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RESEARCH ARTICLE

Evaluation of Oxidative Stress, Immune System and Mineral Concentrations in Milk and Serum of Cows with Clinical and Subclinical Mastitis Naturally Infected by *Staphylococcus aureus*

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Abstract

The aim of this study was to investigate effect of Staphylococcus aureus on oxidative stress status (TAS, TOS, OSI), immune system (IL-1β, IL-6, TNF-α), and mineral (Mg, Fe, Zn, Cu, Na and Ca) concentrations in milk and serum of cows with mastitis. The cows were allocated to three groups according to mammary health status as follows: healthy (Group 1), clinical (Group 2) and subclinical mastitis cows (Group 3). IL-1β levels in serum and milk increased in Group 2 compared to Group 1 and Group 3 (P<0.001; P<0.05). Milk IL-6 level was greater in Group 3 and Group 2 than in Group 1 (P<0.01). Blood TNF-α (P<0.001), TOS and OSI levels (P<0.01) were higher in Group 2 than other groups. Milk TNF-α level increased and blood TAS level decreased in Group 2 compared to Group 1 (P<0.05). Milk TOS (P<0.01) and OSI (P<0.05) levels increased in Group 3 compared to Group 1. Blood and milk Mg levels increased in Group 1 (P<0.05) and Group 2 (P<0.001), respectively. Milk Fe (P<0.01) and Na levels were greater in Group 2 (P<0.001). Blood Zn level was lower in Group 2 compared to Group 3 (P<0.05). While blood Cu level decreased in Group 1 compared to other groups, milk Cu level decreased in Group 3 compared to other groups (P<0.001). In conclusion, S. aureus had significant effects on oxidative stress, cytokine and mineral levels in milk and blood serum of cows with clinical and subclinical mastitis. However, since there were specific changes only in milk OSI and IL-6 levels according to other parameters, it is thought that milk IL-6 and OSI levels may be a diagnostic tool in the detection of subclinical mastitis.

Keywords: *Cytokine, Mastitis, Mineral substance, Oxidative stress, Staphylococcus aureus*

Staphylococcus aureus İle Doğal Olarak Enfekte Olan Klinik ve Subklinik Mastitisli İneklerin Süt ve Serumlarında Oksidatif Stres, Bağışıklık Sistemi ve Mineral Konsantrasyonlarının Değerlendirilmesi

Öz

Bu çalışmanın amacı, *Staphylococcus aureus*'un mastitisli ineklerin süt ve kan serumlarında oksidatif stres durumu (TAS, TOS, OSI), bağışıklık sistemi (IL-1β, IL-6, TNF-α) ve mineral (Mg, Fe, Zn, Cu, Na ve Ca) konsantrasyonları üzerine etkisini araştırmaktı. Çalışma sağlıklı (Grup 1), klinik (Grup 2) ve subklinik mastitisli (Grup 3) inekler olmak üzere üç grup üzerinde yürütüldü. Grup 1 ve Grup 3'e göre Grup 2'de kan ve sütteki IL-1β düzeyleri arttı (P<0.001; P<0.05). Grup 1'e göre Grup 3 ve Grup 2'de süt IL-6 düzeyi daha yüksekti (P<0.01). Diğer gruplara göre Grup 2'de kan TNF-α (P<0.001), TOS ve OSI düzeyleri (P<0.01) daha yüksekti. Grup 1'e göre Grup 2 de süt TNF-α düzeyi yükseldi ve kan TAS düzeyi azaldı (P<0.05). Grup 1'e kıyasla Grup 3'te süt TOS (P<0.01) ve OSI (P<0.05) düzeyleri yükseliş gösterdi. Diğer gruplara göre kan ve süt Mg düzeyleri sırasıyla Grup 1 (P<0.05) ve Grup 2'de (P<0.001) arttı. Diğer gruplara göre Grup 2'de süt Fe (P<0.01) ve Na düzeyleri arttı (P<0.001). Grup 3'e göre Grup 2'de kan Zn düzeyi düşüş gösterdi (P<0.05). Kan Cu düzeyi diğer gruplara göre Grup 1'de düşerken, süt Cu düzeyi diğer gruplara kıyasla Grup 3'te azaldı (P<0.001). Sonuç olarak, *S. aureus*'un klinik ve subklinik mastitisli ineklerin süt ve kan serumlarında oksidatif stres, sitokin ve mineral seviyeleri üzerinde önemli etkileri olduğu görülmüştür. Ancak diğer parametrelere göre sadece süt OSI ve IL-6 düzeylerinde spesifik değişiklikler olduğu için süt IL-6 ve OSI düzeylerinin subklinik mastitisin saptanmasında tanı aracı olabileceği düşünülmektedir.

