 17	200 \pm 27*	205 \pm 20*	135 \pm 17	139 \pm 21	131 \pm 8	158 \pm 21

*– Represents a significant difference between parameter's concentrations on different days with day 0 as control ($P < 0.05$).

of cholesterol on day 0 was (51.80 ± 3.73) mg/dL but decreased gradually up to day 20 with concentration (42.00 ± 2.60) mg/dL, and then increased gradually up to day 38 when the concentration was (51.00 ± 2.88) mg/dL. However, a significant decrease on day 20 compared to day 0 were recorded. Moreover, the mean total protein concentration insignificantly increased from days 15 to 34, but significantly increased on day 38 $[(7.04 \pm 0.12)$ g/dL] compared to day 0 $[(6.34 \pm 0.21)$ g/dL].

Serum globulin concentrations showed a significant increase on days 20 and 27 to 38 compared to day 0 ($P < 0.05$). The highest globulin concentration was (3.50 ± 0.70) g/dL on day 38 in comparison to day 0 $[(2.94 \pm 0.14)$ g/dL]. However, no significant changes were observed in serum albumin ($P > 0.05$).

The Fe amounts increased significantly on days 20 $[(200 \pm 27)$ µg/dL] and 23 $[(205 \pm 20)$ µg/dL] of post infection compared to day 0 $[(146 \pm 14)$ µg/dL]. Serum Fe level was increased in all animals on day 23. After day 23, there was a decline in the serum Fe level.

Hp concentration significantly increased from days 5 to 34, and maximum values of Hp were observed on days 27 $[(430 \pm 9)$ µg/mL] and 30 $[(400 \pm 4)$ µg/mL] in comparison to day 0 $[(220 \pm 2)$ µg/mL]. It should be noted that Hp was measured up to day 34. Furthermore, in comparison to the first day (day 0), the mean of TIBC insignificantly decreased from days 5 to 38. There was also not any statistically significant difference in mean Fib level on different days of experiment (Figure 1).

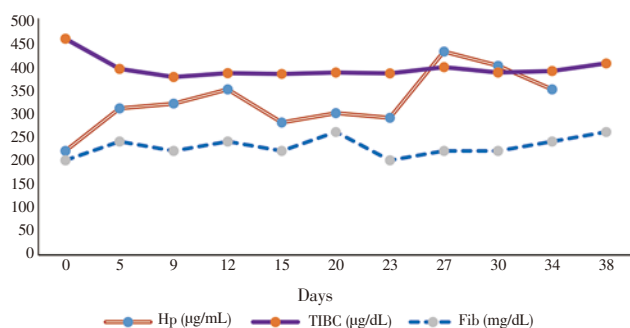


Figure 1. Changes in concentrations of serum Hp, TIBC and plasma Fib during experimental period.

4. Discussion

Ovine tick-borne diseases are widespread in Iran, causing high economic losses[1]. There is limited information about experimental ovine anaplasmosis in the world. In addition, there were no published reports about the biochemical and APP parameters on experimental ovine anaplasmosis by *A. ovis*. Therefore, this study was carried out to ascertain the changes in blood biochemistry and APP parameters of sheep experimentally infected with Iranian strain of *A. ovis* based on microscopic and molecular identification. We hope that the results of this study help better understanding of the pathogenesis and find a useful tool for leveling of disease in sheep experimentally infected

with *A. ovis*.

Our experiment indicated that parasitemia significantly increased along with the progress of infection and displayed its highest level on days 12 and 15 of post infection. Afterwards, the parasitaemia decreased slowly, but the number of organism did not reach zero in none of the sheep until day 38 (the end of experimental anaplasmosis study). A similar observation was reported in experimental anaplasmosis caused by *A. ovis*[14,15].

In our study, the progressive increase in the percentage parasitemia was associated with a progressive anemia and decrease in the percentage of PCV with lowest levels on days 15 and 20 of post infection. According to previous report, in the sheep experimentally infected with *A. ovis*, anemia occurred and negative correlations existed between parasitemia and PCV value[14]. Extra-vascular hemolytic anemia is a key feature of anaplasmosis[16]. The reason for anemia is related to erythrophagocytosis, activation of a complement system, immune-mediated destruction of erythrocytes and oxidative stress[17]. Besides, no significant clinical signs (except anemia) were seen in infected sheep because anaplasmosis is usually a mild condition and sometimes even be asymptomatic. Co-infection of *A. ovis* with other blood parasites was reported in sheep previously. However, *A. ovis* can be activated and induces its pathogenesis in the presence of other pathogens[1].

Furthermore, in the present study, simultaneously with severe anemia, we observed a significant increase of urea concentration on days 15 and 20 compared to day 0 and other days of the experiment. In this respect, Sandhu *et al.*[18] reported a significant increase in the level of blood urea nitrogen and a significant decrease in Hb concentration, PCV and RBC count from day 16 on experimentally infected bovine theileriosis. Also, Alsaad[19] reported a significant increase of blood urea nitrogen concentration in naturally infected camel anaplasmosis. It has been demonstrated that the increase in urea levels observed in infected animals with *Theileria* or *Anaplasma* might be due to increased catabolism of proteins such a erythrophagocytosis[18,20]. In the current study, no significant changes were observed in serum creatinine. However, Coskun *et al.*[21] and Alsaad[19] reported a significant increase of creatinine concentration in bovine and camel anaplasmosis respectively. In contrast, Omer *et al.*[22] reported a significant decrease in the creatinine concentration in Friesian cattle naturally infected with *Theileria annulata*.

