Investigation of blood and milk lactoferrin concentrations in lactating ewes with subclinical mastitis

Cevat NİSBET[1], Gül Fatma YARIM[1], Gülay ÇİFTCİ[1], Nilgün GÜLTİKEN[2], Sena ÇENESİZ[1]

[1] Department of Biochemistry, Faculty of Veterinary Medicine, University of Ondokuz Mayıs, Samsun
[2] Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, University of Ondokuz Mayıs, Samsun

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Summary: The aim of the present study was to determine the availability of blood and milk lactoferrin concentrations in the diagnosis of subclinical mastitis in lactating Karayaka ewes. The milk samples obtained from 43 mid-lactating Karayaka ewes, aged between 2 and 3, were examined by California Mastitis Test (CMT) and somatic cell count (SCC) methods. Animals (n=23) had positive CMT score and SCC between 300.000-1.000.000 cells/ml were defined as subclinical mastitis group, whereas ewes with CMT negative and SCC between 100.000-200.000 cells/ml were described as control (healthy) group. The serum were obtained from blood and milk samples of ewes and ELISA was performed to detect blood and milk lactoferrin concentrations. The mean blood lactoferrin concentrations in subclinical mastitis group and control group were 0.90±0.08 µg/ml and 0.85±0.08 µg/ml, respectively, however the difference was not significant ($p>0.05$). The mean milk lactoferrin concentrations in ewes with subclinical mastitis and healthy ewes was 23.98±3.45 µg/ml and 16.84±1.29 µg/ml, respectively ($p<0.01$). Consequently, it is suggested that the concentration of milk lactoferrin might be a useful marker in the diagnosis of ewes with subclinical mastitis.

Key words: Lactoferrin, mid-lactation, milk, ewe, subclinical mastitis.

Laktasyon periyodundaki subklinik mastitisli koyunlarda kan ve süt laktoferrin konsantrasyonlarının araştırılması

Özet: Bu çalışmada, laktasyon periyodundaki subklinik mastitisli ve sağlıklı Karayaka ırkı koyunlarda süt ve kan serumlarında laktoferrin konsantrasyonlarının belirlenmesi amaçlandı. Araştırmada, ortalama 2-3 yaşlarında ve laktasyonlarının orta döneminde bulunan Karayaka ırkı koyunlar, Kaliforniya mastitis test ve sütte somatik hücre sayımı yöntemleri ile subklinik mastitis yönünden incelendi. Sütleri Kaliforniya mastitis test pozitif ve somatik hücre sayımı 300.000-1.000.000 olan 23 adet koyun subklinik mastitis grubunu, Kaliforniya mastitis test negatif ve somatik hücre sayımı <300.000 olan 20 adet koyun ise kontrol grubunu oluşturdu. İneklerden kan ve süt örnekleri alınarak serumları çıkarıldı. Koyunların kan ve süt serumlarında laktoferrin ölçümleri ELISA test kitleri kullanılarak gerçekleştirildi. Subklinik mastitisli ve sağlıklı koyunların serum laktoferrin değişimleri sırasıyla ile 0.90±0.08 µg/ml ve 0.85±0.08 µg/ml olarak tespit edildi ve gruplar arasında istatistiksel olarak önemli bir fark bulunamadı ($p>0.05$). Süt serumu laktoferrin konsantrasyonları subklinik mastitisli ve kontrol gruplarında sırasıyla 23.98±3.45 µg/ml ve 16.84±1.29 µg/ml olarak bulundu. Subklinik mastitisli grubun süt serumu laktoferrin düzeylerinin kontrol grubununkine göre istatistiksel olarak yüksek olduğu belirlendi ($p<0.01$). Sonuç olarak, koyunlarda süt serumu laktoferrin konsantrasyonunun subklinik mastitisin teşhisinde faydalı bir belirteç olarak kullanılabileceği kanaatine varıldı.

Anahtar kelimeler: Koyun, Laktasyon, Laktoferrin, Subklinik mastitis, Süt.

