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A different approach to diagnosis of subclinical mastitis: milk arginase activity

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ABSTRACT

The aim of this study was to determine the relationships between subclinical mastitis and milk arginase activity in dairy cows. Thirty, various breed 4-8 year old cows from Firat University Animal Hospital were used in the study. Subclinical mastitis was diagnosed by the California Mastitis Test (CMT) combined with bacteriological examination of milk samples. The subclinical mastitis group consisted of fifteen clinically healthy but CMT and microbiologically positive animals. CMT and microbiologically negative animals (n = 15) served as the control group. Arginase activity in milk samples was measured by spectrophotometer using the thiocemicarbazide diacetylmonoxime urea (TDMU) method and protein was determined with the folin phenol reagent. Arginase activity in the milk of animals with subclinical mastitis (0.26 ± 0.07 U/mg protein) significantly increased compared to the arginase activity in the milk from healthy animals in the control group (0.10 ± 0.03 U/mg protein). Milk enzyme activity in the mildly severe disease (0.26 ± 0.01 U/mg protein) was significantly lower than in the moderately severe disease (0.30 ± 0.01 U/mg). No relationship was found between the nature of the bacterial infection of the cows with subclinical mastitis and their milk arginase activity. It was concluded that assay of milk arginase activity may be used as an additional laboratory method for the diagnosis of subclinical mastitis.

Key words: subclinical mastitis, arginase, cow, California mastitis test

Introduction

Mastitis is the inflammation of all structures forming the mammary tissue and the surrounding connective tissue. The disease is the reaction of the mammary gland to

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irritants and significantly influences the quality and quantity of mammary tissue and milk (YÜKSEL et al., 2009). Mastitis has various underlying causes and degrees of severity, with subclinical mastitis being the most widely encountered form. A decrease in milk production and a reduction in milk quality are seen in subclinical mastitis, without signs of macroscopic inflammation or visible changes in the milk (YAGCI, 2008; JAIN et al., 2012). The disease results in changes in the composition of milk and shortening of the productive lives of cows. About 70-80% of mastitis-induced milk production losses originate from subclinical mastitis (GURBULAK et al., 2009).

In subclinical mastitis, some changes may be observed in the milk, including the increased presence of plasma proteins, changes in ion concentration, a reduction in the synthesis capacity of the mammary epithelium, the passage of intracellular components to the milk due to the destruction of local cells, and an increase in somatic cells (SANDHOLM and MATILLA, 1986). The first pathological change seen in animals with mastitis is the passage of albumin from the blood to the milk. Sodium, chloride and bicarbonate passage also increases, with subsequent changes in the pH of the milk (ALACAM, 1990). The concentration of milk histamine and glycogen content are higher than in healthy milk. The increase in the number of leucocytes results in an increased glycogen content. Enzymes associated with inflammation reaction increase during the inflammation (YAGCI, 2008).

Arginase (E.C. 3.5.3.1, L- arginine-amidino hydrolase) is the final enzyme of the urea cycle (KANDEMIR and OZDEMIR, 2009). Catalyzing the hydrolysis of L-arginine to ornitine and urea, arginase has two isoforms. While arginase I is localized in the cytoplasm, arginase II is found in the mitochondria (KEPKA-LENHART et al., 2008). Although the urea cycle is present only in hepatocytes, the arginase enzyme is seen in many other cells. The liver has the highest content and it is active in the urea cycle to transform ammonia to non-toxic components (SPEKTOR et al., 1982; FUENTES et al., 1994; BER and MUSZYNSKA, 1979). It has been reported to be present at low concentrations in many tissues, such as the kidneys, brain, intestines and the mammary gland, and serves special functions, such as polyamine synthesis and the production of the proline required for protein biosynthesis, in addition to its functions in the urea cycle (OZCELIK and OZDEMIR, 2003). BASCH et al. (1997) suggested proline synthesis in the mammary gland, where it has been reported to synthesize the amino acid proline required for casein synthesis.

It has been found that arginase has many metabolic functions and plays an important role in certain diseases (KANDEMIR and OZDEMIR, 2009), and arginase activity increases in inflammatory processes (BACHETTI et al., 2004). The purpose of this study was to study the relationship between subclinical mastitis and milk arginase activity, and investigate the value of milk arginase activity in the diagnosis of subclinical mastitis.

Materials and methods

Animal and clinical examination. The study material consisted of 30 cows of varying breeds, aged between 4 and 8 years, admitted to the Obstetrics and Gynecology Clinic of the Animal Hospital, Faculty of Veterinary Medicine, Fırat University. Samples of milk were collected from all mammary quarters of the cow for the California Mastitis Test (CMT), and for bacteriological examinations 5 mL of milk samples were collected in sterile tubes, observing asepsis-antisepsis rules. Animals with no clinical changes in their milk, but positive for CMT and/or found be bacteriologically positive, were classified as having subclinical mastitis (n = 15), whereas those who were CMT and bacteriologically negative formed the control group (n = 15).

