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Evaluation of antioxidant profile in subclinical mastitis in dairy buffaloes

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Abstract
The present research investigation was taken up to find out level of oxidative stress in dairy buffaloes suffering from subclinical mastitis with emphasis on *Staphylococcus aureus* infection at Pantnagar, Uttarakhand, India. Buffaloes were screened for subclinical mastitis (SCM) based on physical examination of udder, California mastitis test (CMT), Somatic cell count (SCC) and Differential cell count (DCC) on quarter's milk samples. Intramammary infection (IMI) was confirmed by cultural examination of milk. A total of 30 buffaloes were included in the study and divided randomly in 3 groups consisting of healthy control, buffaloes affected with subclinical mastitis with IMI caused by *S. aureus* and ten buffaloes infected with pathogens other than *S. aureus*. There was significant increase in levels of Lipid peroxidation (LPO), superoxide dismutase (SOD), catalase (CAT), lactate dehydrogenase (LDH) and Alkaline phosphatase (ALP) while significant reduction in reduced glutathione (GSH) was observed in buffaloes with SCM. No significant difference was observed in blood urea nitrogen (BUN) concentration in buffaloes with SCM. Micromineral estimation revealed higher serum zinc and iron in buffaloes with subclinical mastitis than that of healthy control.

Keywords: Buffaloes, subclinical mastitis, oxidative stress, *Staphylococcus aureus*, microminerals

Introduction
Mastitis is the inflammation of the mammary gland. Pathological changes at the gland level along with physical, chemical and bacteriological alterations in milk are prominent features of mastitis [1]. It can affect all lactating mammals but presents a major health crisis in dairy animals [2]. In dairy animals, it is a highly prevalent disease not only in dairy cattle but also in dairy buffaloes [3], though traditionally it is considered that buffaloes are less susceptible to mastitis than cattle [4]. However, more pendulous udder and longer teats may predispose buffaloes to greater risk of mastitis [3]. In recent years, buffalo health has gained priority and research has been focused on buffalo specific health problems. Bubaline mastitis is one such ailment and it also has capacity to adversely affect our economy. Bubaline mastitis inflicts financial losses on owing to decreased milk yield, increased labour, high treatment costs, withholding milk for human consumption following treatment and premature culling [5].
The specificity of oxidative stress and its relation to various diseases including mastitis is a complex phenomenon. Production of free radicals is a normal occurrence in living tissues. Under normal homeostasis, the reactive oxygen species (ROS) are effectively counteracted by cellular defense mechanisms (enzymatic-antioxidants) or non-enzymatic antioxidants [6]. Imbalance due to surplus ROS and/or absence of substantial amounts of antioxidants leads to oxidative stress [7]. The oxidative stress is the prime factor that leads to immune dysfunction and impaired the inflammatory response [8], enabling the establishment of microbial pathogens in mammary glands. The most commonly used markers for evaluation of oxidative stress in biological fluids are lipid peroxidation and glutathione. Alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) serve as general indicators of severity of tissue damage. The present research investigation was taken up to assess the role of SCM on the alterations in levels of oxidative stress markers *viz.* catalase (CAT), reduced glutathione (GSH), Lipid peroxidation (LPO) and superoxide dismutase (SOD); trace minerals *viz.* copper (Cu), iron (Fe) and zinc (Zn) and the activity of serum enzymes such as alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and blood urea nitrogen (BUN) in Murrah buffaloes and evaluate them as being a probable indicator of SCM in buffaloes.

Materials and Methods

Study area: The research investigation was conducted on lactating buffaloes of Murrah breed raised at Instructional Dairy Farm (IDF), GBPUA & T., Pantnagar which is located in Terai region of Uttarakhand state in North India. The climate in Pantnagar is warm and temperate and summers have most of the rainfall. As per Köppen-Geiger system climate of Pantnagar is classified as Cwa.

Selection of animals: Murrah buffaloes were screened for subclinical mastitis on the basis of physical examination of udder, California mastitis test (CMT), Somatic cell count (SCC) and Differential cell count (DCC) on quarter's milk samples. Intramammary infection (IMI) was confirmed by cultural examination of milk.

