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Original article

PRE- AND POST-TREATMENT OXIDATIVE STRESS MARKERS, SERUM AMYLOID A AND METABOLIC VARIABLES IN DAIRY COWS WITH *STAPHYLOCOCCUS AUREUS* SUBCLINICAL MASTITIS

N. GOLSHAN[1], B. KHORAMIAN TOOSI[1] & M. HEIDARPOUR[1,2]

[1]Department of Clinical Sciences, School of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran; [2]Center of Excellence in Ruminant Abortion and Neonatal Mortality, School of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran

Summary

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The objective of the current research was to compare oxidative stress markers, metabolic variables and serum amyloid A (SAA) in dairy cows with *Staphylococcus aureus* subclinical mastitis before and after treatment and to evaluate their correlation with treatment outcome. Oxidative stress biomarkers, including total antioxidant capacity (TAC) and malondialdehyde (MDA) and SAA were measured in milk samples and non-esterified fatty acids (NEFA), glucose, calcium and magnesium were determined in blood serum samples of two groups of cows: 1) *S. aureus* subclinical mastitis cows with unsuccessful treatment (UST group; *S. aureus* isolated in bacterial culture and somatic cell count (SCC) ≥ 200,000 cells/mL in milk samples obtained before and after treatment; n=26), and 2) *S. aureus* subclinical mastitis cows with successful treatment (ST group; negative bacterial culture and SCC < 200,000 cells/mL in milk samples obtained after treatment; n=26). Successful treatment significantly decreased milk SCC and SAA and serum NEFA ($P<0.05$), while significantly increased serum calcium, magnesium and glucose levels ($P<0.05$) in ST cows. After unsuccessful treatment, mean milk SAA dropped significantly ($P<0.05$) while milk TAC and SCC and serum calcium levels increased ($P<0.05$) in UST cows. The findings of the present study showed that the improvement of negative energy balance (observed as decreased NEFA and increased glucose concentration following successful treatment) might be an important variable for the treatment outcome of *S. aureus* subclinical mastitis in dairy cows

Key words: oxidative stress, metabolic variables, *Staphylococcus aureus,* subclinical mastitis, treatment

INTRODUCTION

Staphylococcus aureus contributes to one of the most prevalent types of mastitis in dairy cows. The *S. aureus* mastitis is frequently subclinical, resulting in increased

somatic cell counts (SCC) and other indicators of inflammation with no visible alterations in either milk or the mammary gland. The mastitis caused by *S. aureus* is an important problem in dairy herds, resulting in significant economic losses and negative impacts on animal welfare (Monistero *et al.*, 2018). Mastitis is known as one of the major reasons for antibiotics use in dairy herds (Royster & Wagner, 2015). However, a considerable number of *S. aureus* infections do not respond appropriately to antibiotic therapy and the infected cows should be culled from the herd. The reported cure rates were about 24 to 34% when the infected cows were treated for subclinical *S. aureus* mastitis (Sol *et al.*, 1997; Oliveira & Ruegg, 2014; Ruegg, 2018). By hiding within leukocytes, *S. aureus* can escape from the antimicrobial effects of some antibiotics. The ability to generate enzymes such as beta-lactamase and leukocidins also enables *S. aureus* to acquire antibiotic resistance (Petersson-Wolfe *et al.*, 2010; Algharib *et al.*, 2020). So, it is advisable to develop some criteria for the detection of cows with *S. aureus* subclinical mastitis which are more likely to respond to antibiotic therapy and also to find efficient ways to improve antibiotic treatment efficiency (Ruegg, 2018). Age, the number of infected quarters, SCC, previous treatments, the stage of lactation and environmental factors are among factors that influence the success of antibiotic therapy (Hektoen *et al.* 2004; Barkema *et al.*, 2006; Oliveira & Ruegg, 2014). Nutritional and metabolic variables, such as minerals, glucose and non-esterified fatty acids (NEFA), also affect the immune response in dairy cattle (Suriyasathaporn *et al.*, 2000; Burvenich *et al.*, 2007; Nyman *et al.*, 2008; LeBlanc, 2010). Nearly all peripartum dairy cattle experience negative energy balance, insulin resistance, lipolysis, as well as hormonal changes, contributing to impaired immune response, especially neutrophil activities (LeBlanc, 2010).