Enzyme assay. The milk samples collected were centrifuged for 15 minutes at 15000 g. The fat layer was removed and the supernatant portion was used for assay. Milk arginase activity was measured by spectrophotometer, using a modification of the thiosemicarbazide-diacetylmonoxime urea (TDMU) method, described by GEYER and DABICH (1971). Measurements were made in duplicate.

Briefly, 0.1 mL of supernate was diluted with 2 mM $MnCl_2$ at the rate of 1:10 (v/v), and preincubated for 14 minutes at 58 °C. Tubes containing 0.3 mL enzyme source, 0.3 mL L-arginine (120 mM, pH 9.5) and 0.4 mL carbonate buffer (200 mM, pH 9.5) were incubated for 10 minutes at 37 °C. The reaction was stopped by adding 3 mL acid reagent, and 2 mL color reagent was added to the tubes, which were kept in the water bath for 10 minutes. Then tubes were taken from the water bath, cooled, and their absorbance was measured at 520 nm. The principle of arginase activity determination was based on spectrophotometric measurement of urea produced by hydrolysis of L-arginine by arginase. One unit of arginase activity was expressed as the amount of enzyme catalyzing the formation of one micromole of urea/h at 37 °C. The results (specific activity) were presented as units/mg protein.

The protein concentration was determined by the method of LOWRY et al. (1951), using bovine serum albumin as standard. Briefly, tubes including 1 mL alkaline copper reagent and 0.1 mL supernatant samples were mixed and incubated for 10 minutes at room temperature. Following this, 4 mL of the folin and Ciocalteu's phenol reagent were added to the tubes, and mixed and incubated for 5 min at 55 °C. The absorbance of the samples was measured at 650 nm using a Shidmadzu UV 240 spectrophotometer.

Statistical analyses. Data were analyzed statistically by analysis of variance (ANOVA). Differences between the means were statistically estimated by the Independent Sample T Test. All values were expressed as mean and standard error of the mean (SEM). Statistical significance was judged at a significant level of $P<0.05$.

Results

Among the subclinically infected animals, 8 cows had staphylococcus infection, while streptococcus infection was revealed in 7 cows. The mean milk arginase activity in healthy animals (Table 1) was 0.10 ± 0.03 U/ mg protein, while cows with subclinical mastitis showed a mean value of 0.26 ± 0.07 U/mg protein in milk (P<0.01).

Table 1. Milk arginase activities (mean ± SEM) of subclinical mastitis and healthy cows

Parameter	Mastitis Group	Control Group	P
Milk arginase activity (U/mg protein)	0.26 ± 0.07	0.10 ± 0.03	<0.01

An examination of milk arginase activity by the severity of mastitis (Table 2) showed that the enzyme activity was 0.26 ± 0.01 U/mg protein at mild (++), increasing up to a concentration of 0.30 ± 0.01 for cows with moderate severity (+++). There was a positive correlation (P<0.05) between the severity of the mastitis and milk arginase activity.

Table 2. Correlation between California Mastitis Test severity and milk arginase activity.

Parameter	Mild severity CMT^{+2}	Moderate severity CMT^{+3}	P
Milk arginase activity (U/mg protein)	0.26 ± 0.01	0.30 ± 0.01	<0.05

No correlations (P>0.05) were found between the species of bacteria in milk and enzyme activity (Table 3).

Table 3. Relationships between bacterial species and milk arginase activity.

Parameter	*Staphylococcus aureus* (n = 8)	*Streptococcus agalactiea* (n = 7)	P
Milk arginase activity (U/mg protein)	0.30 ± 0.16	0.29 ± 0.10	>0.05

Discussion

The early diagnosis and appropriate treatment of mastitis are recommended as solving one of the major problems of dairy farming which causes significant economical losses (SMITH, 1983). For this reason much research has been done into the early diagnosis and treatment of the condition. Currently, direct methods such as microscopic somatic cell count (SCC), fossomatic and Coulter Counter are commonly used, as well

as indirect methods such as: the California Mastitis Test, White Side Test, Catalase Test and Wisconsin Mastitis Tests (ANONYM., 1981). Measurement of the milk's electrical conductivity and microbiological examinations are also methods used in diagnosing subclinical mastitis (YAGCI, 2008) and the biochemical changes caused by this disease in the blood and body fluids can be detected using various laboratory tests (KITCHEN et al., 1980). Today, biochemical analyses are one of the most commonly used methods for following the course of a disease. The determination of the disease's impacts, particularly on the biochemical parameters in the blood and serum, has an important role in the treatment of the disease and in the use of protective and control measures for this disease (ALTUNTAS and FIDANCI, 1993).