Introduction

Lactoferrin (LF) is an iron-binding glycoprotein of the transferrin family present in most biological secretions in the organism and it plays a key role immunity [22]. LF is synthesized from genital tract and mammary tissue [32] or from neutrophils by cell differentiation. It is also secreted by epithelial cells and can be found in exocrine fluids (saliva, pancreas, bile juice, gastric juice, lacrima, semen, bronchial and nasal mucus) as apolactoferrin [22,36]. Transferrin is a glycoprotein and found in plasma for an iron transporter [14].

It is also characterized as a metalloprotein with molecular mass of 80 kDa [21]. It is well known that iron binding areas of lactoferrin and transferrin have the same composition and geometry but have differences in molecular structure [5,26]. Lactoferrin's capability of binding iron is two times higher than

Yazışma adresi / Correspondence: *Cevat Nisbet, Department of Biochemistry, Faculty of Veterinary Medicine, University of Ondokuz Mayis, Samsun Turkey E-posta: cevatnisbet@gmail.com*

that of transferrin [1]. The ability to keep iron bound even at low pH (pH~2) is important, especially at sites of infection and inflammation where, due to the metabolic activity of bacteria, the pH may fall under 4.5. In such a situation lactoferrin also binds iron released from transferrin, which prevents its further usage for bacterial proliferation [34]. Transferrin releases its iron at pH 5 [25]. Transferrin releases iron in low pH environment due to infection and lactoferrin binds transferrin to prevent bacterial proliferation [8]. Therefore, lactoferrin concentration increases during inflammation [17]. It has been stated that measurement of lactoferrin concentration in fluids, such as milk, is to be important in the investigation of mammary gland infections [10]. Since lactoferrin has antimicrobial [21], antifungal, antiparasitic [34], antiviral [22], immun-modulator and antineoplastic activities, evaluation of its concentrations in pathological conditions was proved to be an important tool in the diagnosis of the presence of infection [32, 35]. It is well known that mastitis is one of the main diseases that causes economic loss in dairy industries, as it alters physical and chemical composition of milk [23, 33]. Moreover, it is already described that the incidence of clinical mastitis was 5%, while subclinical mastitis was 5-30% in small ruminants [7,13] hence, early diagnosis of subclinical mastitis is of particular importance to preclude contamination and economic loss [15,30]. Therefore, the study was designed to blood and/or milk lactoferrin concentrations as an indicator in diagnosis of subclinical mastitis in lactating Karayaka ewes.

Material and Method

A total of 43 Karayaka ewes in mid-lactation period, aged between 2 and 3 were used in the study. One hundred fifty ewes were investigated in private farms in Tekkekoy, Samsun province, Turkey. Milk samples from each quarter were tested by California mastitis test (CMT) [29]. For this purpose, fresh milk sample obtained from each quarter was mixed with CMT reagent in the CMT test plate and gently rotated, gel formation and differentiation of the colour were investigated. CMT results were scored as negative (0), weak positive (+1), distinct positive (+2) and strong positive (+3). CMT positive or negative udder halves of ewes were recorded. In order to confirm CMT results microscopic method of somatic cell count (SCC) was performed. After SCC analysis [19], 20 ewes with negative CMT results and SCC between 100.000-200.000 cell/ml were included in the control group and 23 ewes with CMT positive results and SCC between 300.000-1.000.000 were included in subclinical mastitis group. Blood samples were collected from the jugular vein of each ewe into vaccunated tubes and centrifuged at 3000 rpm to separate serum. Ten ml quarter milk samples were centrifuged at 14.000 rpm for 15 minutes. After removal of lipid layer and sediment, casein was precipitated by acetic acid (0.1 M) reducing pH into 4.5 using pH meter. The extract centrifuged at 25.000 rpm, +2°C for 30 minutes to remove casein completely and to obtain approximately 2 ml of clear milk serum [37]. Blood and milk LF were measured by Bovine Lactoferrin ELISA test kits (Alpha Diagnostic International, 8090, USA) using ELISA reader (Digital and analog systems, Das, 2004, ITALY). In the present study bovine ELISA kit was used since amino acid composition of both ovine and bovine lactoferrins show that %80 of homology [9]. All steps were performed at room temperature. After each reagent addition, the plate was gently tapped to mix the well contents prior to beginning incubation. 100 µl of standards, samples and controls were added each to pre-determined wells. The plate was tapped gently to mix reagents and incubated for 60 min. The plate was washed four times and pat dry on fresh paper towels. 100 µl of diluted anti-bovine lactoferrin-HRP conjugate were added to each well and incubated for 30 min. The plate was washed five times. 100 µl tetramethylbenzidine (TMB) liquid substrate was added to each well and incubated for 15 min in the dark place. 100 µl of stop solution was added to each well. Absorbance of the entire plate was read at 450 nm using a single wavelength within 30 min after addition of stop solution. Significances of differences were analysed by Student t test [28]. Data of the groups are presented as mean±StD. A value of $p<0.05$ was considered as statistically significant (SPSS Inc., Chicago, IL, USA).

