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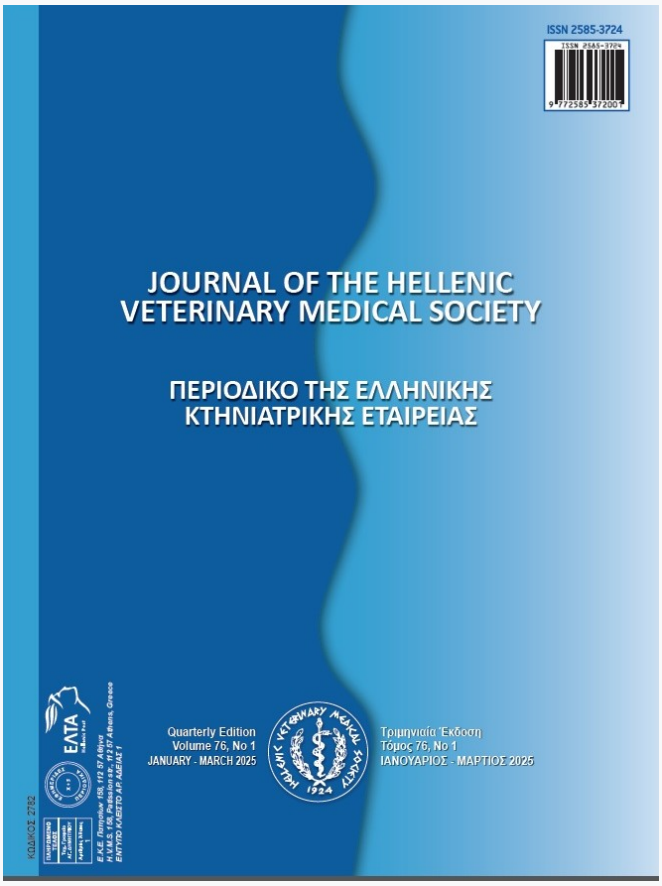

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Research article
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The effect of subclinical mastitis on milk transforming growth factor-beta level in Anatolian water buffaloes

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ABSTRACT: This study aims to evaluate the relationship between transforming growth factor beta (TGF-β) and somatic cell count (SCC) in Anatolian buffalo milk. The study material comprised 80 milk samples obtained from the udder quarter of 40 healthy and 40 subclinical mastitic nonpregnant lactating Anatolian water buffaloes. The California mastitis test and SCC were performed on milk samples milked from four udders of buffaloes to diagnose subclinical mastitis. The milk TGF-β level was measured by a bovine-specific enzyme-linked immunosorbent assay kit. The milk TGF-β levels in subclinical mastitic and control groups were 11.7±2.6 (8.0 to 18.3) ng/ml and 4.8±1.3 (2.4 to 7.0) ng/ml. The milk TGF-β level of subclinical mastitic buffaloes was significantly higher than healthy ones ($P<0.01$). A significant positive correlation was determined between the milk TGF-β level and SCC in subclinical mastitic buffaloes ($r^2 = .936$, $P < 0.001$). This is the first study to determine the relationship between TGF-β concentrations and SCC in milk obtained from mammary quarters with subclinical mastitis in Anatolian water buffaloes. This study has the potential to contribute to studies on cytokine changes in milk in subclinical mastitis of buffaloes.

Key words: buffalo; milk; subclinical mastitis; transforming growth factor-beta

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INTRODUCTION

Buffalo milk is essential in human nutrition, especially in developing countries. It is richer in almost all major milk nutrients than cow's milk (Zicarelli et al., 2004; Stocco et al. 2016; Pegolo et al., 2017; Becskei et al., 2020). Moreover, some specific classes of gangliosides are only present in buffalo milk (Colarow et al., 2003). Mastitis is one of the most common and costly diseases on dairy farms worldwide. In particular, subclinical mastitis is insidious without clinical signs of infection. To monitor the health of milking animals' udders and assess the quality of their milk, it is crucial to count somatic cells, which include immune cells and milk-producing mammary cells. This parameter is influenced by various variables, including animal breed, milk yield, udder health, and lactation period/number (Boutinaud et al., 2002; Sundekilde et al., 2013; Alhussien et al., 2018). The amount of some components in milk are candidates to be potential biomarkers of subclinical mastitis. Although there are many scientific studies on the level of biomarkers in cow's milk with subclinical mastitis, a few are in buffalo milk.