Anahtar sözcükler: *Mastitis, mineral madde, Oksidatif stres, Sitokinler, Staphylococcus aureus*

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Introduction

Mastitis, which is one of the costliest problems for the dairy industry [1], is defined as inflammation of the mammary gland against infectious and non-infectious factors [2]. It affects the milk composition and quality [3], and accounts for about 38% of the total costs caused by major production diseases in dairy cows [2]. One of the most important bacterial agents that cause bovine mastitis is *Staphylococcus aureus* (*S. aureus*) [2,4], and it is associated with the clinical and subclinical mastitis [4,5]. Since *S. aureus* found on the normal skin flora, it is impossible to eradicate from the herds. It is also very difficult to combat because it can produce exotoxins, biofilms, bacterial superantigens and proteases, and adhere to mammary epithelial cells [6]. It can also survive within the phagocytes and epithelial cells, so antibiotic treatments are often ineffective [7]. Therefore, alternative methods are needed to increase the treatment rate for mastitis caused by *S. aureus* [6]. In order to achieve success in the combat against *S. aureus*, some studies have been conducted to investigate its effects on the defense system [8,9]. Immune response of a mammary gland infected by *S. aureus* is controlled by lymphocyte subpopulations, other leukocytes and some cytokines released by them. The virulence factors of S. *aureus* such as enterotoxin and toxic shock syndrome toxins allow it to deceive and escape from the immune system [10], however it can affect the levels of proinflammatory cytokines [11] such as interleukin 1 beta (IL-1β), interleukin 6 (IL-6) [12] and tumor necrosis factor alpha (TNF-α) [11]. These cytokines induce an immune response by stimulating phagocytic cells to the inflammation [13]. Therefore, the production of reactive oxygen species (ROS), the most abundant oxidant in the biological system, increases as a result of the destruction of pathogens during mastitis [14]. If the level of ROS produced in the body exceeds the antioxidant capacity, oxidative stress occurs. Oxidative stress causes damage to cells' macromolecules such as DNA, lipids and proteins [15,16], and thus it may exacerbate mastitis. More clearly, there is a relationship among oxidative stress, cytokine release and mastitis [15,17], and these changes can be observed in both milk and systemic circulation [18]. On the other hand, since mastitis causes deterioration in the blood-milk barrier, it causes extracellular fluid to enter the mammary gland [19]. Therefore, it affects the mineral and trace element level in the milk and blood [20]. Some mineral substances are involved in the antioxidant system [21,22] and are important in monitoring udder health [23]. In recent studies, it has been emphasized that proinflammatory cytokines [24] and oxidative stress [25] are critical in the fight against mastitis. However, this needs to be further elaborated in order to effectively combat *S. aureus* mastitis.

We hypothesized that mastitis caused by *S. aureus* has significant influences on oxidative stress, some cytokine and mineral levels in milk and blood serum of cows. Therefore, the presented study aimed to investigate oxidative stress status, immune system (IL-1β, IL-6, TNF-α), and mineral (Mg, Fe, Zn, Cu, Na and Ca) concentrations in milk and serum of cows with clinical and subclinical mastitis caused by *S. aureus*.

Material and Methods

Ethical Statement

The present study was carried out in compliance with the National Research Council's guide for animal use and approved by the Local Ethics Committee of Ceyhan Veterinary Faculty, Cukurova University, Adana, Turkey (Approval no: 27/07/2020-04/01).