Findings

The mean blood and milk lactoferrin concentrations in healthy ewes (n=20) and ewes with subclinical mastitis (n=23) are shown in Table 1. Accordingly, the mean milk lactoferrin concentrations was

16.84±1.29 µg/ml in control group, while it was 23.98±3.45 µg/ml in ewes with subclinical mastitis ($p<0.01$). However, the mean blood lactoferrin concentrations in healthy ewes and ewes with subclinical mastitis were 0.85±0.08 and 0.90±0.08 µg/ml, respectively ($p>0.05$).

Table 1. The mean blood and milk lactoferrin concentrations (µg/ml) in healthy ewes and ewes with subclinical mastitis (mean±StD).

	Control group (n=20)	Subclinical mastitis group (n=23)	Significance
Blood LF	0.85 ± 0.08	0.90 ± 0.08	p>0.05
Milk LF	16.84 ± 1.29	23.98 ± 3.45	p<0.01

Discussion and Conclusion

Mastitis reduces not only the milk yield but also alters the chemical composition of the milk, hence it is a major problem for dairy industry. Clinical mastitis can be defined with the symptoms of the teat, udder and macroscopic changes in milk composition, whereas laboratory analysis is needed to diagnose subclinical mastitis [20]. After the beginning of inflamation in the mammary gland, polimorphonuclear cells pass through the endothelial cells to the inflammation site, therefore SCC, widely used method, is a quick and effective method in the determination of subclinical mastitis [2]. Moreover, CMT is also a reliable method to detect subclinical mastitis in ewes in veterinary practice [20]. In the present study, the CMT score was negative in healthy ewes and positive in ewes with subclinical mastitis, respectively. SCC<300.000 was evaluated as CMT negative in a healthy group and SCC 300.000 to 1.000.000 was evaluated as CMT+1 in a subclinical mastitis group. These results are consistent with the results of other studies [11,19].

LF is known to contribute to specific defense mechanisms of mammary gland in subclinical mastitis [6]. Therefore, it has been indicated that the measurement of milk lactoferrin concentration may be a useful method in the diagnosis of subclinical mastitis [12,31]. Moreover, a strong relationship has been reported between milk LF levels and SCC [16]. It was detected that milk LF and somatic cell count increased concurrently in both natural and experimental mastitis of goats compared to healthy ones [10]. The range of LF concentration is reported to be wide that is between 31.78-485.63 mg in healthy animals related to lactation periods and increases in inflammation [12]. Blood and milk LF concentrations were stated to be stable during lactation period in the Ankara goat [4]. Kawai et al. [18] detected that milk LF concentration was higher in cows with clinical mastitis than cows with subclinical mastitis. In this study, the results revealed that milk LF concentrations in ewes with subclinical mastitis was higher than those in healthy ewes ($p<0.01$) (Table 1). Similarly, Hagiwara et al. [16] reported that cows with subclinical mastitis had higher milk LF concentrations as compared to healthy cows (2.23±0.39, 2.70±0.39). Al-Majali et al. [3] reported that concentration of milk lactoferrin from mastitic camels (3.8 ± 0.67) was significantly higher than that in normal camels (2.65 ± 0.88).

