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Status of lipid peroxidation and some antioxidants in sheep with acute ruminal lactic acidosis*)

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Summary

The purpose of this study was to evaluate the status of lipid peroxidation (LPO) and some antioxidants in sheep with acute ruminal lactic acidosis (ARLA). The material consisted of 20 Akkaraman sheep which had been diagnosed with ARLA and 20 healthy sheep of the same breed as the control group. The sheep were 1-2 years old. Ruminal fluid was obtained from the animals from both groups by means of a stomach tube and examined immediately. Blood samples were obtained from the jugular vein of the animals. The activites of catalase (CAT) and superoxide dismutase (SOD) as well as the levels of reduced glutatione (GSH) and malondialdehyde (MDA, a marker of LPO) were determined. Compared with the control group, the sheep with ARLA had a significantly higher level of MDA ($p < 0.0001$), whereas the activities of CAT and SOD and the level of GSH were significantly lower ($p < 0.0001$). In conclusion, this study highlights the occurrence of oxidative stress with important differences in antioxidant status, as reflected by the assessment of selected enzymatic and nonenzymatic antioxidants in sheep with ARLA and healthy controls. This indicates that the serum activities of SOD and CAT, as well as the levels of GSH and MDA, may be useful as indicators of LPO and antioxidant status in sheep with ARLA.

Keywords: Acute ruminal lactic acidosis, antioxidant status, lipid peroxidation, sheep

Acute ruminal lactic acidosis (ARLA) is a nutritional disorder suffered by ruminants. It is an acute indigestion resulting from a rapid, excessive digestion of foods containing easily fermentable carbohydrates, such as barley, wheat, potatoes, and sugar beet (6, 11, 19). When the quantity of highly fermentable carbohydrates in the ration is increased abruptly, normal fermentation patterns change. Gram-positive streptococcus and lactobacillus organisms become predominant, and lactic acid becomes the principal fermentation end product (19, 40). Lactic acid production increases osmotic pressure within the rumen so that fluid is drawn into the rumen from the circulatory system and thus from other tissues as well. The rumen pH decreases, resulting in rumen stasis, and a large percentage of the normal rumen microflora is destroyed. Most of the Gram-negative microorganisms and protozoa disappear. Clinical signs become severe when the pH reaches 4 to 5. Lactic acid isomers (D and L) in the rumen contribute to hyperosmolality and acidity. They are absorbed into the circulation and contribute to the depression of the blood pH and to the depression of the animal itself (32, 37, 46). Additional lactate is absorbed from the omasum, abomasum, and intestine as a result of the continuing fermentation of carbohydrates passing from the rumen. An osmotic gradient is also established within the intestine, drawing additional fluid into the lumen and contributing to profuse diarrhea (6, 39, 46). Chemical damage occurs to the surface epithelium of the rumen mucosa, bacterial and mycotic organisms begin to invade the rumen wall, and absorption patterns are changed. ARLA is often followed by bacterial or mycotic rumenitis (10, 15, 36, 37). The whole disease process is complex and variable, and its another component may be an endotoxic shock caused by the toxins released after the destruction of large numbers of Gram-negative microorganisms in the rumen content. Cardiovascular collapse, renal failure, muscular weakness, shock, and death are significant pathophysiological results seen

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in ARLA (11, 19, 40). Enterotoxemia, liver abscess, peritonitis, thiamine deficiency, hypocalcemia, and laminitis can develop in animals surviving the disease (11, 19, 27, 32, 37).

Components of reactive oxygen species (ROS) are produced during physiological and metabolic functions and can cause harmful oxidative reactions under conditions of excessive production. The situation in which the rate of ROS production is matched by the rate ROS removal from the organism is referred to as oxidative balance (7, 31). Oxidative stress may develop during situations such as protozoal (18, 35) and viral infections (28), toxications (9), gastric reperfusion injury (30, 33), and osteoarthritis (43). Primary targets of ROS are polyunsaturated fatty acids (PUFAs) in cell membranes. The resulting lipid peroxidation (LPO) may lead to the damage of the cell structure and functions (26, 45). Additionally, the decomposition of lipid hydroperoxides yields a wide variety of end-products, including malondialdehyde (MDA). One of the most frequently used ROS biomarkers, providing an indication of the overall LPO intensity, is MDA, which is a by-product of LPO (13, 20, 26, 38). The thiobarbituric acid (TBA) assay is the most common and easiest method used as an indicator of LPO and free radical activity in biological samples. The assay is based on the reaction of two molecules of TBA with one of MDA (8, 38).

