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BLOOD METABOLIC PROFILE AND OXIDATIVE STATUS OF ENDANGERED MEDITERRANEAN SHEEP BREEDS DURING PREGNANCY

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Abstract

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The aim of the present research was to determine metabolic profile and oxidative status of critically endangered Dubrovnik sheep and Zeta Zuja breeds of sheep and compare them to physiological values, which could help in understanding quality of feeding. The study was conducted on 40 sheep (20 Dubrovnik sheep in Croatia and 20 Zeta Zuja in Montenegro). Sheep were an average age of three years, in the last third of pregnancy. Feeding of sheep was based on local grazing pasture, with addition of 150 g of corn/day in feeding Dubrovnik sheep. In blood of both breeds following indicators were determined: leukocyte number – WBC, erythrocytes – RBC, platelet – PLT, content of hematocrit, hemoglobin, mean corpuscular volume – MCV, mean corpuscular hemoglobin – MCH and mean corpuscular hemoglobin concentration – MCHC, Ca, P – inorganic, Na, K, Mg and Fe; urea, glucose, total protein, albumin, cholesterol; HDL-cholesterol, LDL-cholesterol, triglycerides, NEFA, BHB; and activity of enzymes: ALT, AST, CK, GGT, GSH-Px and SOD. Analyzing hemato – chemical indicators, pH and mineral content in both breeds there were no major deviations from the sheep's reference values, except higher concentrations of total protein, CK activity, slightly lower pH in the blood of Zeta Zuja, as well as a higher concentration of albumin, triglyceride, MCV content, lower Ca, Fe, GSH-Px and SOD activity. Significantly higher concentrations of K, Na, Fe, glucose, NEFA and content of RBC, hemoglobin and hematocrit and pH value were determined in blood of Dubrovnik sheep. In blood of Zeta Zuja significantly higher concentrations of urea, total protein, BHB, platelet content, activity of AST and CK, and lower GSH-Px were determined. In this research specific effect of breed was determined. If sheep are in the food deficit their rations must be supplemented with certain amount of concentrate feed and feed rich in selenium, especially in advanced stage of pregnancy.

Key words: endangered sheep, Mediterranean Region, blood metabolic profile, oxidative status, pregnancy

Abbreviations: ALT - Alanin aminotransferase; AST - Aspartate aminotransferase; BHB - Beta-hydroxybutyrate; CK - Creatine kinase; GGT - Gamma-glutamyl transferase; GSH - Px-Glutathione peroxidase; HDL cholesterol - High density lipoprotein cholesterol; K_3EDTA - three-potassium ethylenediaminetetraacetic acid; LDL cholesterol - Low density lipoprotein cholesterol; MCH - Mean corpuscular hemoglobin; MCHC - Mean corpuscular hemoglobin concentration; MCV - Mean corpuscular volume; NADPH - Nicotinamide adenine dinucleotide phosphate; NEFA - Non-esterified fatty acids; PLT - Platelet count; RBC - Red blood cells; SOD - Superoxide dismutase; WBC - White blood cells