Due to metabolic changes and pathogen challenges, cattle often encounter tremendous oxidative stress at the periparturient period, which enhances the impairment of the immune system (LeBlanc, 2010). Furthermore, oxidative stress also occurs in mastitis as phagocytic cells demonstrate their bactericidal activities through producing free radicals, particularly reactive oxygen species (ROS). In polymorphonuclear cells (PMNs), oxygen is converted to superoxide anion by a membrane-bound enzyme complex called NADPH oxidase. The ROS are consequently converted to hydrogen peroxide (H_2O_2) and hypochlorite free radical (HOCl) (Halliwell & Gutteridge, 1999). Due to the involvement of PMNs in inflammatory reactions, the production of ROS may increase during inflammation (Mudron *et al.*, 2007). Important inflammatory diseases in dairy cows, such as endometritis and mastitis, are thought to be associated with oxidative stress (Kleczkowski *et al.*, 2005; Ranjan *et al.*, 2005; Heidarpour *et al.*, 2012). Alterations in milk oxidative stress markers were revealed in cows with subclinical mastitis (Suriyasathaporn *et al.*, 2006; Atakisi *et al.*, 2010). Malondialdehyde (MDA) and total antioxidant capacity (TAC) are oxidative stress biomarkers that have been recently used for the diagnosis of subclinical mastitis (Sadek, 2017; Amiri *et al.*, 2019). Mastitis induces acute phase response, resulting in increased acute-phase proteins such as serum amyloid A (SAA) in milk. Such changes occur even before visible changes in the milk or clinical signs (Safi *et al.*, 2009). SAA is synthesised in the mammary gland; its level is substantially elevated in both

clinical and subclinical mastitis. Therefore, the measurement of milk SAA can be used for subclinical mastitis diagnosis. There is also an association between the SAA level and the severity of mastitis (Petersen *et al.*, 2004).

To our knowledge, no study has evaluated oxidative stress markers, metabolic variables and acute-phase proteins before and after treatment in dairy cows with *S. aureus* mastitis nor their association with treatment outcome. Therefore, the objectives of the present study were to investigate these factors in two groups of dairy cattle: those recovered from *S. aureus* subclinical mastitis after treatment and those showing no cure after treatment. Such information would be beneficial to detect variables influencing the efficiency of treatment.

MATERIALS AND METHODS

Animals and housing

Fifty-two Holstein cows were examined during 3 months from May to August 2019. The study was conducted in a commercial dairy herd (Mashhad, Iran) with more than 1,500 Holstein cows producing about 8,000 kg of fat-corrected milk per cow on average. The cows were milked three times a day, received a well-balanced total mixed ration and were kept and fed in a free stall. The first clinical examination was carried out between 20 and 30 days in milk. If the cows showed any apparent clinical signs, they were excluded from the study, and if not, the first milk samples were obtained. Second sampling and examination were performed two weeks after treatment. Cows suffering from diseases such as milk fever, retained foetal membranes, ketosis, lameness, displaced abomasum, metritis and clinical mastitis were excluded from the study.

Detection of subclinical mastitis

Bacterial culture and SCC count on quarter milk samples were used to detect subclinical mastitis caused by *S. aureus*. The milk sample for bacterial culture was obtained aseptically (National Mastitis Council, NMC), cultured on McConkey and blood agar plates, and incubated for 24–48 h at 37 °C. The SCC was measured with Fossomatic device 5000 (Hillerød, Denmark). Based on the results of SCC and bacterial culture, animals with SCC count more than 200,000 cells/mL and isolation of *S. aureus*, but no other bacteria, were considered as *S. aureus* subclinical mastitis.

Treatments and trial groups

The subclinical mastitis cows were treated with five intra-mammary ointments for lactating cows containing cefquinome (Cobacter MC; Kimia Biotechnology Co, Iran) every 24 h and an intramuscular injection of 5 mg/kg body weight enrofloxacin every 24 h for four days.

The second milk samples were collected two weeks after treatment. Regarding the results of SCC and bacterial culture of second samples, subclinical mastitis cows were divided into two groups: 1) cows with unsuccessful treatment (UST group; *S. aureus* isolated in bacterial culture and SCC≥200,000 cells/mL; n=26), and 2) cows with successful treatment (ST group; negative bacterial culture and SCC<200,000 cells/mL; n=26). It should be noted that the studied animals showed no clinical signs throughout the study.