Animal and Management

This study was carried out on a total of 30 multiparous Holstein dairy cows at a medium-scale commercial farm in Adana, Turkey, in August. All cows had a similar age, milk yield, body condition score and parity, and they were managed in free stall barns under the same conditions, milked twice a day using an automatic milking system and had free access to water. All cows were in mid-lactation period and fed with same total mixed ration (TMR) arranged for mid-lactation period. The mid-lactation ration content is given in the *Table 1*. The farm had evaporative and ventilated air-cooling systems against heat stress. Moreover, pre- and post-milking udder disinfection procedures [26], and mastitis screenings were routinely performed in the farm.

Groups and Experimental Design

The dairy cows were divided into three groups as healthy (Group 1; n = 10), clinical (Group 2; n=10) and subclinical (Group 3; n = 10) mastitis. No treatment was performed before and during the study. First, the days in milking (DIM) of all cows were recorded to evaluate the similarity in terms of lactation days between the groups. The groups were

Table 1. *The ration content of the cows in the mid lactation period*

Ration content	Percantage (%)
Corn silage	46.16
Clover	13.02
Wheat straw	4.73
Soy meal	6.62
Cottonseed	5.20
Barley	2.60
Corn grain	11.83
Corn gluten meal	0.47
Corn bran	1.18
Soybean grain	5.77
Other	2.36
Total	100

formed according to California mastitis test (CMT), udder examination and bacteriological identification in milk. The CMT was performed according to the manufacturer's instructions (California mastitis test kit, ImmuCell). The CMT reaction of each quarter was recorded as negative (0), trace, 1+, 2+ and 3+ [27,28]. If one of the 1+, 2+, and 3+ values was obtained, it was considered positive. All tests were performed for 4 teats of each cow. The clinical symptoms of mastitis, as previously described, were considered as changes in milk appearance, heat, swelling and pain of affected udder [18]. Before collecting the milk, the udder and the teat ends were washed, cleaned and dried, and samples were taken after the foremilk was discarded [23]. The healthy group consisted of cows with the negative CMT test, no clinical signs of mastitis and no bacteria was isolated in their milk samples. Subclinical mastitis group consisted of cows with the positive CMT test, no clinical signs of mastitis and only *S. aureus* was isolated in their milk samples. Clinical mastitis group consisted of cows with the positive CMT test, typical signs of clinical mastitis [12] and only *S. aureus* was isolated in their milk samples. A total of 560 teats were examined until the number of animals in all groups was completed.

Blood and Milk Samples

All blood and milk samples were taken before morning milking and feeding. In all groups, blood samples (5 mL) were collected from the jugular vein into sterile vacutainer tubes with clot activator (Hema & Tube®, Italy). Immediately after the blood collection process, milk samples (15 mL; two samples per cows) were taken from individual quarters with healthy, subclinical and clinical mastitis groups into sterile falcon tubes (Isolab®, Germany) under aseptically conditions, according to the Laboratory Handbook on Bovine Mastitis of the National Mastitis Council [29]. One milk sample per cow was transported to the laboratory at + 4°C for microbiological and mineral substance analyzes. Other milk and blood samples were centrifuged at 4.000 ×g (for 15 min) and 1500 x g (for 10 min), respectively. After the milk and blood serums were harvested, they were immediately stored at −20°C until IL-1β, IL-6, TNF-α, TAS, TOS, mineral substance analyzes. While milk analyzes were performed on the same day, serum analyzes were performed within 14 days after storage at −20°C.

IL-1β, IL-6 and TNF-α Analysis in Milk and Blood Serum

Milk and blood serum IL-1β, IL-6 and TNF-α levels were determined using a microplate reader (Stat Fax-2100, Awareness®, USA) and commercial kits (IL-1β cat no: CSB-E12019B-bovine IL-1 ELISA kit; IL-6 cat no: CSB-E12899B-bovine IL-6 ELISA kit; TNF-α cat no: CSB-E12020B-bovine TNF-α ELISA kit).