The circulating blood LF concentrations is normally very low; but it might increase due to LF secreted from the mammary gland endometrium and decidua during the pregnancy [24,27] due to neutrophil activation [8,17]. Indeed, the similar blood LF concentrations in both groups as shown in the results of the study suggested that the concentrations of blood LF might not be effected by the local defense of mammary gland. Moreover, it is predictable that the subclinical mastitis does not lead to a systemic infection, since it is a local inflammation and blood LF concentration does not increase.

In conclusion, based on the findings of the study, it is suggested that the determination of milk LF concentration is more reliable than blood LF concentration. Moreover, the milk LF concentration might be used in the diagnosis of subclinical mastitis in ewe.

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References

1. **Adlerova L, Bartoskova B , Faldyna M,** (2008). *Lactoferrin: a review*.Veterinarni Medicina. 53, 457-468.
2. **Alacam E,** (1989). *Diseases of cattle,* Veterinary Medicine Teknografik Matbaa, İstanbul, Turkey. 459-482.
3. **Al-Majali A, Ismail ZB, Al-Hami Y, Nour Ay,** (2007). *Lactoferrin concentration in milk from camels (camelus dromedarius) with and without subclinical mastitis.* Int J Appl Res Vet Med. 5, 120-124.
4. **Avci G, Sel T,** (2004). *Milk and serum lactoferrin levels in Angora Goats during lactation period.* Ankara Üniv Vet Fak Derg, 51, 181-187.
5. **Baker EN,** (2005). *Lactoferrin: a multi-tasking protein par excellence*. Cell Mol Life Sci. 62 (22), 2529-2530.
6. **Baveye S, Elas E, Mazurier J, Spik G, Legrand D,** (1999). *Lactoferrin: a multifunctional glycoprotein involved in the modulation of the inflammatory process*. Clin. Chem. Lab. Med. 37, 281-286.
7. **Bergonier D, Berthelot X,** (2003). *New advances in epizootiology and control of ewe mastitis.* Livest Prod Sci. 79, 1-16.
8. **Brock JH,** (2002). *The physiology of lactoferrin*. Biochem Cell Biol. 80, 1-6.
9. **Buchta R,** (1991) *Ovine lactoferrin: isolation from colostrum and characterization*. J Dairy Res. 58(2):211-218.
10. **Chen Gh, Yin Li, Chiang JH, Jiang ST,** (2008). *Cloning and expression of antibacterial goat lactoferricin from Escherichia coli AD494(DE3)pLysS expression system.* J Food Prot. 71 (12), 2523-2525.
11. **Chen PW, Shyu C, Mao FC,** (2003). *Antibacterial activity of short hydrophobic and basic-rich peptides.* Am J Vet Res. 64 (9), 1088-1092.
12. **Cheng JB, Wang JQ, Bu DP, Liu GL, Zhang CG, Wei HY, Zhou LY, Wan JZ,** (2008). *Factors Affecting the Lactoferrin Concentration in Bovine Milk.* J Dairy Sci. 91, 3, 970-976.
13. **Contreras A, Sierra D, Sanchez A, Corrale JC, Marco JC, Paape MJ, Gonzalo C,** (2007). *Mastitis in small ruminants*. Small Rum Res. 68, 145-153.
14. **De Jong G, Van Dijk JP, Van Eijk HG,** (1990). *The biology of transferrin.* Clin Chim Acta. 190, 1-46.
15. **Graaf T, Dwinge RH,** (1995*). Estimation of milk production losses due to subclinical mastitis in dairy cattle in Costa Rica. In proceeding of the third IDF international mastitis seminar*, 11, 28 May-1 June, Tel-Aviv, Israel.
16. **Hagiwara SI, Kawa K, Anri A, Nagahata H,** (2003). *Lactoferrin concentrations in milk from normal and subclinical mastitic cows.* J Vet Med Sci. 65 (3), 319-323.