Biochemical analysis

Blood samples were obtained from the jugular vein of all cows at the time of diagnosis (before treatment) and two weeks after treatment. Serum was separated after spinning at 1800×g for 10 minutes and stored at −20 °C until analy-

sis. The concentrations of NEFA, glucose, calcium and magnesium were determined with commercial kits (Randox, UK for NEFA; Pars Azmoon, Iran for glucose, calcium and magnesium) using an autoanalyzer (Mindray, BS 200, China).

For SAA measurement, whole milk samples were diluted with diluent buffers for SAA assay, as recommended by the manufacturer. The concentration of SAA was measured using an ELISA assay (Bioassay Technology Laboratory, Shanghai, China). The ELISA automatic washer (BioTek, ELX-50, Winooski, USA) and reader (BioTek, ELx-800, Winooski, USA) were utilised.

Defatted milk samples were used for the measurement of oxidative stress markers and SAA. The milk samples were defatted via centrifugation at 4000×g at 4 °C for 20 minutes. TAC was measured through the ferric reducing antioxidant power (FRAP) method described by Chen *et al.* (2003). Trichloroacetic acid assay was used for the measurement of MDA (Suriyasathaporn *et al.,* 2012).

Statistical analysis

Repeated measure ANOVA was used to compare means among the trial groups and to assess the effect of time of sampling throughout the study. The Tukey *post hoc* test was run for group as the between-subject factor. Paired samples t-test was used for comparison of measured parameters before and after treatment in each trial group. SPSS software (version 16, USA) was used for data analysis. A P value less than 0.05 was considered statistically significant.

RESULTS

The results of repeated measures ANOVA demonstrated that time had significant effects on the values of SAA, magnesium and calcium ($P<0.05$). The group had significant effects on the values of SCC and glucose ($P<0.05$). Significant interactions between sampling time and group were observed for SCC, TAC and magnesium. UST cows showed significantly higher SCC but lower glucose than subclinical mastitis cows with successful treatment ($P<0.05$; Table 1). The value of TAC after treatment in UST cows was significantly higher than that in the ST cows ($P<0.05$).

Table 1. Means ± SEM of metabolic variables, SAA, minerals and oxidative stress markers in unsuccessful treatment (UST) and successful treatment (ST) cows.

	UST cows (n=26)	ST cows (n=26)
Somatic cell count ($\times 10^3$/mL)	269.0±132.8*	451.2±132.8
Milk SAA (µg/mL)	14.6±0.10	14.2±0.10
NEFA (mmol/L)	0.316±0.021	0.268±0.021
Glucose (mmol/L)	1.83±0.20*	1.24±0.20
Calcium (mmol/L)	1.02±0.02	1.08±0.02
Magnesium (mmol/L)	2.65±0.05	2.88±0.05
Milk TAC (mmol/L)	0.945±0.087	1.156±0.087
Milk MDA (nmol/L)	0.177±0.018	0.216±0.018

* Significant difference between UST and ST cows ($P<0.05$); SAA, serum amyloid A; NEFA, non-esterified fatty acids, TAC, total antioxidant capacity; MDA, malondialdehyde.

Table 2. Means ± SD of metabolic variables, SAA, minerals and oxidative stress markers in unsuccessful treatment cows (n=26) at the time of diagnosis (before treatment), and after treatment

	Before treatment	After treatment
Somatic cell count ($\times 10^3$/mL)	377.16±44.53	517.01±166.16*
Milk SAA (μg/mL)	15.83±0.41	12.71±1.02*
NEFA (mmol/L)	0.25±0.10	0.27±0.17
Glucose (mmol/L)	1.11±0.92	1.46±1.30
Calcium (mmol/L)	2.64±0.28	3.15±0.47*
Magnesium (mmol/L)	1.10±0.11	1.07±0.23
Milk TAC (mmol/L)	0.798±0.101	1.488±0.112*
Milk MDA (nmol/L)	0.158±0.023	0.196±0.023*

* Significant difference between before and after treatment (P<0.05); SAA, serum amyloid A; NEFA, non-esterified fatty acids, TAC, total antioxidant capacity; MDA, malondialdehyde.