Study design: To evaluate the oxidative stress markers in peripheral blood, 30 buffaloes were included in the study. Out of these 30 buffaloes, 10 apparently healthy buffaloes which were negative for SCM served as healthy control. Ten buffaloes affected with subclinical mastitis with intramammary infection (IMI) caused by *S. aureus* and ten buffaloes infected with pathogens other than *S. aureus* in one or two quarters were included to investigate oxidative stress markers in peripheral blood. The results of these two groups which are affected with SCM were compared with each other and with those of healthy buffaloes.

Approximately 10 ml of blood sample was collected from affected animal in a sterile syringe aseptically. Four ml of blood was poured into heparinized glass tubes to measure the oxidative stress markers which included the measurement of lipid peroxidation as regards to malondialdehyde (MDA) levels, potential antioxidant reduced glutathione (GSH) concentration and the activities of antioxidant enzymes *viz*. catalase (CAT) and superoxide dismutase (SOD). Approximately 3 ml of heparinized blood from each animal was centrifuged at 400 g for 10 min; the plasma and buffy coat were removed to collect the red blood cells (RBC). The RBC pellet so obtained was washed thrice with ice-cold normal saline solution (NSS). Then, one part of RBC pallet was diluted with ice-cold distilled water in ratio of 1:10 to prepare 10% stock homosylate. This stock homosylate was used to determine the MDA level and activities off CAT and SOD. Another part of RBC pellet was kept separately was diluted with ice cold NSS to get 50% RBC suspension. This was used spectrophotomectrically for estimation of GSH using cyanomethaemoglobin method [9]. To harvest serum, left over i.e. 6 ml of blood was transferred into a clean and dry glass tube without any anticoagulant in it. The tube was kept undisturbed in slanting position to allow proper clotting. It was then centrifuged at 3000 rpm for 10 minutes. The serum was then harvested, labeled clearly and stored in clean and dry eppndorf tubes at -20 degree centigrade till used further for biochemical and serological estimation of enzymes' activity such as lactate dehydrogenase (LDH), alkaline phosphatase (ALP) and blood urea nitrogen (BUN) activity and to evaluate the antioxidant micromineral status *viz*. copper (Cu), iron (Fe) and zinc (Zn).

Statistical analysis: Statistical analysis of results was performed using Graph pad Prism statistical software version 8. One-way ANOVA was used to compare the mean activity between groups at p-value of <0.05.

Results

Mean CMT point score and SCC recorded in milk of healthy lactating dairy buffaloes were 0.16± 0.16 and 73.33 ± 16.01 *1000/ml respectively. Mean CMT point score and SCC recorded in the milk of subclinical mastitic buffaloes were 2.67±0.21 and 416.67±28.62 *1000/ml respectively; which were significantly higher in comparison to normal healthy buffaloes. The blood antioxidant profile and lipid peroxides in buffaloes with subclinical mastitis (table 1). The mean activities of LPO, GSH, SOD and CAT in blood from healthy were found to be 2.601 nmol MDA/mg Hb, 0.793 µmol DTNB-GSH conjugate/ml packed RBC, 0.455 µmol MTT formazan/mg Hb and 89.991 µmol H_2O_2 decomposed/min/mg Hb respectively. There was significant increase in levels of LPO, SOD and CAT while significant reduction in GSH was observed in buffaloes with SCM. Mean activities serological enzymes *viz*. LDH, ALP and BUN from healthy were evaluated to be 963.308 IU, 60.816 IU and 23.302 IU. Results revealed there was considerable increase in LDH and ALP concentrations in buffaloes with SCM while no significant alterations was observed in BUN concentration in buffaloes with SCM (table 2). Mean concentration of zinc, copper and iron in blood serum from healthy murrah buffaloes were found to be 71.58 mg/dl, 144.90 mg/dl and 66.59 mg/dl correspondingly. Micromineral estimation revealed higher serum zinc and iron levels and non-significant (P<0.05) increase in serum copper level (table 3). However, no significant (P<0.05) differences were observed in the concentration of any of the stress markers in blood or serum of buffaloes with *S. aureus* IMI and infection with pathogens other than *S. aureus.*

