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Some biochemical parameters and oxidative stress biomarkers in sheep with paratuberculosis

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Summary

The aim of this study was to investigate the oxidative stress biomarkers and some biochemical parameters for subclinical and clinical paratuberculosis in sheep. A number of 24 sheep positive for ELISA and PCR were categorized into 2 classes. According to the results of biochemical parameters, total protein, albumin, Ca and Mg levels of the diseased sheep decreased significantly ($P < 0.05$) in clinical and subclinical groups when compared to the control group. Total protein, albumin, Ca and Mg levels decreased significantly ($P < 0.05$) in the clinical group when compared to the subclinical group. No significant difference was observed for globulin, glucose, BUN, creatinine, total bilirubine, total cholesterol and triglycerides levels in both subclinical and clinical cases compared to control sheep. The levels of SOD, GSH-Px and GSH decreased significantly ($P < 0.05$) in clinical and subclinical groups compared to the control group. The plasma level of TBARS increased significantly ($P < 0.05$) in clinical and subclinical groups compared to the control group. Conclusively oxidative stress and some biochemical parameters could be used as novel biomarkers in differential diagnosis of clinical and subclinical paratuberculosis in sheep.

Keywords: Paratuberculosis, oxidative stress, biochemical parameters, sheep

Paratuberculosis is a chronic infectious enteric disease that affects domestic and wild ruminants. It is an economically important disease seen primarily in cattle, sheep, and goats and is caused by *Mycobacterium avium* subsp. *paratuberculosis* (26, 30). Paratuberculosis in small ruminants is widely distributed (14).

Diarrhea, the cardinal sign of paratuberculosis in cattle, is an uncommon clinical sign in sheep. Tracing paratuberculosis suspected sheep in a flock is difficult because of the absence of a noTab. clinical sign such as profuse diarrhoea. Non-specific symptoms of clinical diseased sheep are progressive weight loss (24) and decreased serum concentrations of calcium (Ca), total protein and albumin (12). The diagnosis of paratuberculosis in sheep requires additional testing. For this purpose serological and skin tests, fecal culture and necropsy are used (8). The nonspecific nature of these clinical signs makes a definitive diagnosis of paratuberculosis in sheep by clinical examination impossible. The disease can be definitively diagnosed antemortem by Ziehl-Neelsen (ZN) staining (acid-fast) of rectal scraping, fecal culture, and polymerase chain reaction (PCR) (8, 10).

Many potentially toxic reactive oxygen species (ROS) are generated through the normal oxidative metabolism, and ROS in low concentrations is necessary for some physiological processes (11). Oxidative stress may be defined as an alteration in the steady-state balance between oxidant and antioxidant agents in the cells, when ROS are accumulated into cells, several physiological processes may be disturbed (9). Oxidative stress is a secondary aggravating factor in most diseases.

Publications specifically regarding the effect of paratuberculosis on oxidative stress markers in sheep are lacking. Therefore, the present study aimed to investigate the oxidative stress biomarkers and some biochemical parameters for subclinic and clinic paratuberculosis in sheep.

Material and methods

Experimental design and animals. In April 2014, paratuberculosis was diagnosed in two 4- and 5-years-old Ivesi sheep afflicted with anorexia, recumbency and weight loss. For the definitive diagnosis, two dead sheep were thoroughly examined postmortem. According to the pathological (15) and microbiological (5) results of laboratory examination, paratuberculosis was diagnosed in both sheep. The herd of these two sheep with paratuberculosis was located in Adıyaman province in Turkey. The herd had 131 animals in total, but only the sheep > 2-years-old were sampled. During the winter season the herd was confined to a free-stall barn, fed with formulated diets to meet or exceed nutritional requirements of the sheep as specified by NRC (19) and the rest of the time it was intensively grazing. The sheep were milked mechanically twice daily. Sera were collected from a total number of 85 sheep > 2-years-old to be analyzed by indirect ELISA (32). ELISA tests were positive for 48 sheep in the herd. Inclusion criteria for diseased animals were PCR-positive fecal samples. A number of 24 sheep positive for ELISA and PCR were categorized into 2 classes, admitted with only inappetence for 2 to 7 d (subclinical group, n = 12), and presented with severe loss of body condition and chronic or intermittent diarrhea for up to 90 d (clinical group, n = 12). Control animals (control group, n = 12) were categorized as healthy based on ELISA and PCR-negative results. Parasitological exemination was carried out in the fecal samples of all animals in the experiment. The Body Condition Score (BCS) values of the animals in the experiment were determined in a 1 (emaciated) to 5 (fat) scale in accordance with the procedure by Russel (23).