Table 3. Means ± SD of metabolic variables, SAA, minerals and oxidative stress markers in successful treatment cows (n=26) at the time of diagnosis (before treatment), and after treatment

	Before treatment	After treatment
Somatic cell count ($\times 10^3$/mL)	399.22±54.38	146.98±30.24*
Milk SAA (μg/mL)	16.12±0.43	13.44±1.10*
NEFA (mmol/L)	0.39±0.18	0.24±0.13*
Glucose (mmol/L)	1.47±1.19	2.09±1.51*
Calcium (mmol/L)	2.56±0.25	2.72±0.21**
Magnesium (mmol/L)	0.9±0.23	1.14±0.14
Milk TAC (mmol/L)	0.865±0.089	1.050±0.112
Milk MDA (nmol/L)	0.205±0.023	0.227±0.023

* Significant difference between before and after treatment (P<0.05); SAA, serum amyloid A; NEFA, non-esterified fatty acids; TAC, total antioxidant capacity; MDA, malondialdehyde.

After unsuccessful treatment, mean milk SAA dropped significantly (P<0.05) while milk TAC and SCC and serum calcium levels were elevated (P<0.05) in UST cows. No significant difference was observed for MDA, magnesium, NEFA and glucose before and after treatment in UST cows (Table 2).

Successful treatment significantly decreased milk SCC and SAA and serum NEFA (P<0.05), while significantly increased serum calcium, magnesium and glucose levels (P<0.05) in ST cows. No significant difference was observed for milk MDA and FRAP before and after treatment in ST cows (Table 3).

DISCUSSION

Milk values of oxidative stress markers and metabolic variables were evaluated before and after treatment in two groups of cows with *S. aureus* subclinical mastitis (cured vs uncured). The possibility that oxidative stress might be a major underlying cause of immune dysfunction in dairy cows was confirmed in various studies (Bernabucci *et al.*, 2005; Castillo *et al.*, 2005; Sordillo *et al.*, 2005). Due to the increased number and activity of immune cells, the production of excess free radicals, such as reactive nitrogen species (RNS) and ROS in mammary glands with subclinical mastitis was enhanced. Such

an increase would result in oxidative stress. The evaluation of oxidative stress biomarkers in milk has been used for the early diagnosis of subclinical mastitis (Ellah, 2013). In the present study, the UST cows showed a significant increase in TAC after treatment, and their values after treatment were significantly higher than those in the ST cows ($P<0.05$). Increased antioxidant capacity assessed by the FRAP method has been revealed in dairy cows with subclinical mastitis (Amiri *et al.*, 2019). Similarly, other studies reported enhanced activity of milk antioxidative enzymes in cows and goats with subclinical mastitis (Seifu *et al.*, 2007; Andrei *et al.*, 2011). However, our finding is not in agreement with the study of Silanikove *et al.* (2014), which reported that milk antioxidant levels were significantly lower in infected quarters of goats vs healthy ones. There are many enzymatic and nonenzymatic antioxidants such as superoxide dismutase, catalase, glutathione peroxidase, lactoferrin, carotenoids and vitamins E, A and C in milk, which can neutralise free radicals very quickly (Yilmaz-Ersan *et al.*, 2018). The increased TAC level in cows with unsuccessful treatment showed that probably higher levels of free radicals had been generated in these cows and up-regulation of antioxidants has occurred in response to oxidative stress. The increase in the antioxidant capacity might be described via the fact that oxidants could activate the expression of genes through antioxidant responsive elements (Rushmore *et al.*, 1991). Furthermore, the FRAP is affected by the concentration of uric acid, total protein, vitamin C, bilirubin and albumin, all of which originate from blood circulation (Bouwstra *et al.*, 2010). Thus, the higher TAC level in *S. aureus* subclinical mastitis cows with unsuccessful treatment might be caused by more severe inflammation in the mammary gland and, in turn, higher vascular permeability, which have been associated with increased release of blood components into the milk. Enhanced lipid peroxidation has been reported in cattle and goats with subclinical mastitis (Silanikove *et al.*, 2014; Amiri *et al.*, 2019). ROS and nitrogen metabolites can promote tissue oxidative injury and disturb udder function (Zhao & Lacasse, 2008). Lipid peroxidation contributes to the disarrangement and finally, disruption of cell membranes, resulting in cell death. However, in the current research, there was no significant difference for the level of MDA, as a marker of lipid peroxidation, before and after treatment in both ST and UST groups. It seems that the over-expression of the antioxidants in subclinical mastitis cows, even in those with unsuccessful treatment, was high enough to prevent lipid peroxidation. However, the effects of oxidative stress on the oxidative damage of proteins and nucleic acids and immune and inflammatory responses are not clear.