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Introduction

The Mediterranean Region is an area rich in genetic diversity in sheep, but a certain number of sheep breeds are considered endangered. Only Dubrovnik sheep (Dubrovnik Ruda) is considered to be critically endangered breed of sheep in Croatia, and in Montenegro Zeta Zuja breed (Antunović et al., 2014). These breeds belong to the group Zackel sheep. Nowdays in Croatia 712 heads of Dubrovnik sheep are raised and in Montenegro about 500 sheep of Zeta Zuja (Markovic et al., 2013). In both countries, protection measures and possible improvement of the economic efficiency of their breeding have been set. Base feeding of sheep in these areas is pasture with very small amounts of a concentrated feed or, more often, without the supplement. The climate is typically Mediterranean with hot and long summers, thus animals are often in deficit due to scarce pastures which can lead to a variety of production and health problems (Di Trana et al., 2006). Blood metabolic profile is an important laboratory diagnostic technique that can be used efficiently to assess the nutritional status and animal health (Herdt et al., 2000; Macrae et al., 2006; Castillo et al., 2006). Determining the concentration of biochemical and hematological parameters, pH and enzyme activity in blood complements previous parameters which were included in the analysis of the blood metabolic profile. The main blood indicators of lipomobilization in ruminants include determining concentration of BHB and NEFA (Gonzalez et al., 2011). Antioxidant enzyme activities are sensitive markers of oxidative stress, as their levels may increase or decrease in response to reactive oxygen species (Mann et al., 2013). Primarily, those are glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD). GSH-Px is antioxidant enzymes that catalyze the reduction of hydrogen peroxide and lipid hydroperoxides to destroy free radicals produced during normal metabolic activity (Yue et. al., 2009). SOD protects the cell integrity structurally and metabolically from pathological conditions by catalysing the conversion of superoxide anions (O_2^-) to hydrogen peroxide (H_2O_2) to protect the cells from the harmful effects of O_2^- (Agarwal et al., 2005; Nisbet et al., 2007). However, there are significant deviations in these indicators of metabolic profile and enzymes activity in the blood of sheep depending on some environmental and genetic factors (Braun et al., 2010; Abdelrahman and Aljumaah, 2012; Donia et al., 2014). Also, it is known that pregnancy and lactation are physiological stages which can cause metabolic stress (Drackley, 1999), thus changing the parameters of metabolic profile, and leading to metabolic diseases. Generally, there are very few research that combine the study of metabolic profile and oxidative stress in sheep, especially in pregnant sheep. So far, a small number of studies that aimed to determine the metabolic profile through a small number of parameters in both breeds (Antunović et al., 2011, 2013, 2014) were conducted, but there is no research on their oxidative status. Therefore, the aim of the present study was to determine the overall metabolic profile and oxidative status of Dubrovnik sheep and Zeta Zuja, and compare them to physiological values. This would also help to understand the quality of nutrition of mentioned sheep breeds and allow the elimination of observed mistakes (Antunović et al., 2002; Whitney et al., 2009).

Materials and Methods

The study was conducted on 40 sheep: 20 Dubrovnik sheep in Dubrovnik-Neretva County, Croatia (42^0 92‘ N, 17^0 68’ E) and 20 Zeta Zuja sheep in Montenegro near Podgorica (42^0 26’ N; 19^0 16’ E) in September 2013. An altitude site in Croatia was 135 m, and in Montenegro 55 meters. Sheep were an average age of three years, in the last third of pregnancy and in satisfying physical condition. The average body weight of sheep Dubrovnik Rude was 52.70 kg and Zetska Zuja 40.00 kg. Feeding of the sheep was based on the local grazing pasture, and sheep of Dubrovnik Rude were also fed with addition of 150 g corn/day. Water and animal feed salt were constantly available. Hot long summers, warm autumns and springs, with average environmental temperature in September of 21.6^0C, rainfall of 87.2 mm/m^2 and 60% of relative air humidity are characteristic for Mediterranean area climate. The above data refer to the average of the last 20 years for the September in Croatia. The average annual rainfall for the site in Montenegro during September is 120 mm, with maximum and minimum environmental temperature of 27.40^0C and 16.50^0C.

Blood sampling was carried out in the morning before sheep were left on pastures and for researching the sheep metabolic profile, in order to determine the hematological parameters, blood was taken in a sterile vacutainer with K_3EDTA as anticoagulants for biochemical indicators and in heparin tubes for determination pH value. Determination of hematological parameters (leukocyte number – WBC, erythrocytes – RBC, platelet – PLT, as well as content of hematocrit, hemoglobin, mean corpuscular volume – MCV, mean corpuscular hemoglobin – MCH and mean corpuscular hemoglobin concentration – MCHC) was performed in the whole blood of sheep on automatic 3 diff veterinary hematology analyzer Sysmex pocH-Vef-100-iV. Blood samples for biochemical parameters were first centrifuged (32 Rotofix A) at 3000 rpm for 10 minutes, when serum was separated in specific eppendorf tube and frozen afterwards. After unfreezing, in serum samples from both breeds concentrations of biochemical indicators (minerals Ca, P-inorganic, Na, K, Mg and Fe, and urea, glucose, total protein, albumin, cholesterol, HDL-cholesterol,