TAS and TOS Analysis in Milk and Blood Serum

Milk and blood serum TAS and TOS levels were measured by same as above (Stat Fax-2100, Awareness®, USA) using commercial kits (Rel Assay Diagnostics®, Turkey), according to the method developed by Erel [30] and Erel [31]. The test principle is a colorimetric method that can be determined spectrophotometrically.

Calculation of Oxidative Stress Index (OSI)

OSI was accepted as percent ratio of TOS level to TAS level. After the resulting unit of TAS (mmol Trolox equivalent/L) was converted to μmol Trolox equivalent/L, the OSI was calculated according to the following formula: TOS (μmol H_2O_2 Eq/L)/TAS (mmol Trolox Eq/L) x 100 [32].

Mineral Substance Analysis

- Preparation of Milk Samples

All milk serum samples were dried in a forced stove at 100°C to obtain a constant weight. They were brought to room temperature, and 4 mL of HNO_3 (65%, Merck, Germany) and 2 mL of H_2O_2 (30%, Merck, Germany) were added onto 2 mL milk serum sample. The samples were solubilized at 180°C and 270 bar pressure. After this process, they were allowed to cool for 15-20 min. Subsequently, they were filtered from filter papers and taken into balloon flasks. Their volumes were completed to 25 mL with ultra-pure water.

- Preparation of Blood Serum Samples

Serum samples in 1 mL volumes were taken into glass tubes. Then, a 3% HNO_3 solution was prepared from 65% HNO_3 (Merck, Germany) and added onto serum samples in a volume of 1 mL. Finally, the resulting mixture was centrifuged at 3000 rpm for 10 min. The remaining particles in the tubes were filtered, 1 mL of 1% Triton-X was added to them and their volume were completed to 10 mL using deionized pure water.

- Mineral Substance Analysis in Milk and Blood Serum

Analysis of copper (Cu), iron (Fe), zinc (Zn), calcium (Ca), sodium (Na), magnesium (Mg) was performed using an atomic absorption spectrophotometer (ICE 3000, Thermo).

Microbiological Examination

Microbiological examinations were performed according to standard procedure of National Mastitis Council [29] to identify gram-negative and gram-positive bacteria. The *S. aureus* identify procedures were summarized below.

Culture

The milk samples were individually inoculated into blood agar (Oxoid, CM0055) and MacConkey agar (Oxoid, CM0007) using a quadrant streaking method, and they were incubated at 37°C for 24-48 h. Blood agar and MacConkey agar were used for bacteriological isolation; morphologic characteristics of isolated microorganisms were observed on these primary cultures. Additionally, Gram staining was

applied to define the gram reaction and shape of the cultures. Coagulase test (Coagulase Plasma Lyophilized. Rabbit plasma w/EDTA, Catalog number: R21052, Thermo Fisher Scientific, MA, USA) was applied to differentiate staphylococcus species.

Statistical Analysis

In this study, the group sizes were determined as 10, according to the results of the power analysis using 80% power and 5% margin of error. All statistical calculations were done with SPSS software (Version: 23.0; IBM, USA). The normality tests of data were performed using the Kolmogorov-Smirnov test. One-way analysis of variance test (ANOVA) was used to compare group means. The differences among all groups were analyzed with Duncan's multiple comparison test. Significance level was accepted as $P<0.05$ in all analyses. The results were given as the mean±standard error of mean (mean±SEM).

Results

It was found that DIM was similar between all groups ($P>0.05$). The DIM averages in Group 1, Group 2, and Group 3 were 143.7±6.7, 146.2±6.1, and 140.0±10.2, respectively ($P>0.05$).

Pro-inflammatory Cytokine Concentrations

IL-1β levels in blood and milk serums significantly increased in the Group 2 compared to Group 1 and Group 3 ($P<0.001$; $P<0.05$). While the IL-6 levels in blood were similar between the groups ($P>0.05$), its level in milk was higher in the Group 3 and Group 2 than in the Group 1 ($P<0.01$). Blood TNF-α level was higher in the Group 2 group than in the other groups ($P<0.001$). Milk TNF-α level was higher in the Group 2 than in the Group 1 ($P<0.05$).