In the present study, significantly lower glucose concentration was observed in subclinical mastitis cows with unsuccessful treatment compared to that in subclinical mastitis cows with successful treatment. Furthermore, NEFA showed a descending whereas glucose demonstrated an ascending trend in ST cows and successful treatment was associated with a significantly decreased serum NEFA ($P<0.05$) and increased serum glucose levels ($P<0.05$); while, this was not the case in UST cows. The immune system function in the peripartum period is affected by the metabolic status. Negative energy balance has an adverse impact on the udder defense system and results in immune response impairments, such as lower bactericidal activities, impaired

phagocytosis, reduced chemotaxis, diminished blood leukocyte count and decreased cytokine production (Suriyasathaporn *et al.*, 2000). Such changes can make dairy cows susceptible to mastitis and influence the severity and duration of mastitis (Duffield *et al.*, 2009). Furthermore, decreased NEFA values in ST cows might be associated with diminished lipid infiltration in the liver, possibly improving the hepatic synthesis of proteins that are involved in innate immune responses (Nyman *et al.*, 2008).

As mammary glands could synthesise some acute-phase proteins like SAA, the measurement of milk SAA has been applied for the diagnosis of subclinical mastitis (Safi *et al.*, 2009). There is an association between its concentration and SCC (Gerardi *et al.*, 2009; Simojoki *et al.*, 2009) and the severity of subclinical mastitis (Eckersall *et al.*, 2006). Recovered dairy cows with *Klebsiella pneumonia* mastitis had significantly lower acute phase proteins than euthanised ones (Hisaeda *et al.*, 2011). In the present study, however, time had significant effects on milk SAA ($P<0.05$) and its concentration significantly decreased in both ST and UST groups after treatment. It seems that SAA concentration diminished by time and was independent of treatment outcome; indeed, there was no relationship between SAA concentration and the outcome of treatment in the subclinical mastitis caused by *S. aureus,* as a Gram-positive bacterium.

The clinical importance of increased blood magnesium concentration after treatment in ST cows and its role in the outcome of subclinical mastitis treatment is not clear. One of the excretion routes of magnesium is mammary glands (Stockham & Scott, 2008); therefore, the higher magnesium level in ST cows might be caused by the fact that resolved inflammation in the mammary gland would decrease vascular permeability, resulting in decreased release of blood magnesium into the milk. An adequate concentration of calcium is necessary for the appropriate closure of the teat sphincter. Lack of teat sphincter closure would result in an enhanced risk of mastitis (Goff, 2008). Furthermore, adequate intracellular calcium concentration is required for signalling in the immune cells. The increased demand for calcium in periparturient cows may negatively influence intracellular ionised calcium stores of peripheral mononuclear cells. This decrease in intracellular calcium in immune cells could affect intracellular calcium release following an activating stimulus, contributing to periparturient immune suppression (Kimura *et al.*, 2006). In the present study, however, time had significant effects on the concentration of serum calcium ($P<0.05$) and its concentration significantly increased in both ST and UST groups after treatment. It seems that calcium concentration increased by time and was independent of treatment outcome.

The findings of the present study showed that the improvement of negative energy balance (observed as decreased NEFA and increased glucose concentration following successful treatment) might be an important variable for the treatment outcome of *S. aureus* subclinical mastitis in dairy cows. However, there was no association between the treatment outcome and the values of minerals (calcium and magnesium), oxidative stress markers (TAC and MDA) and acute phase protein (SAA).

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Correspondence:

M. Heidarpour
P.O.Box 91775-1793
Fax: +98-511-8763852
e-mail: heidarpour@um.ac.ir
ORCID: 0000000205807984