LDL-cholesterol, triglycerides, NEFA, BHB, and activity of enzymes ALT, AST, CK, GGT) in automatic biochemical analyzer Olympus AU400 (Olympus ®, Japan) were simultaneously determined. The activity of glutathione peroxidase (GSHPx) in serum was determined by Ransel ® kits (Randox, UK) on an automatic analyzer Olympus AU 400 (Olympus, Japan) at a wavelength of 240 nm. The principle of the reaction is based on the oxidation of glutathione with cumene peroxide with a catalytic activity of GPx. The resulting oxidized form of glutathione is immediately converted further to a reduced form with the presence of glutathione reductase and NADPH as an oxygen acceptor. Thereby NADPH, receiving oxygen, becomes oxidized form, NADP. The activity of total superoxide dismutase (SOD) in serum was determined by using RANSOD ® kits (Randox, UK) on an automatic analyzer Olympus AU 400 (Olympus, Japan), at a wave length of 510 nm. The method is based on the generation of superoxide radicals from xanthine by xanthine oxidase which react with 2 - (4-iodophenyl) -3 - (4nitrofenil) 5-phenyltetrazole chloride to form formazan red coloration.

Mean values of the research results were calculated by MEANS procedure in the computer program SAS 9.3. Differences between mean values were analyzed with GLM (General Linear Model) procedure analysis of variance (ANOVA). In cases where the analysis of variance showed significant difference Fisher's post hoc test has been made. Data are reported as mean, standard deviation and total standard error. Statistical differences was declared at $P < 0.05$ or lower.

Results and Discussion

Analyzing hemato-chemical parameters in both breeds (Table 1) it can be seen that there were no major deviations from the reference values for sheep (Kaneko et al., 2008; Kramer, 2000).

Higher concentration of total protein in blood of Zeta Zuja, and slightly higher concentrations of albumin and triglycerides, as well as the content of MCV in both breeds were determined. Table 2 shows the pH and mineral content in the blood of Dubrovnik sheep and Zeta Zuja. It is evident that there were no significant deviations from the reference values, except for minor variations in pH of Zeta Žuja and concentration of Ca and Fe in both breeds. Analyzing Table 3, it is evident that higher CK activity and lower GSH-Px and SOD in comparison to reference value were determined. Significantly higher concentrations of K, Na, Fe, glucose and

Table 1
Hemato-chemical indicators in blood of Dubrovnik sheep and Zeta zuja sheep

Indicators	Breed (Mean ± s)		Significance (P value)	Reference value*
	Dubrovnik sheep	Zeta zuja sheep		
Glucose, mmol/L	3.63 ± 0.60	3.30 ±0.40	0.046	2.78-4.44
Urea, mmol/L	4.93 ± 1.17	7.04 ± 1.12	<0.001	2.86-7.14
Total protein, g/L	77.45 ± 2.19	87.00 ± 5.31	<0.001	60-79
Albumins, g/L	31.67 ± 2.19	30.61 ± 2.22	0.136	24-30
Globulins, g/L	47.78 ± 6.14	56.39 ± 6.24	<0.001	>50[1]
Cholesterol, mmol/L	1.82 ± 0.32	1.84 ± 0.35	0.834	1.35-1.97
HDL-cholesterol, mmol/L	0.95 ± 0.12	0.93 ± 0.14	0.618	-
LDL-cholesterol, mmol/L	0.73 ± 0.19	0.80 ± 0.25	0.287	-
Tryglicerides, mmol/L	0.33 ± 0.18	0.26 ± 0.07	0.114	0.0-0.2
NEFA, mmol/L	0.46 ± 0.17	0.032 ± 0.01	<0.001	>0.4[1]
BHB, mmol/L	0.087 ± 0.17	0.31 ± 0.07	<0.001	>0.6[1]
WBC (x 10^9 L)	9.11 ± 2.53	8.51 ± 1.74	0.391	9-15**
RBC (x 10^{12} L)	9.89 ± 1.14	8.83 ± 0.763	0.002	9-15**
PLT (x 10^9 L)	223.79 ± 81.06	426.30 ± 114.09	<0.001	500**
Hemoglobin, g/L	110.57 ± 9.59	98.95 ± 7.40	<0.001	90-150**
Hematokrit	0.44 ± 0.04	0.38 ± 0.03	<0.001	0.41[2]
MCV, fL	44.69 ± 2.34	43.62 ± 2.72	0.189	28 – 40
MCH, pg	11.23 ± 0.64	11.22 ± 0.44	0.917	8 – 12
MCHC, g/L	251.30 ± 9.88	258.05 ± 14.60	0.095	292[2]

*Kaneko et al. (2008), **Kramer (2000); [1]Whitaker et al. (1995)- for cows; [2]Dias et al. (2010); s –standard deviation