Sample collection and biochemical assays. Blood samples were taken from the jugular vein with 10 mL heparinized test tube and silicone (for serum) vacutainer tubes. Samples were centrifuged at 3.000 × g at 4°C for 10 min to separate the plasma and serum from the erythrocytes. To obtain packed erythrocytes, the remaining erythrocytes were washed twice with an isotonic solution of sodium chloride. To obtain erythrocyte hemolysates, 500 μL of packed erythrocytes were destroyed by adding four volumes of cold redistilled water. The resulting suspension was centrifuged twice: first for 10 min in the tube centrifuge at 1.500 × g at 4°C and then in an Eppendorf centrifuge at 5.000 × g for 5 min at 4°C. Clear supernatant was obtained as hemolysates to determine glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and glutathione (GSH). The lysate was frozen at –20°C until the time of analysis. The other sample was collected in plain tubes to obtain serum for biochemical parameters. Serum total protein, albumin, glucose, total bilirubin, blood urea nitrogen (BUN), creatinine, total cholesterol, triglycerides, Ca and magnesium (Mg) concentrations were determined according to Olympus Kits in Olympus AU600 autoanalyzer (Olympus Corp., Tokyo, Japan). Globulin concentration was calculated by subtracting albumin from total protein concentration.

The activities of antioxidant enzymes GSH-Px and SOD were measured in hemolysate. GSH-Px activities were measured by the oxidation of glutathione using tert-butyl hydroperoxide. Oxidized glutathione was converted to reduced form in the presence of glutathione reductase and NADPH, while NADPH was oxidized to NADP. The reduction in absorbance of NADPH at 340 nm was measured. The absorbance change per minute and the molar extinction coefficient of NADPH were used to calculate glutathione peroxidase activity, which was expressed as international units per deciliter (20). SOD activity was measured in the hemolysate (28). Hemolysate was assayed for SOD activities using the xanthine/xanthine oxidase system for superoxide radical generation. This anion reduced nitroblue tetrazolium to a red formasone compound. SOD activity was measured at 560 nm by detecting the inhibition of this reaction. One unit of SOD activity was defined as the activity that caused half-maximal inhibition of the nitroblue tetrazolium reduction rate. The determined enzymatic activity was converted into Hb concentration and presented for SOD and GSHPx as U/g Hb.

GSH concentration was detected in the blood hemolysate by the titration with 0.1 mmol/L dithiobis in a 0.1-mol/L disodium phosphate buffer solution. The formation of the reduced product, thionitrobenzene, was measured spectrophotometrically at 412 nm. The GSH content was expressed as milligrams per deciliter of hemolysate (4).

Plasma levels of thiobarbituric acid reactive substances (TBARS) were analyzed spectrophotometrically after extraction with nbutanol according to the optimized method of Yagi (34) by adding 100 μL of plasma to a 0.37% thiobarbituric acid solution.

Serological testing. Sera were analyzed by indirect ELISA, with a slight modification of the technique described by Turnquist et al. (32). The antigen used was a Paratuberculosis Protoplasmic Antigen (PPA-3 Allied Monitor, USA), a sterile filtered, lyophilized protoplasmic cell extract of *Mycobacterium sp*., recommended for use in ELISA screening for the detection of antibodies produced against *Mycobacterium avium* subsp. *paratuberculosis*.