17. **Kanyshkova TG, Buneva VN, Nevinsky GA,** (2001). *Lactoferrin and its biological functions.* Biochemistry (Moscow). 66, 1-7.
18. **Kawai K, Hagiwara S, Anri A, Nagahata H,** (1999). *Lactoferrin concentration in milk of ovine clinical mastitis.* Vet Res Commun. 23, 391-398.
19. **Kilicoglu Ç, Alacam E, Izgur H, Akay O, Wiesner HU,** (1989). *Eutergesundheitskontrolle von Milchkülen im Gebiet von Ankara (Turkei)*. Dtsch Tierärztl Wschr. 96, 486-488.
20. **Lafi SQ,** (2006). *Use of somatic cell counts and California Mastitis Test results from udder halves milk samples to detect subclinical intramammary infection in Awassi sheep.* Small Rum Res. 62, 83-86.
21. **Legrand D, Elass E, Carpentier M, Mazurier J,** (2005). *Lactoferrin: a modulator of immune and inflammatory responses.* Cell Mol Life Sci. 62, 2549-2559.
22. **Legrand D, Pierce A, Elass E, Carpentier M, Mariller C, Mazurier J,** (2008). *Lactoferrin structure and functions.* Adv Exp Med Biol. 606, 163-94.
23. **Leitner G, Chaffer M, Shamay A, Shapiro F, Merinu E, Ezra A, Saran A, Silanikove N,** (2004). *Changes in milk composition as affected by subclinical mastitis in sheep*. J Dairy Sci. 87, 46-52.
24. **Levay PF, Viljoen M,** (1995). *Lactoferrin: a general review*. Haematologica. 80, 252-267.
25. **Mazurier J, Lhoste JM, Montreuil J, Spik G,** (1983). *Comparative study of the iron-binding properties of human transferrins. II. Electron paramagnetic resonance of mixed metal complexes of human lactotransferrin.* Biochim Biophys Acta. 30, 745 (1), 44-49.
26. **Moguilevsky N, Retegui La, Masson PL,** (1985). *Comparison of human lactoferrins from milk and neutrophilic leucocytes. Relative molecular mass, isoelectric point, iron-binding properties and uptake by the liver*. Biochem J. 229 (2), 353-359.
27. **Oberg G, Lindmar G, Moberg L, Venge P,** (1983). *The peroxidase activity and cellular content of granule proteins in PMN during pregnancy*. Br J Haematol. 55 (4), 701-708.
28. **Rao CR,** (1973). *Linear Statistical Inference and Its Applications*, John&Sons, New York
29. **Schalm OW, Carroll Ej, Jain NC,** (1971). *Bovine Mastitis,* Lea and Febiger, Philadelphia. P, 24.
30. **Silanikove N, Shapiro F, Leitner G, Merin U,** (2005). *Subclinical mastitis affects the plasmin system, milk composition and curd yield in sheep and goats: comparative aspects.* In: Hogeveen, H. (Ed.), Mastitis in Dairy Production. Wageningen Academic Press Publishers, The Netherlands, 511-516.
31. **Sordillo LM, Streicher KL,** (2002). *Mammary Gland Immunity and Mastitis Susceptibility*. Journal of Mammary Gland Biology and Neoplasia, 7, 2, 135-146.
32. **Teng CT, Beard C, Gladwell W,** (2002). *Differential expression and estrogen response of lactoferrin gene in the female reproductive tract of mouse, rat and hamster.* Biol Reprod. 67, 1439-1449.
33. **Tripathi BN,** (2000). *Diseases of the mammary glands of goats and sheep.* Vet Bul. 70, 1117-1142.
34. **Valenti P, Antonini G,** (2005). *Lactoferrin: an important host defense against microbial and viral attack.* Cell Mol Life Sci. 62, 2576-2587.
35. **Vorland LH,** (1999). *Lactoferrin: a multifunctional glycoprotein*. APMIS. 107, 971-981.
36. **Weinberg ED,** (2003). *The therapeutic potential of lactoferrin.* Expert Opin Investig Drugs. 12 (5), 841-51.
37. **Welty FK, Smith KL, Schanbacher FL,** (1976). *Lactoferrin concentration during involution of the bovine mammary gland*. J Dairy Sci. 59, 224-231.