It is known that inflammation cytokines play critical roles in the innate immune response to intramammary infection (Bannerman et al., 2005). Transforming growth factor beta (TGF-β) is an indispensable cytokine in maintaining immune homeostasis that shows proinflammatory properties locally and has a strong immunosuppressive effect systemically. TGF-β which has three isoforms as TGF-β1, TGF-β2, and TGF-β3 plays an essential role in physiological processes associated with mammary gland development and pathological processes related to inflammation, host immune responses, and also tissue regeneration and remodeling as well as in fibrosis. It does all this by influencing cell proliferation, differentiation, and migration and stimulating extracellular matrix protein production (Chockalingam et al., 2005; Ingman et al., 2008; Letterio and Roberts, 1998; Marek et al., 2002). Şafak and Rişvanlı (2021) reported that cytokines/growth factors play a role in innate immune responses against mastitis and maintain homeostasis due to their pleiotropic nature in regulating mammary gland development and inflammation. The anti-inflammatory properties of TGF-β include these processes: 1) limiting IFN-γ production; 2) increasing the expression of (IL)-1 receptor antagonist; 3) inhibiting macrophage production of chemokines, proinflammatory cytokines, nitric oxide, and reactive oxygen intermediates 4) inhibiting macrophage clearance of bacterial debris and damaged parenchymal cells (Şafak and Rişvanlı, 2021).

Mastitis is a multi-etiological and multifactorial disease, and the immune response to this disease shows wide individual variation depending on the bacterial strain, the stage of subclinical mastitis, and the host. Subclinical mastitis is more critical as it is 15-40 times more common and challenging to diagnose than clinical mastitis. Therefore, it stays in the herd longer and causes production losses (Ali et al., 2015). Early diagnosis of subclinical mastitis is crucial in terms of this feature and preventing its transformation into clinical and severe forms. In subclinical mastitis, no systemic or local symptoms are observed. It can be diagnosed by an increase in the number of somatic cells in milk, measurement of electrical conductivity, lactate dehydrogenase (LDH), N-acetyl-β-D-glucosaminidase (NAGase), ultrasonography echotexture of the mammary parenchyma, isolation of pathogenic factors, and other changes in biochemical values in milk and blood (Adkins and Middleton, 2018; Bobbo et al., 2017; Hovinen et al., 2016; Themistokleous et al., 2023; Viguer et al., 2009; Zhang et al., 2022; Zhao, 2008). The California mastitis test, SCC, cultural examination, and electrical conductivity are used to diagnose subclinical mastitis (Tripaldi et al., 2003; Tripaldi et al., 2005; Viguer et al., 2009; Varatanović et al., 2010; Sharma et al., 2011; Gültiken et al., 2012; Galfi et al., 2015). Since these tests have limitations, examining biomarkers in milk for early and rapid detection of subclinical mastitis. Researchers have reported the level of growth factors (Bruckmaier et al., 2004; Chockalingam et al., 2005; Gürler et al., 2019; Gökçeoğlu et al., 2020) and cytokines (Chockalingam et al., 2005; Şafak and Rişvanlı, 2021). In milk with subclinical mastitis, it varied compared to healthy milk. To our knowledge, no studies evaluated the effect of subclinical mastitis on milk TGF-β levels in Anatolian water buffaloes. That is why this study aimed to investigate the milk TGF-β change in Anatolian water buffaloes that were linked to subclinical mastitis susceptibility.

Studies have shown that changes in cytokine expression in cases of mastitis are associated with disease activity. It has been reported that changes in the expression pattern of proinflammatory cytokines of the mammary gland in healthy and sick animals can help in the early detection of infection (Lee et al., 2006; Wenz et al., 2010; Bhatt et al., 2012; Singh et al., 2016). However, no evidence exists between milk

TGF-β levels and SCC in buffaloes with subclinical mastitis. This study evaluated the relationship between TGF-β and SCC, known to increase bacterial infections, regardless of the bacterial type. The aim was to determine the change in TGF-β depending on the number of somatic cells in Anatolian water buffalo milk with subclinical mastitis.

MATERIALS AND METHODS

Animals and sampling

The raw milk from Anatolian water buffalo was collected between May 2021 and September 2021 from individual farmers in Samsun province. The study was conducted on 80 milk samples from 40 healthy and 40 mastitic mammary quarters of clinically healthy and nonpregnant lactating Anatolian water buffaloes. The number of milk samples used in the study was calculated as 89.9% power when the effect size d: 0.8 and the margin of error α: 0.05 were accepted; control group n= 40 and subclinical mastitis group n= 40. Power analysis was performed with the help of the G Power 3.1.9.4 program (Faul et al., 2007). Batavani et al. (2007) was used as the reference article. For this purpose; approximately 500 buffaloes were screened in the above-mentioned date range, and the criterion that the milk obtained from 4 quarters of each 40 healthy buffaloes was negative for subclinical mastitis was taken into consideration. Similarly, the criterion that the milk obtained from 4 quarters of each 40 buffaloes constituting the subclinical mastitis group was + for subclinical mastitis was taken into consideration. The buffaloes were 3-5 years old and in different stages of the lactation period. Milk samples were collected from buffaloes with manual milking. After teat ends were disinfected with cotton swabs with 70 % alcohol and then allowed to dry and discarded the foremilks from quarters. The California Mastitis Test (CMT) was carried out on the milk samples from each quarter on the farm using Schalm et al. (1971). CMT was evaluated separately from four mammary quarters with negative (-) and positive (+) (Schalm et al., 1971). After CMT evaluation, milk samples were transported to the laboratory by maintaining a cold chain in a cool box. Before bringing it to the laboratory for somatic cell counting, a chemical tablet (Microtabs), which inhibits microbial growth without affecting the SCC, was added to each tube, as it will be one tablet for a 10 ml milk sample. At the same time, some examples of stored frozen at -40 °C until the TGF-β assay.