Oxidative Stress Markers

Milk TAS levels were found to be similar between groups ($P>0.05$); however, blood TAS level significantly increased in the Group 1 compared to the Group 2 ($P<0.05$). Blood OSI and TOS levels were significantly higher in the Group 2 compared to the Group 1 and Group 3 ($P<0.01$). On the other hand, an increase was observed in the milk TOS ($P<0.01$) and OSI ($P<0.05$) levels of the Group 3 compared to the Group 1.

Mineral Substance Concentrations

Blood and milk Mg levels were significantly higher in the Group 1 ($P<0.05$) and Group 2 ($P<0.001$), respectively, than in the other group. While the blood Fe levels were similar between the groups ($P>0.05$), milk Fe level was found higher in the Group 2 than in the Group 1 and Group 3 ($P<0.01$). There was no significant difference between the groups in terms of milk Zn levels ($P>0.05$), but the blood Zn level was lower in the Group 2 than in the Group 3 ($P<0.05$). Blood and milk Cu levels were found to be significantly lower in the Group 1 and Group 3, respectively, compared to the other group ($P<0.001$). Blood Na levels were similar in all groups; however, milk Na levels was found to be higher in the Group 2 compared to other groups, and also significantly higher in the Group 3 than in the Group 1 ($P<0.001$). No difference was found between the groups in terms of blood and milk Ca levels ($P>0.05$). The levels of proinflammatory cytokines (IL-1β, IL-6, TNF-α), oxidative stress markers (TAS, TOS, OSI) and mineral substance (Mg, Fe, Zn, Cu, Na, Ca) obtained from blood and milk serums of all groups are presented in *Table 2* and *Table 3*, respectively.

Table 2. *The levels of some proinflammatory cytokines and oxidative stress markers obtained from blood and milk serums of all groups*

Parameters		**Group 1**	**Group 2**	**Group 3**
IL-1β ng/L	Blood	$0.96\pm0.05^{b***}$	$1.59\pm0.22^{a***}$	$0.62\pm0.08^{b***}$
	Milk	$0.76\pm0.04^{b*}$	$1.09\pm0.11^{a*}$	$0.69\pm0.13^{b*}$
IL-6 ng/L	Blood	3.31±0.07	3.09±0.09	3.11±0.02
	Milk	$3.01\pm0.03^{b**}$	$3.16\pm0.02^{a**}$	$3.1\pm0.00^{a**}$
TNF-α ng/L	Blood	$0.97\pm0.14^{b***}$	$2.0\pm0.03^{a***}$	$0.96\pm0.12^{b***}$
	Milk	$0.58\pm0.06^{b*}$	$0.89\pm0.06^{a*}$	$0.76\pm0.07^{b*}$
TAS mmol/L	Blood	$1.22\pm0.02^{a*}$	$1.07\pm0.02^{b*}$	$1.12\pm0.04^{ab*}$
	Milk	1.07±0.00	1.07±0.03	1.12±0.01
TOS µmol/L	Blood	$0.96\pm0.04^{b**}$	$1.49\pm0.20^{a**}$	$0.78\pm0.15^{b**}$
	Milk	$1.03\pm0.01^{b**}$	$1.20\pm0.07^{ab**}$	$1.46\pm0.14^{a**}$
OSI	Blood	$0.08\pm0.00^{b**}$	$0.14\pm0.01^{a**}$	$0.07\pm0.01^{b**}$
	Milk	$0.10\pm0.00^{b*}$	$0.12\pm0.00^{ab*}$	$0.13\pm0.01^{a*}$

*^{a,b,c} Different letters in the same column indicate the statistical difference ($P<0.05$); * $P<0.05$, ** $P<0.01$, *** $P<0.001$; **IL-1β:** Interleukin 1 beta; **IL-6:** Interleukin 6; **TNF-α:** Tumor necrosis factor alpha; **TAS:** Total antioxidant status; **TOS:** Total oxidant status; **OSI:** Oxidative stress index; **Group 1:** Healthy; **Group 2:** Clinical mastitis; **Group 3:** Subclinical mastitis*