Table 2
Blood pH values and minerals concentration in Dubrovnik sheep and Zeta zuja sheep

Indicators	Breed (Mean ± s)		Significance	Reference values
	Dubrovnik sheep	Zeta zuja sheep	(P value)	Kaneko et al. (2008)
pH values	7.44 ± 0.05	7.31 ± 0.07	<0.001	7.35–7.50
Ca, mmol/L	2.65 ± 0.15	2.68 ± 0.13	0.571	2.88-3.20
P-inorganic, mmol/L	1.68 ± 0.45	1.88 ± 0.27	0.092	1.62-2.60
K, mmol/l	5.36 ± 0.55	5.11 ± 0.26	0.003	3.90-5.40
Na, mmol/l	150.11 ± 3.27	143.40 ± 1.54	<0.01	139.00-152.00
Mg, mmol/L	1.10 ± 0.08	0.96 ± 0.07	<0.001	0.91-1.31
Fe, µmol/L	29.20 ± 9.15	20.66 ± 5.59	0.002	29.70 do 39.70

s –standard deviation

Table 3
Blood enzymes activity in Dubrovnik sheep and Zeta zuja sheep

Indicators	Breed (Mean ± s)		Significance	Reference value
	Dubrovnik sheep	Zeta zuja sheep	(P value)	Kaneko et al. (2008)
AST, U/L	94.52 ± 14.09	118.17 ±16.30	<0.001	60-280
ALT, U/L	17.99 ± 5.33	18.80 ± 5.25	0.631	6-20
GGT, U/L	49.50 ± 6.77	34.54 ± 12.15	<0.001	20-52
CK, U/L	115.20 ± 35.26	301.20 ± 140.63	<0.001	106-168
GSH-Px, U/L	235.34 ± 116.76	322.28 ± 147.17	0.045	>600*
SOD, U/mL	0.234 ± 0.31	0.245 ± 0.223	0.915	0.184**

*Pavlata et al. (2012)- whole blood; **Maan et al. (2013) – goats; s –standard deviation

NEFA, content of RBC, hemoglobin and hematocrits as well as the pH were determined in Dubrovnik sheep blood compared to Zeta Zuja. In blood of Zeta Zuja significantly higher concentrations of urea, total protein, BHB, platelet content and activity of AST and CK, as well as lower GSH-Px, compared to Dubrovnik sheep were determined. Lower concentrations of K, Na, Fe, glucose and NEFA and higher BHB in the blood of Zeta Zuja indicate the possibility of pregnant toxemia.

The above is indicated and identified by significantly higher urea concentration and activity of CK and AST as well. Specifically, these changes indicate the beginning of degradation of body reserves (proteins) in order to ensure the needs of the growing fetus. Urea is created in the liver from ammonia produced during degradation of proteins in fore stomachs during protein catabolism and amino acid usage in the process of gluconeogenesis, which is probably due to metabolic redirection (Belic et al., 2011). Sheep are usually protected from hypoglycaemia by eating large quantities of food, particularly grain, which increases the exogenous glucose supply. Then liver transforms glycogen to glucose, and body tissues of pregnant sheep perform the hydrolysis of fats, where the glycerin is transformed into glucose, and fatty acids oxidized due to the production of energy.

During oxidation process ketone bodies (acetone, aceto acetic acid, beta-hydroxy butyric acid, etc.) are formed, which can accumulate to the concentrations that result in hyperketonemia. Beginning of hypoglycemia is accompanied by increased catabolism of fat, leading to the accumulation of fat, increase of liver fat content and ketone bodies in blood and urine of sheep. Dubrovnik sheep received a ration containing of corn, rich in non-fiber carbohydrates. Minor et al. (1998) concluded that in ruminant during high non-fiber carbohydrates feeding leads to increased ruminal levels of propionate which is the major glucose precursor. High level of non-esterifed fatty acids and glucose concentration are indicators of lipid metabolization and fatty acid oxidation (Wathes et al., 2009). If an animal is unable to consume enough feed to meet mainatenance requirements, it uses body reserves, which results in increase of serum NEFA and urea due to adipose and protein catabolism (Caldeira et al. 2007). Concentration of NEFA higher than 0.40 mmol/L indicates problems with energy balance and subsequent intensive lipomobilization (Oetzel, 2004; Djoković et al., 2013).