The direct microscopic SCC

Somatic cells in raw milk were counted by microscopic method (Kılıçoğlu et al., 1989). Firstly, the top milk cream was removed from milk samples and centrifuged at 1550 g for 10 min, and the tubes were then left upside-down in the port tube for 20 min. A thin smear of 0.01 ml of each sediment was prepared on a 1 cm diameter circle on a microscopic slide and allowed to air dry. Slides were then immersed in a covered trough containing the 0.2 % toluidin blue staining solution for 2 min. At least 15-20 fields on the slide were counted under a compound microscope (Nikon Eclipse E600, Nikon Instruments Inc., Tokyo, Japan) with oil immersion (objective lens, 100x magnifications). The average cell count was computed by dividing the total number of cells by the total number of fields and SCC was presented as 10^5 cells/ml.

TGF-β assay

The milk level of the TGF-β was analyzed using a bovine-specific enzyme-linked immunosorbent assay (ELISA) kit, following the manufacturer's instructions (E2049Bo, Bioassay Technology Laboratory, Shanghai, China). The kit's sensitivity was 0.32 ng/ml; the range was 0.5- 150 ng/ml. Briefly, 50 µl standards to the standard wells, and 40 µl sample and 10 µl TGF-β antibody to the sample wells were added. Then 50 µl streptavidin-horseradish peroxidase was added to standard and sample wells, not blank control wells. The plate was covered with a sealer and incubated for 60 min at 37 °C. After five washing steps, 50 µl substrate solution A and 50 µl substrate solution B were added to each well and incubated at 37 °C in the dark. Finally, the reaction was stopped after 10 min by 50 µl of stop solution, and the absorbance of each well was determined at 450 nm with a microtiter plate reader (Infinite F50, Tecan Austria GmbH, Grödig, Austria). The milk TGF-β levels were determined using the standard curve. The intra- and inter-assay variations were 5.4 % and 8.9 %, respectively. All sample measurements were performed in duplicate.

Statistical analysis

Statistical analysis was performed using the statistical package program (SPSS Statistics V22.0, IBM Corporation, Armonk, NY). All data are given as mean ± standard deviation (SD). The Shapiro-Wilks test was used to evaluate the normality of data. Data showed normal distribution. Student's t-test was used to compare between the two groups. Pearson correlation analysis was used to evaluate the relationship

between TGF-β and SCC. Statistical significance was considered as $P < 0.05$.

RESULTS

The milk TGF-β level in subclinical mastitis and control groups was presented (Figure 1). The mean serum TGF-β level in the subclinical mastitis group was 11.7±2.6 (8.0 to 18.3) ng/ml, and in the control group was 4.8±1.3 (2.4 to 7.0) ng/ml ($P<0.01$). A significant positive correlation was determined between the milk TGF-β and SCC (Figure 2). The mean (±SD) SCC of the healthy and subclinical mastitis groups are shown in Table 1.

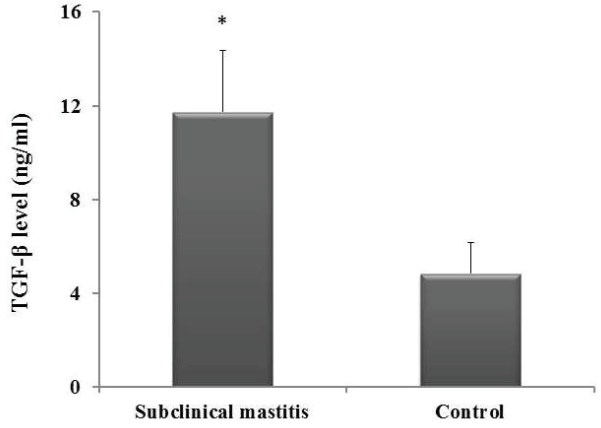

Figure 1. TGF-β level in the milk of the subclinical mastitis and control groups. * $P < 0.01$, Student's t-test.