Statistical analysis. All results were expressed as mean ± standard deviation (SD). SPSS/PC software one-way repeated measure analysis of variance (ANOVA) was used to determine statistical differences between mean values of the studied parameters among the groups. Differences were considered as significant at $P < 0.05$.

Results and discussion

The diseased sheep showed diarrhea, dehydration, anorexia and weight loss in the clinical group. Compared to a mean BCS of 3.7 ± 0.3 in controls, the mean BCS of the diseased sheep in the clinical group and subclinical group were 1.4 ± 0.5 and 2.4 ± 0.3 respectively. Twelve sheep had normal fecal pellets, 8 had intermittent diarrhea, and only 4 had chronic watery diarrhea in clinical group.

Compared to controls, some biochemical parameters in sheep with paratuberculosis are presented in Tab. 1. Total protein, albumin, Ca, Mg levels decreased significantly ($P < 0.05$) in the clinical and subclinical groups when compared to the control group. Total protein, albumin, Ca and Mg levels decreased significantly ($P < 0.05$) in the clinical group when compared to the subclinical group. Total bilirubin levels increased insignificantly ($P > 0.05$) in clinical and subclinical groups when compared to the econtrol group. No significant difference was observed for globulin, glucose, BUN, creatinine, total cholesterol and triglycerides levels in both subclinical and clinical groups compared to the control group (Tab. 1).

The levels of SOD, GSH-Px and GSH decreased significantly ($P < 0.05$) in the clinical and subclinical groups compared to the control group. The plasma level of TBARS increased significantly ($P < 0.05$) in the clinical and subclinical groups compared to the control group (Tab. 1).

Tab. 1. Levels of biochemical and oxidative stress parameters in the groups: clinic paratuberculosis, subclinic paratuberculosis and healthy sheep ($\bar{x} \pm SD$; n = 12)

Parameters	Groups		
	control	subclinic	clinic
Total proteins, g/dL	6.52 ± 0.16^a	5.33 ± 0.15^b	4.85 ± 0.21^c
Albumin, g/dL	3.74 ± 0.19^a	2.90 ± 0.18^b	2.47 ± 0.16^c
Globulin, g/dL	2.78 ± 0.11	2.43 ± 0.09	2.38 ± 0.09
BUN, g/dL	12.30 ± 0.34	14.20 ± 0.29	15.84 ± 0.48
Creatine, mg/dL	1.14 ± 0.09	1.17 ± 0.08	1.19 ± 0.11
Glucose, mg/dL	67.37 ± 3.10	55.52 ± 4.24	52.04 ± 3.83
Total bilirubin, mg/dL	1.24 ± 0.06	1.41 ± 0.07	1.52 ± 0.08
Total cholesterol mg/dL	64.16 ± 2.24	61.35 ± 3.39	57.84 ± 3.22
Triglycerides, mg/dL	34.24 ± 2.85	37.08 ± 2.32	40.12 ± 3.10
Ca, mg/dL	9.20 ± 0.63^a	7.94 ± 0.66^b	6.26 ± 0.45^c
Mg, mg/dL	1.28 ± 0.08^a	0.92 ± 0.09^b	0.84 ± 0.08^c
SOD, U/g Hb	1058.46 ± 25.45^a	853.52 ± 36.22^b	748.10 ± 30.42^b
GSH-Px, U/g Hb	72.16 ± 5.26^a	54.73 ± 3.84^b	38.24 ± 3.78^b
GSH, mg/dL	77.52 ± 3.56^a	49.82 ± 3.48^b	36.26 ± 2.25^b
TBARS, nmol/L	1.84 ± 0.06^a	3.21 ± 0.11^b	4.36 ± 0.16^b

Explanations: a, b, c – means with different superscript in the same line significantly differ ($p < 0.05$)

During the last 10 years it appears that the prevalence of paratuberculosis has been increasing in sheep (3) and the disease causes economic losses (13, 30). A successful control program and prevention depend on animal health authorities and livestock industries acquiring a good understanding of the nature and epidemiology of the infection, and of the application of tools for diagnosis and control (33). An effective diagnosis is more important than an expensive treatment. However, additional biochemical markers are essential for control of paratuberculosis in animals. Domestic sheep do not generally show clinical signs until 2-3-years-of-age (2). Thus, the sheep > 2-years-of-age were sampled and tested for paratuberculosis in the study.