Superoxide dismutase (SOD) is the first line of defense against ROS and is active in the detoxification of superoxide radicals (8, 22, 29). Under normal conditions, catalase (CAT) is of no great importance to most cell types, but in the presence of oxidative stress it is the most adaptive antioxidant enzyme and plays a significant role in cell defense against oxidative damage (25). Reduced glutathione (GSH) is the most important cellular antioxidant, which plays a major role in protecting cells against oxidative stress caused by ROS (3, 8, 41). There have been no studies on LPO and antioxidants in sheep with ARLA. The purpose of the present study was therefore to evaluate the levels of serum malondialdehyde (MDA) as a marker of lipid peroxidation (LPO), as well as that of some enzymatic (SOD and CAT) and nonenzymatic (GSH) antioxidants in sheep with ARLA.

Material and methods

Animals and clinical examination. The study involved 20 Akkaraman sheep with ARLA (ARLA group) from one herd, aged 1-2 years, admitted to the Veterinary Teaching Hospital School of Veterinary Medicine at Firat University, and 20 healthy Akkaraman sheep aged 1-2 years, randomly selected from a different herd (control group). The herds (from which the two groups originated) were composed of sheep obtained by the owners from different farms.

In a routine clinical examination of all animals (ARLA group and control group), the pulse and respiratory rates, the number of rumen contractions, rectal temperatures, and dehydration degrees were measured. Also mucous membranes and conjunctiva were examined.

Rumen fluid collection and examination. After the clinical examination, samples of rumen fluid were taken from the sheep with ARLA and from the healthy sheep through a stomach tube. The first portion coming from the tube was discharged to minimize the saliva contamination of these samples, and the rest (200-250 ml) was immediately examined (19, 27). These ruminal fluid samples were analyzed in terms of odor, color, pH, methylene blue reduction time, and protozoal activity. The odor of the samples was physically examined and classified as aromatic (normal) or acidic (abnormal). The color was classified as oily brownish green (normal) or milky grey (abnormal). The pH of the samples was measured with commercial test strips (ColorpHast, Merck KGaA, Darmstadt, Germany) and classified as normal (6-7), moderately low (5-5.9), or very low (4-4.9). For the methylene blue reduction time of the samples, 1 ml of 0.03% methylene blue was mixed with 20 ml of rumen fluid and compared for color with another unaltered tube of the fluid. Methylene blue reduction time was classified as 3-6 minutes (normal), 6-9 minutes, and over 9 minutes. The protozoal activity in the rumen fluid was evaluated microscopically (at × 40 magnification) and classified as normal, reduced, or absent.

Blood sampling. Blood samples were collected from the jugular vein of all animals. The blood samples were centrifuged at 3000 *g* for 10 min at room temperature. Serum was separated and stored at –30°C until analyses.

Biochemical analysis

MA measurement. Serum MDA levels were measured by the thiobarbituric acid (TBA) reaction according to the method described by Yoshioka et al (47). In this method, the lipid content, which has a low pH, is heated in the presence of TBA. One molecule of MDA with two TBA molecules form a stable chromogen which absorbs at 532 nm due to its red-pink color. For the calibration, 2.5, 5-10, and 20 μmmol/l concentrations of 1.1.3.3-tetraethoxypropan dissolved in alcohol were used.

SOD measurement. Serum SOD activity was assayed according to the method of Sun et al. (42). In this method, xanthine-xanthine oxidase system was used to generate a superoxide flux, and nitroblue tetrazolium (NBT) was used as an indicator of superoxide production. SOD activity was then measured by the degree of the inhibition of the reaction. One unit of the enzyme causes a 50% inhibition of NBT reduction. Results were expressed as U/ml.