Many biochemical studies had been conducted on cows with subclinical mastitis and different enzyme activities analyzed to assist the diagnosis process. Lactate dehydrogenase (LDH), N-acetyl-β-D glucosaminidase (NAGase) and alcaline phosphatase (ALP) are some of the enzymes used for diagnosis of mastitis (NIZAMLIOGLU et al., 1989; NIZAMLIOGLU et al., 1992).

ALP and alanine aminotransferease (ALT) enzyme activities were studied in both the blood serum and the milk in animals with subclinical mastitis, and efforts were made to associate these enzymes with the condition (YÜKSEL et al., 2009; CETIN et al., 2005; BATAVANİ et al., 2003; NAK, 1999; ATROSHI et al., 1996; MERT et al., 1992). It was determined that ALP synthesis from the damaged cells was increased (TURGUT, 2000). MERT et al. (1992) and WADA et al. (2002) reported that the serum ALP activity was higher in animals with mastitis than in healthy animals. The serum and milk activity of ALP was found to be 15 times higher in animals with mastitis compared to controls and 6 times higher in the case of ALT activity (TRIPATHI, 2000). N-acetyl β-D glicosaminidase, a lysosomal enzyme have been found to be an indicator of tissue destruction, and although its role in the mammary gland remains unknown, it was found to be synthesized at an increased level during mastitis (YAGCI, 2008), with reports of a positive correlation between the enzyme activity in milk and CMT findings (NIZAMLIOGLU et al., 1992). It was also found that the lactate dehydrogenase (LDH) enzyme was a determining enzyme in the diagnosis of mastitis, and that the LDH level in milk with mastitis was 3 times higher than that in the serum of normal animals, and 16 times higher than in normal milk (BOGIN and ZIV, 1973).

In the present study, we focused on the arginase activity in milk and found that the arginase activity in milk with subclinic mastitis was about 2.5 times higher than the arginase activity in the control group. Although arginase enzyme is known to be present in the mammary gland (OZCELIK and OZDEMIR, 2003; BASCH et al., 1997), no studies have been undertaken of changes in the enzyme activity in milk under different circumstances.

L-Arginin is metabolized to nitric oxide by the nitric oxide synthase enzyme and is also metabolized to urea and ornitine by the arginase (COOK et al., 1994). In inflammatory cases, these two pathways compete with each other. Arginase is also present in the region of inflammation. It is stated that arginase functions play a role in regulating apoptosis in the inflammatory region, and produce structural and cellular proteins (WADDINGTON and CATTELL, 2002). Arginase is necessary for production of polyamines and proline, which are required for cell proliferation and matrix production (WADDINGTON et al., 1998).

Arginase contributes to the healing process, because NO formation is suppressed by an increase in the arginase activity in the inflammatory region. It is proposed that arginase passes into the milk in the event of mammary gland inflammation, with a subsequent rise in milk arginase activity. This may be of importance in the diagnosis of subclinic mastitis. This study concludes that milk arginase activity may be used as an additional laboratory method for the diagnosis of subclinical mastitis.

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SAŽETAK

Cilj istraživanja bio je utvrditi povezanost između supkliničkog mastitisa i aktivnosti arginaze u mlijeku krava. U istraživanje je uključeno 30 krava u dobi od 4 do 8 godina. Krave su bile različitih pasmina i pacijenti bolnice Firat Sveučilišta. Supklinički mastitis dijagnosticiran je kombinacijom kalifornijskog mastitis testa (KMT) i bakteriološke pretrage uzoraka mlijeka. Skupinu sa supkliničkim mastitisom činilo je 15 klinički zdravih krava pozitivnih pretragom s KMT i bakteriološki pozitivnim nalazom. Petnaest krava s pozitivnim KMT i bakteriološki negativnim nalazom poslužilo je kao kontrolna skupina. Aktivnost arginaze u uzorcima mlijeka utvrđena je spektrofotometrijski, uz primjenu tiocemikarbazid diacetilmonoksim ureja metode (TDMU), a bjelančevine su bile određene pomoću folin fenol reagensa. Aktivnost arginaze u mlijeku krava sa supkliničkim mastitisom (0,26 ± 0,07 U/mg bjelančevina) bila je značajno povišena u usporedbi s uzorcima iz mlijeka zdravih krava kontrolne skupine (0,10 ± 0,03 U/mg bjelančevina). U krava sa slabo izraženim simptomima bolesti, aktivnost enzima u mlijeku (0,26 ± 0,01 U/mg protein) bila je značajno niža u odnosu na krave s umjerenim simptomima (0,30 ± 0,01 U/mg). Nije utvrđena povezanost između prirode bakterijske infekcije i supkliničkog mastitisa odnosno aktivnosti arginaze u mlijeku. Zaključak je da analiza aktivnosti arginaze može poslužiti kao dodatna laboratorijska metoda za dijagnostiku supkliničkog mastitisa.

Ključne riječi: supklinički mastitis, arginaza, krave, kalifornijski mastitis test