Table 3. *The levels of some mineral substance obtained from blood and milk serums of all groups*

Parameters		Group 1	Group 2	Group 3
Mg	Blood	$14.45\pm0.9^{a*}$	$12.30\pm0.45^{b*}$	$11.76\pm0.27^{b*}$
	Milk	$43.37\pm0.70^{b***}$	$50.02\pm3.24^{a***}$	$37.40\pm1.49^{b***}$
Fe	Blood	0.47±0.04	0.64±0.11	0.63±0.02
	Milk	$0.07\pm0.00^{b***}$	$0.13\pm0.01^{a***}$	$0.08\pm0.00^{b***}$
Zn	Blood	$0.23\pm0.01^{ab*}$	$0.21\pm0.00^{b*}$	$0.26\pm0.01^{a*}$
	Milk	0.45±0.02	0.51±0.10	0.58±0.04
Cu	Blood	$0.03\pm0.00^{b***}$	$0.15\pm0.00^{a***}$	$0.14\pm0.00^{a***}$
	Milk	$0.05\pm0.01^{a***}$	$0.04\pm0.00^{a***}$	$0.01\pm0.00^{b***}$
Na	Blood	372.39±0.15	371.05±1.66	373.78±1.78
	Milk	$158.67\pm3.82^{c***}$	$303.96\pm3.68^{a***}$	$200.97\pm5.74^{b***}$
Ca	Blood	45.73±1.0	42.66±1.38	42.21±0.75
	Milk	300.13±3.9	213.08±41.43	273.23±17.08

*^{a,b,c} Different letters in the same column indicate the statistical difference (P<0.05); * P<0.05; *** P<0.001;* ***Group 1:*** *Healthy;* ***Group 2:*** *Clinical mastitis;* ***Group 3:*** *Subclinical mastitis*

Discussion

In the present study, healthy dairy cows and those diagnosed with subclinical and clinical mastitis naturally infected by *S. aureus* were used. Levels of oxidative stress markers (TAS, TOS, OSI), proinflammatory cytokines (IL-1β, IL-6, TNF-α) and mineral substances (Mg, Fe, Zn, Cu, Na, Ca) were compared in the blood and milk serums of all groups. Since environmental and management differences such as heat stress, lactation period, and ration content can affect both oxidative stress and cytokine levels [13,33-35], the present study was carried out in mid-lactation cows fed the same ration in same season.

During mastitis, immune cells struggle to eliminate invading pathogens [13]. Cytokines are the important mediators in establishing immune response against intramammary infection [36], and proinflammatory cytokines including TNF-α, IL-1β and IL-6 are critical for activation of the innate immune system in epithelial cells, macrophages or monocytes [37]. It was reported that levels of TNF-α, IL-1β and IL-6 are significantly elevated in infected mammary glands [38] within clinical and subclinical mastitis [18], and they are not only produced locally but also released into the circulation [24]. Guo et al.[39] noted that there is an association between *S. aureus*-induced mastitis and TNF-α, IL-1β and IL-6 levels. Early changes in cytokine transcription may be useful as a predictive tool in the detection of *S. aureus*-induced mastitis [10]. Alluwaimi [11] reported that *S. aureus* may affect TNF-α release due to their immunosuppressive effect, while Osman et al.[12] stated that IL-1 and IL-6 levels increased in the early stage of *S. aureus* mastitis. Considering the findings of the presented study, it was observed that *S. aureus*-induced mastitis did not affect IL-1β and TNF-α levels in blood and milk serum of the Group 3, but their levels increased significantly in the Group 2. Similarly, Yang et al.[40] stated that *S. aureus,* which causes subclinical infections, results in only a very low level of proinflammatory cytokine response. On the other hand, it was reported that milk IL-6 level could be used in the early diagnosis of subclinical mastitis and significantly increased in the milk of cows with subclinical mastitis [41]. In addition, Kleczkowski et al.[17] noted that IL-6 is required to initiate the systemic inflammatory response. To clarify this situation, many studies have investigated the changes in cytokine levels, especially IL-6, in cases of mastitis caused by different bacteria [42-45]. Hagiwara et al.[43] reported that IL-6 is an important inflammatory mediator in cases of endotoxin-induced mastitis. On the other hand, Shaheen et al.[45] reported that IL-6 levels were similar between cows with healthy and subclinical mastitis without bacterial isolation. Similarly, Ohtsuka et al.[46] stated that the severity of the disease did not affect the level of IL-6 in cases of mastitis caused by coliform bacteria. However, staphylococcal enterotoxins stimulate lymphocytes and leukocytes rapidly and thus immediately trigger IL-6 release in the early stage of mastitis [12]. Therefore, we think that IL-6 level is more specific in *S. aureus*-induced subclinical mastitis because of the strong stimulation of IL-6 release as a defense mechanism in the early stage of staphylococcal mastitis. However, the relationship between IL-6 and mastitis cases caused by *S. aureus* or other bacterial species has not been clarified in terms of specificity and sensitivity. In a previous study, milk IL-6 levels were found higher in cows with *S. aureus*-induced subclinical mastitis compared to healthy cows and those with clinical mastitis. In the present study, while blood IL-6 level was similar in all groups, its milk serum level was higher in the Group 3 and Group 2 compared to the Group 1. It is known that systemic inflammation and oxidative stress occurs in cows with subclinical and clinical mastitis [14], and inflammatory response and oxidative stress are closely related [47]. Similarly, it was informed that there is a relationship between mastitis and oxidative stress [48].