CAT measurement. In the Goth method (23) CAT is measured by spectrophotometry. The spectrophotometric measurement of hydrogen peroxide is based on the principle that it creates a stable complex with ammonium molybdate.

GSH measurement. The GSH concentration was measured by the method described by Tietze (44).

Statistical analysis. Statistical analysis of the data obtained in the study was performed by the SPSS 11.5 statistical program package (SPSS Inc, Chicago, IL, USA). Differences between the groups were calculated by the Mann Whitney U test, which is nonparametric. The significance level between the two groups was determined to be $p < 0.05$. The data was presented with mean and standard deviations (Mean ± SD).

Tab. 1. Clinical signs in the sheep with acute ruminal lactic acidosis (ARLA) and in the control group

Parameter	ARLA group (n = 20)	Control group (n = 20)
Rectal temparature (T/°C)	Increased in all animals	Normal in all animals
Heart rate (P/min.)	Increased in all animals	Normal in all animals
Respiration rate (R/min.)	Increased in all animals	Normal in all animals
Ruminal stasis	In all animals	In none of the animals
Ruminal atony	In all animals	In none of the animals
Ruminal contractions (Rh/5 min.)	Decreased in all animals	Normal in all animals
Mucous membranes	Dirty hyperemic in all animals	Normal in all animals
Scleral congestion (engorged scleral vessels)	In all animals	In none of the animals
Diarrhea	In ten animals	In none of the animals
Dehydration	From moderate to severe in all animals	In none of the animals
Teeth grinding	In some animals	In none of the animals
Death	In seven animals	In none of the animals

Results and discussion

Clinical signs. The clinical signs observed in the sheep with ARLA and in the control group are shown in Tab. 1. In the clinical examination, all the sheep with ARLA were characterized by engorged scleral vessels, dirty hyperemic mucous membranes, moderate to severe dehydration, increased heart and respiratory rates, ruminal atony, ruminal stasis, and increased rectal temperature. Moreover, seven sheep died, ten suffered from diarrhea, and in some animals teeth grinding was observed. In the clinical examination of the control group, rectal temperature, heart and respiratory rates and rumen contractions were normal.

Ruminal fluid findings. The results of the ruminal fluid analyses for the sheep with ARLA and for the control group are given in Tab. 2.

Biochemical findings. The activities of CAT, SOD and the levels of GSH and MDA in the serum of the sheep with ARLA and of the control group are presented in Tab. 3. Compared with the control group, in the sheep with ARLA the level of MDA is significantly higher ($p < 0.0001$), whereas the activities of CAT and SOD, and the level of GSH are significantly lower ($p < 0.0001$).

ARLA is increasingly recognized as a significant disorder in ruminants that increases the morbidity and mortality of animals, especially for dairy cattle and sheep (46). The disease has been usually observed after a sudden or accidental feeding, depending on the errors that can be based on easily fermentable carbohydrate diets shaped by the result of taking herd sheep, rather than on the basis of individual cases (40).

In sheep, oxidant/antioxidant status has been identified in metabolic (1), toxic (9), trematodal (12), protozoal (18, 35) and viral diseases (28), as well as in physiological conditions, such as age (2) and pregnancy (17). However, oxidative stress and antioxidant status in sheep with ARLA has not been studied.