Dubrovnik Ruda is a breed of sheep that is physically more developed than Zeta Zuja (Marković et al., 2013) and therefore has a greater genetic potential for growth, which indicates a higher nutritional needs that are not fulfilled by this kind of rations. This is particularly expressed because they were in the last third of pregnancy when the nutritional needs are increased. Higher concentration of NEFA in the blood of Dubrovnik sheep (0.46 mmol/L) indicate mentioned above. Whitaker et al. (1999) found that high concentrations of BHB before calving in cows indicate a loss of condition in late pregnancy. Whitaker et al. (1995) have pointed out that the reference value for the BHB concentration in blood of cows before calving was > 0.6 mmol/L and after calving > 1.0 mmol/L. Duffield (2000) found that for the body energy imbalance of prepartum animals determining concentration of NEFA in blood is most used, while in postpartum period BHB concentrations. Stress has been shown on inducing energy mobilization in the form of lipid catabolism and protein degradation (Richardson and Herd, 2004). The fact that pregnancy, especially high pregnancy is very stressful period for sheep goes in favor of this (Hajdarević et al., 1989). Higher content of globulin and lower hemoglobin indicate possible parasitic invasion in sheep breed of Zeta Zuja. Lower GSH-Px activities indicate a lack of selenium in feed of both breeds. The reason for this is that the pasture areas where sheep graze are among the ones with a lack of selenium in soil, and consequently in herbs from pastures that is basis for sheep ration (Antunovic et al., 2010; Marković et al., 2011, unpublished data). Investigation of Andres et al. (1997) conducted with lambs in Spain showed a relationship between blood glutathione peroxidase (GSH-Px) activity in grazing sheep and the soil and pasture characteristics. Petazzi et al. (2009) found higher AST activity and albumin concentration, lower content of hematocrit, total protein and globulin as well as s similar hemoglobin content in the blood of Mediterranean sheep breeds (Comisana, Valle del Bela and Sardinian sheep). In investigation in Bulgaria, Angelov et al. (2013) determined with two Bulgarian indigenous sheep breeds (Karakachanian and Copper Red Shumen) higher blood activity of AST, ALT and CK enzymes. Table 4 presents available data on the research of metabolic profiles of Mediterranean sheep breeds. It is evident that the determined indicators of the blood metabolic profile, with minor deviations, are similar to breeds investigated in this research. The above indicates a significant influence, besides breeds, of non-genetic factors as well (nutrition, reproductive status and age).

Conclusion

The parameters of the metabolic profiles and oxidative status of Dubrovnik sheep and Zeta Zuja sheep in the present study indicate a specific effect of breed and non-genetic factors. If sheep are in condition of food deficit their rations have to be supplemented with certain amount of concentrate

Table 4
Blood metabolic indicators in different bredds of sheep in Mediterranean region

Indicators, mmol/L	Breed of sheep, country of breeding and references				
	Comisana Italia[1]	Chios Greece[2]	Churra Portugal[3]	Sarda Italia[4]	Comisana or Sardinian or Valle del Belice Italia[5]
Ca	-	2.18-2.75	2.1-3.2	-	9.96 mg/dl
P-inorganic	-	1.62-3.33	1.29-3.19	-	6.03 mg/dl
K	-	3.90-5.70	3.87-5.67	-	-
Na	-	143-156	145.6-160.8	-	-
Urea	9.83-12.47	4.28-14.28	2.7-11.3	4.5-6.0	41.12 mg/dl
Glucose	-	2.39-4.44	1.7-4.53	5.08-5.70	62.59 mg/dl
Total protein, g/L	72.1-73.95	58-89	63.1-88.1	65-72.1	74.8
Albumine, g/L	25.06-27.02	34-46	-	37.3-40.6	34.9
Globuline, g/L	-	21-45	-	27.7-31.4	39.9
Cholesterol	1.45-1.46	-	0.8-2.6	5.611-6.833	-
Tryglicerides	-	-	-	1.2-1.41	-
AST, U/L	-	-	58-300	-	164.33
ALT, U/L	-	-	5-46	-	-
GGT, U/L	-	58	10-90	67.75-70.93	-

[1]Piccione et al. (2009.); [2] Roubies et al. (2006.); [3] Dias et al. (2010.); [4]Annicchiarico et al. (2007.); [5]Petazzi et al. (2009)

feed and feed rich in selenium, especially in advanced stage of pregnancy.

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