It was also reported that total oxidant capacity increased and total antioxidant capacity decreased in clinical and subclinical mastitis [21]. Moreover, it was stated that total oxidant capacity of milk increased in cow with subclinical mastitis [25]. However, Sadek et al.[18] reported that while serum total antioxidant capacity decreased in subclinical mastitis, both milk and serum total antioxidant capacity decreased in clinical mastitis. So, oxidative stress status can be evaluated as a systemic or local finding in cases of mastitis caused by different bacterial species, including *S. aureus* [18,47,49-51]. However, since *S. aureus* can survive for a long time in host cells [52], it has a greater importance than other bacteria in terms of creating oxidative stress in subclinical mastitis cases. Nevertheless, sensitivity and specificity of OSI in the diagnosis of subclinical mastitis cases caused by *S. aureus* or other bacteria need to be investigated in further studies. In our study, *S. aureus*-induced mastitis did not affect milk TAS levels. However, it decreased the blood TAS level in the Group 2 compared to the Group 1. The reason for this is considered to be an intense antioxidant transition from blood to milk in cows with *S. aureus* mastitis, but the milk TAS level does not change as they are used to inactivate ROS in udder. TOS and OSI results obtained in this study support the above-mentioned information. *S. aureus*-induced mastitis resulted in increased blood TOS and OSI levels in the Group 2 than in other groups. In addition, it was associated with increased milk TOS and OSI levels in the Group 3 compared to the Group 1. On the other hand, it has been reported that there is a relationship between the antioxidant defense system and the number of bacterial colonies in cows with mastitis [53]. It is also known that the milk quality of treated or recovered cows is improved and oxidative stress is reduced by increasing the antioxidant defense system [54]. This can be assessed by taking a second milk sample after the first bacteriologically positive sample for infection stability. However, since the main purpose of our study was to instantly evaluate some biochemical changes in blood and milk levels in cows with mastitis caused by *S. aureus*, and no treatment was applied, repeated sampling was not performed. In addition, in the presented study, only milk samples from which aureus was clearly isolated were included in the study. In doubtful cases, cows from which milk samples were taken were excluded from the study. It was recorded that antioxidant levels decreased, a systemic oxidative stress and inflammation response were observed in cows with subclinical and clinical mastitis [14], and it is thought that the increase in oxidant level of milk with mastitis is related with the increase of epithelial cells such as macrophages, eosinophiles, neutrophiles and lymphocytes and some cytokines (IL-1β, IL-6, IL-8, TNF-α) [55].