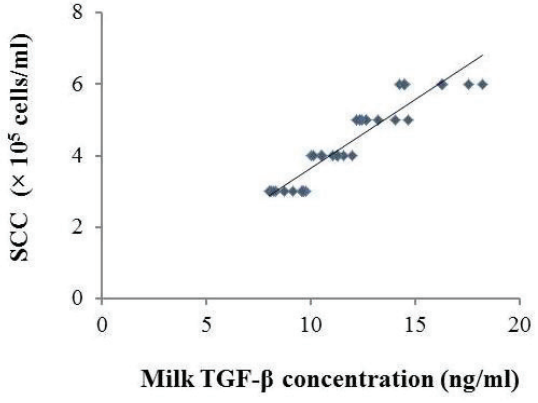

Figure 2. Correlation between milk TGF-β level and SCC in the subclinical mastitis group (r^2= .936, $P < 0.001$, Pearson's correlation).

Table 1. SCC of the subclinical mastitis and control groups.

Groups	SCC (Mean±SD; x 10^5 cells/ml)
Control	1.88±0.85
Subclinical mastitis	4.33±1.07 *

* $P < 0.01$, Student's t-test.

DISCUSSION

Mastitis is the most costly and vital disease in the dairy industry. It is quite common in dairy animals and is usually caused by intramammary bacterial infection. Somatic cells, which include macrophages, neutrophils, lymphocytes, and specific mammary epithelial cells, are used as a diagnostic indicator for subclinical mastitis (Fox, 2009; Viguer et al., 2009; Tripaldi et al., 2010). Macrophages predominate in a healthy udder, while neutrophils predominate in the early stages of mastitis (Rainard and Riollet, 2006). In clinical mastitis, changes in milk's physical and chemical characteristics occur together with the inflammatory reactions seen in the udder. Subclinical mastitis causes severe financial damage to dairy industries in developed and developing countries by causing decreased milk production, deterioration in milk quality, and a threat to cow health (Goncalves et al., 2018). Early diagnosis of subclinical mastitis is vital to prevent clinical mastitis progression and transmission to other animals.

TGF-β not only acts on mammary gland growth but also slightly reduces inflammation. TGF-β primarily suppresses immunological and inflammatory responses but may also have proinflammatory effects depending on where and how much the cells it stimulates are activated, as well as the presence of other cytokines. These effects may explain the non-significant change in TGF-β in cows with subclinical mastitis because of its primary function in regulating the inflammatory response (Letterio and Roberts, 1998; Ashcroft, 1999; Bannerman, 2009; Shaheen et al., 2020). The increase in TGF-β, which has occurred by many different bacterial species, has been reported in many studies (Marie et al., 1996; Marek et al., 2002; Ling et al., 2003; Bannerman et al., 2005; Safak and Risvanli, 2021). A study evaluating the impact of TGF-β1 on the susceptibility of bovine mammary epithelial cells to Staphylococcus aureus showed that TGF-β1 treatment dramatically enhanced S. aureus adhesion and invasion of the treated cells (Zhang et al., 2019). This study evaluated the relationship between TGF-β and SCC, known to increase bacterial infections, regardless of the bacterial type. It would require a separate paper to discuss the current change in the amount of TGF-β depending on the bacterial species. Since there is no available data on the subject, not Anatolian water buffaloes, but even buffaloes in general, the situation in cows will be discussed. There are articles on cytokine changes in bovine mastitis in clinical or subclinical forms studies (Bannerman et al., 2005; Şa-

fak and Rişvanlı, 2021; Shaheen et al., 2020). Chockalingam et al. (2005) and Şafak and Rişvanlı (2021) reported that TGF-β1 concentration increased during mastitis. Although TGF-β showed a significant decrease in clinical mastitis cases ($P<0.001$), it was reported that there was no significant variation ($P<0.01$) in normal and subclinical lactating cows (Shaheen et al., 2020). Similar to this study, Andreotti et al. (2014) found that chronically infected mammary tissue had considerably increased protein expression of TGF-β1, TGF-β2, and TGF-β3 compared to uninfected controls in a cattle study. This study detected a significant increase in TGF-β levels and a positive correlation between milk TGF-β and in Anatolian buffalos with subclinical mastitis.

CONCLUSION

The current investigation shows the relationship between TGF-β and SCC in subclinical mastitis cases in Anatolian buffaloes. The prevalence and severity of subclinical mastitis in Anatolian buffaloes increases with TGF-β severity due to its positive correlation with the number of somatic cells, which gives an idea about the presence and severity of subclinical mastitis. As a result, it should not be overlooked that further studies should be conducted on Anatolian buffalo milk with mastitis on whether the changes in cytokine ratio are due to bacterial species. Additionally, changes in TGF-β (TGF-β1, TGF-β2, and TGF-β3) isoforms in Anatolian water buffalo should also be taken into consideration.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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