Sheep rarely show persistent watery diarrhea, possibly in the terminal stages of the disease (22). Goats can become persistent fecals hedders about 1 year post-infection without any clinical signs of paratuberculosis (16). During the clinical disease, the only consistent finding is weight loss despite apparently normal food intake (27). In the present study, 12 sheep had normal fecal pellets, 8 had intermittent diarrhea, and only 4 had chronic watery diarrhea. No parasiter infection was found after a parasitological examination in the sheep with diarrhea. So diarrhea in this study is thought to be directly on account of paratuberculosis. All the diseased sheep in clinical group showed dehydration, anorexia and weight loss.

There are several studies available reflecting the association between paratuberculosis and biochemical parameters of serum, especially in cattle, goats and camels (7, 21, 31), but such studies are very limited for sheep (12). In these studies, especially low albumin, Ca and Mg levels were reported to be in connection with cases of clinical disease (7, 12, 31). Hypoproteinemia are likely to be present in advanced clinical cases (12). The hypoproteinemia might be attributed to decrease of albumin (7). In the present study, there was a significant decrease of albumin that might be due to leakage of albumin through damaged tissues and a destructive granulomatous inflammatory response develops that eventually leads to intestinal malabsorption and protein losing enteropathy (29). And this case was more specific in the clinical group than the subclinical group because of the presence of intermittent and chronic watery diarrhea in sheep in the clinical group. Morever, hypocalcemia in sheep in the present study has also been previously reported in affected sheep and can be associated with hypoalbuminemia and decreased fraction of calcium bound with albumin (6). In contrast, serum concentrations of calcium, albumin and total protein were reported not to be different between the infected, suspected and negative sheep (18). Although, the previous studies determined significant increases in total bilirubin (12), in our study total bilirubin increased insignificantly. Also other determined parameters concerning hepatic functioning like globulin, glucose, total cholesterol and triglycerides were found to be at normal levels (22). However, the levels of BUN and creatinine were also in normal levels (22).

ROS might be produced as a result of bacterial infection and if they are not removed by endogenous enzymatic (SOD, GSH-Px) and non-enzymatic (GSH) antioxidant defenses of the organism, oxidative stress will be produced as demonstrated in the present findings (25). In the present study, the levels of SOD, GSH-Px and GSH decreased significantly ($P < 0.05$) in clinical and subclinical groups compared to the control group and the plasma level of TBARS increased significantly ($P < 0.05$) in clinical and subclinical groups compared to the control group. Increased oxidative stress can lead to changes in SOD, GSH-Px and GSH activities (7, 21). Also, in the present study the highest percentage of ELISA positive animals were represented by those with low GSH-Px activity. A decrease in SOD, GSH-Px and GSH activities in the study can be explained by a serious damage that occurred in the erythrocyte membrane and other cellular structures depending on inability to fully detoxify oxygen free radicals (1). The reduction in SOD, GSH-Px and GSH activities shows that an anti-oxidant defence system due to an increased oxidative stress remains inadequate. As SOD, GSH-Px and GSH are involved in the conversion of radicals into less effective metabolites, these changes coupled to an increase in TBARS concentrations confirmed the occurrence of an oxidative stress during paratuberculosis (17).

In conclusion, the parameters related with antioxidants and oxidative stress such as SOD, GSH-Px, GSH and TBARS concentrations and some biochemical parameters such as total protein, albumin, Ca, Mg levels in the present study are rather cognitive than practical but may be considered in the differential diagnosis and prognosis of both clinical and subclinical paratuberculosis in sheep.

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