Infections and inflammations activate a variety of inflammatory cells that play important roles in the host's defense (14, 45). These cells are capable of generating large amounts of highly toxic molecules, such as ROS (including superoxide anion, hydrogen peroxide, and hydroxyl radicals) (8). In addition, ROS are capable of damage and peroxidation of numerous biomolecules, including DNA, carbohydrates, lipids, and proteins (5). ROS-induced oxidation of PUFAs in biological systems results in the formation of LPO products. One of the many biological targets of oxidative stress are lipids. PUFAs, in particular, are the most frequently targeted class of biomolecules. LPO

Tab. 2. The results of the ruminal fluid analyses for the sheep with acute ruminal lactic acidosis (ARLA) and for the control group

Parameter	Classification	Frequency (%)	
		ARLA group (n = 20)	Control group (n = 20)
Odor	Aromatic	0	100
	Acidic	100	0
Color	Olive, brownish-green	15	100
	Milky grey	85	0
pH	Normal (6-7)	0	100
	Moderately low (5-5.9)	15	0
	Very low (4-4.9)	85	0
Protozoal activity	Normal	0	100
	Reduced	20	0
	None	80	0
Methylene blue reduction time	3-6 minutes (normal)	0	100
	6-9 minutes	30	0
	> 9 minutes	70	0

Tab. 3. The mean levels and standard deviations (± SD) of serum MDA, enzymatic antioxidants (SOD and CAT), and non-enzymatic antioxidants (GSH) in the sheep with acute ruminal lactic acidosis (ARLA) and in the control group

Parameter	ARLA group (n = 20)	Control group (n = 20)	P-value
MDA (mol/l)	10.29 ± 3.51	7.06 ± 1.13	< 0.0001
CAT (KU/l)	36.96 ± 8.30	46.19 ± 4.55	< 0.0001
SOD (U/ml)	4.90 ± 0.61	5.69 ± 0.57	< 0.0001
GSH (nmol/l)	0.339 ± 0.160	0.560 ± 0.122	< 0.0001

gives rise to a number of secondary products. MDA, the principal and most studied product of the peroxidation of PUFAs, is a highly toxic molecule, and an increase in free radicals causes its overproduction (13, 20). MDA is a breakdown product that is frequently quantified as a measure of lipid hydroperoxides, and is accepted as an indicator of an elevated oxidative stress in the body (20, 26, 45).

Because major inflammatory cells, such as neutrophils and/or macrophages, generate a variety of ROS and release various proteases, tissue damage and destruction occur (5, 14). Peroxidation is not the same, so oxidative stress occurs because of either the excessive production of free radicals or inadequate availability of antioxidants or a combination thereof. The measuring of the activities of nonezymatic antioxidants, such as GSH, and of antioxidant enzymes, such as SOD and CAT, is an appropriate indirect way of assessing the status of antioxidant defense (7, 8, 31).

It has been shown that in bacterial diseases, such as traumatic reticuloperitonitis (TRP) (4), peritonitis (16, 34), and osteoarthritis (43), the oxidant/antioxidant balance is disturbed. Gazi et al. (21) state that the defaunation of ruminal fluid causes the antioxidant level in ruminal fluid to decrease and that the oxidant/antioxidant imbalance can deteriorate the performance and health of ruminants. Guo et al. (24) showed that in cattle with subacute rumen acidosis (SARA), the plasma MDA level increased because of an increase in carbohydrates, which easily fermented in the ration, whereas the antioxidant status had a positive impact when fodder with a high fiber content was added to the ration. In the present study, serum MDA concentrations were found to be increased in the sheep with ARLA, compared to those in the control group ($p < 0.0001$), whereas GSH concentrations, as well as SOD and CAT activities were lower than those in the control group ($p < 0.0001$). Because GSH, SOD, and CAT are involved in the conversion of radicals into less effective metabolites, these changes, coupled with the increase in MDA concentrations, suggest that an excessive ROS production occurred during the disease caused by easily fermentable carbohydrates. Moreover, ARLA was associated with oxidative stress, evidenced by an increase in serum MDA concentrations coupled with decreased SOD and CAT activities and GSH concentrations.

In conclusion, this study has highlighted the occurrence of oxidative stress with important differences in antioxidant status, as reflected by the assessment of some enzymatic and nonenzymatic antioxidants in the sheep with ARLA and in the healthy controls. This indicates that the serum activities of SOD and CAT, as well as the levels of GSH and MDA may serve as useful indicators of LPO and antioxidant status in sheep with ARLA. Additionally, changes in these parameters may provide ideas for further studies on ruminants with rumen acidosis.

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