Amiri et al.[25] stated that oxidative stress indices are current markers used in the determination of subclinical mastitis, and the antioxidant and oxidant levels in milk can be a diagnostic tool for the early diagnosis of mastitis. However, systemic oxidative stress level can increase in many diseases stress [48], so we considered that the milk OSI level would be more specific for the evaluation of mastitis. In addition, we believe that blood and milk mineral and macro element levels are important in mastitis studies. It is known that the blood-milk barrier and function of epithelial cells are disrupted during mastitis, and accordingly, it changes the levels of most components in udder secretion [20,56]. Therefore, in the presented study, the level of some mineral substances in blood and milk serums was measured to evaluate the effect of clinical and subclinical mastitis caused by *S. aureus* on the blood-milk barrier. A previous study indicated that while milk and blood levels of Ca, Mg, Fe and Zn significantly decreased in cows with subclinical mastitis, concentration of Na significantly increased; however, Cu level was not affected [23]. Another study demonstrated that while plasma Ca levels increased, Na and Mg levels did not change in cows with clinical and subclinical mastitis [20]. Moreover, Al-Autaish [57] revealed that serum Mg, Ca and Fe levels decreased, but Zn level increased in cows with subclinical mastitis. However, our study revealed that Ca level did not change in any group. Gera et al. [58] also reported that mastitis increased milk Zn and Fe levels, but not Cu level. As can be understood from the information above, mastitis may have different effects on the mineral values in milk and blood. In presented study, while non-significant changes in levels of Mg, Fe and Zn in the milk serum and in levels of Fe, Zn and Na the blood serum were observed in the Group 3 compared to the Group 1, Mg level in blood and Cu level in milk decreased, and Na level in milk increased. Cu is associated with humoral immune response [59], its deficiency reduces neutrophil killing capacity and increases susceptibility to bacterial infections [49]. Therefore, it is thought that decreased milk Cu level may affect the case of subclinical mastitis caused *by S. aureus*. It was also stated that the change in milk Na level in mastitis may be related with the mastitis pathogen, the severity of inflammation, milk fraction and a decrease in lactose production [28]. In this study, significant increases were observed in only milk levels of Mg, Fe and Na in the Group 2, while a decrease in blood level of Mg was observed. Since Na level has an effect on milk osmolality [60], we assumed that it could also affect the level of other minerals. Considering the above information, it is understood that *S. aureus* has different degrees of local and systemic effects on these observed parameters in cows with clinical and subclinical mastitis.

In conclusion, the present study revealed that *S. aureus* increased IL-1β and TNF-α levels in blood and milk serum of cows with clinical mastitis. IL-6 level increased only in milk serum of cows with both clinical and subclinical mastitis. So, while differences in IL-1β, IL-6 and TNF-α levels were observed in cows with clinical mastitis, only IL-6 level was the discriminant factor in cows with subclinical mastitis. On the other hand, *S. aureus* caused an increase in blood OSI levels in cows with clinical mastitis. OSI level increased in milk serum of cows with subclinical mastitis, according to healthy cows. Therefore, it can be emphasized that the

milk OSI level is of critical importance in terms of subclinical mastitis. *S. aureus* also caused some important changes in Mg, Fe, Na and Cu levels. While these parameters were affected at different levels in milk or blood serum, more specifically, milk Mg and Fe levels increased only in cows with clinical mastitis and milk Cu level decreased only in cows with subclinical mastitis.

Availability of Data and Materials

The data that support the findings of the present study are available from the corresponding author upon reasonable request.

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Competing Interests

The authors declare that they have no conflicts of interest.

Author Contributions

This work was carried out in collaboration between all authors. Serdal KURT and Funda ESKI conceptualized the hypothesis of this manuscript. Serdal KURT and Funda ESKI conducted research. Leyla MIS conducted laboratory experiments and analyzed data. Serdal KURT and Funda ESKI together wrote the manuscript. Pinar AYVAZOGLU DEMIR made statistical analysis. Serdal KURT and Funda ESKI critically reviewed the manuscript. All authors read and approved the final manuscript.

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