Biochemical analysis of the sheep experimentally infected with *A. ovis* revealed a significant rise in total, direct and indirect bilirubin simultaneously with severe anemia on days 15 and 20. The increase of indirect bilirubin concentration was higher than that of direct bilirubin, which usually occurred in hemolytic anemia[12]. It has been suggested that a significant increase in total bilirubin levels is concurrent with the appearance of infected RBCs with *Anaplasma*. This condition could be attributed to RBC lysis in the reticuloendothelial system and probably hepatic cell damage[6,21]. In the current experiment, there was no evidence of intravascular

hemolysis.

Some studies have showed increased AST activity in calves and cattle during *Theileria annulata* infection[18,23], but our research indicated that AST activity was significantly lower in infected animals from days 20 to 30 post infection than day 0. Kumar *et al.*[24] reported a significant decrease in AST levels on days 7 and 21 (the end of the investigation period) in goats infected experimentally with tick and naturally infested goats which is agreement with our findings. The reason for this decrease is not yet clear and requires further investigation.

In terms of cholesterol concentration, it showed a significant decrease on day 20. Hypocholesterolemia has been described in human with chronic anemia and increased erythropoietic activity. However, the etiology of hypocholesterolemia in anemic patients is not clear[25].

Our results showed that mean serum total protein and globulin concentration significantly increased on some days ($P < 0.05$), but no significant changes were observed in serum albumin levels. Therefore, increased protein was caused by an increase in globulin concentrations in response to antigenic stimulation[12], and probably increased haptoglobin[26]. Coskun *et al.*[21] reported an insignificant increase in total protein in bovine anaplasmosis. However, Alsaad[19] reported a significant decrease in serum protein level during camel anaplasmosis.

We also found that serum Fe amounts showed a significant increase on days 20 and 23 post infection compared to day 0. On the other hand, the lowest levels of PCV could be seen on days 15 and 20. Some studies have reported increased serum iron during extravascular hemolytic anemia due to destruction of RBCs in mononuclear phagocyte system[12].

APP production continues in chronic and acute infections[27]. It is known that Hp is considered to be a major APP in sheep[28–30], and binds free hemoglobin[21], which is toxic and has oxidative activity[28]. Although, Hp changes is not specific for any disease but Hp is useful as a marker for the occurrence of inflammatory diseases in sheep[31,32]. Our data showed that serum Hp could be increased in the early and chronic stages of ovine anaplasmosis. Thus, Hp concentration significantly increased from days 5 to 34 post infection in comparison to day 0, with its concentration higher than 400 µg/mL in 60% and 80% sheep on days 27 and 30 respectively. In addition, serum Hp only in one sheep (20% infected sheep) was 420 µg/mL on day 34. It should be noted that the Hp concentration in one sheep was 450 and 420 µg/mL on days 9 and 12, respectively. Consequently, measurement of Hp presumably could be a useful tool for evaluating the staging of disease. Further investigation may be required to confirm it.

TIBC and albumin are negative acute-phase proteins. The TIBC is a measure of the total serum transferrin concentration[12,33]. TIBC is decreased or in the low normal range in inflammatory diseases[33]. Moreover, Watanabe *et al.*[34] previously reported that during severe extravascular hemolytic anemia, serum iron was markedly increased,

while the TIBC was unremarkable or decreased slightly in calves infected with *Theileria sergenti*. However, in the current study, serum TIBC level insignificantly decreased in infected sheep. Although the mean albumin concentration decreased from days 5 to 20 in our study, but it was not significant, because the concentration of serum albumin as a negative APP consistently decreased by 10%-30%[12]. Furthermore, Non-significant decrease of albumin concentration was reported previously in sheep naturally infected with *Theileria annulata*[35].

The synthesis of Fib increases during the acute phase response[36]. It has been reported that measurement of Fib and Hp might be more useful in the diagnosis of infectious disease than the number of circulating neutrophils[12]. But observations indicate that the measurement of Hp is better than Fib in inflammatory disease[16]. However, in the current study, there was not any statistically significant difference in mean Fib concentrations on different days of the disease. Thus measurement of Fib and albumin could not be a useful tool for evaluation on staging of the disease in sheep experimentally infected with *A. ovis*.

In brief, data analysis of the sheep experimentally infected with *A. ovis* revealed a substantial reduction in PCV and a significant rise in parasitemia, urea and bilirubin concentrations on day 15. Moreover, as the disease progresses, on day 20, in addition to the changes mentioned, a significant reduction in serum cholesterol and rise in serum iron occurred.

Based on the mentioned data in this study, it can be concluded that ovine anaplasmosis caused by *A. ovis* could be associated with some changes in parasitemia, PCV, biochemical parameters and APP concentrations during experimental period, which could be helpful for evaluation on staging of the disease.

Conflict of interest statement

Authors declare that there are no conflicts of interest.

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