Discussion

The mammary gland health is determined by assessing the means CMT point score and somatic cell count. Normal healthy udder milk shows low CMT point score and SCC and showing no abnormal constituents [10]. The present study revealed that *Staphylococcus aureus* is the predominant cause of subclinical mastitis in buffaloes. Lipids are easily oxidized and lipid peroxides are formed. They decompose to form aldehydes, out of which malondialdehyde (MDA) is prominent [11]. The present investigation revealed significantly higher levels of erythrocytic MDA in buffaloes with subclinical mastitis irrespective of bacterial isolates. Similar findings have been reported by Mahapatra *et al.* (2018) [12] and Zigo *et al.* (2019) [13] both in subclinical and clinical mastitis in buffaloes and dairy cattle, indicating the involvement of udder related oxidative stress and the possible oxidative damage. This peroxidative damage to membranes might be related with too much production of reactive oxygen species such as nitric oxide (NO) by mammary epithelial cells and macrophages during inflammation [14], evidenced by increased concentrations of nitrite and nitrate both in plasma and milk following intramammary infection [15, 16]. Glutathione, is a a thiol-containing tripeptide. Its reduced form i.e. GSH is present at high concentrations in living cells. Upon reaction with reactive oxygen species, it gets oxidized to glutathione radical which can be regenerated to its reduced form by glutathione reductase [11]. Significant reduction in GSH concentration in buffaloes with SCM could be credited to over consumption of this cytosolic enzyme to scavenge overproduced free radicals. This is suggestive that milk of those buffalo suffering from SCM might have higher PMN along with low concentration of GSH [17]. Irrespective of

bacterial isolates, present investigation revealed significantly higher SOD and catalase activities in blood of infected buffaloes. Similar findings have been reported by Andrei *et al.* (2010) [18] in subclinical mastitis in dairy cows. This might reflect a compensatory response to excessive production of ROS by mammary epithelial cells and macrophages during inflammation [14].

The origin of LDH in SCM milk is attributed to the presence of leucocytes and epithelial cells from the udder [19]. High levels of LDH in the blood serum suggests an increase rate of systemic tissue destruction or remodulation, perhaps including demineralization of bone as mastitis is usually associated with reduced serum calcium levels [20]. ALP activity is being considered as reliable biomarkers in early subclinical mastitis [21]. ALP is an enzyme that is naturally found in biological tissues and fluids. Significant increase in ALP concentration in SCM milk might be due to both mammary epithelial damage and a breach in the blood-milk barrier selectively damaged by bacterial toxins [22]. Blood urea nitrogen is the only reliable biochemical marker used which is positively correlated with the type of bacteria present in mastitis. Cows suffering from gram negative infected mastitis have higher level of blood urea nitrogen as compared to gram positive infected cows [23]. In the present study comparable levels of urea in the blood suggests that prolonged infection by gram positive bacteria. Moreover, significant increase in the mean concentration of Fe and Zn in SCM milk also indicates the higher prevalence of Gram positive infections in the present study. The increase of these trace minerals is attributed to breach in mammary epithelial function [24] as gram positive bacteria are more pathogenic in the destruction of the mammary gland epithelia [25].

Table 1: Mean ± SEM activities of LPO, GSH, SOD and CAT in blood from healthy and subclinical mastitis affected buffaloes

Parameter	Healthy control	*S. aureus* Infection	Infection other than *S. aureus*
LPO (nmol MDA/mg Hb)	2.601 ± 0.088	3.458 ± 0.093 [a]	3.492 ± 0.083 [a]
GSH (µmol DTNB-GSH conjugate/ml packed RBC)	0.793 ± 0.032	0.542 ± 0.033[a]	0.591 ± 0.034 [a]
SOD (µmol MTT formazan/mg Hb)	0.455 ± 0.049	0.696 ± 0.033 [a]	0.723 ± 0.030 [a]
CAT (µmol H_2O_2 decomposed/min/mg Hb)	89.991 ± 2.166	146.876 ± 4.881[a]	144.769 ± 6.655[a]

a=Significant ($P \leq 0.05$) difference as compared to healthy buffaloes within same row.

Table 2: Mean ± SEM (IU/L) activities of LDH, ALP and BUN in blood serum from healthy, subclinical mastitis affected buffaloes

Parameter	Healthy control	*S. aureus* Infection	Infection other than *S. aureus*
LDH (IU/L)	963.308 ± 13.770	1116.915 ± 10.083 [a]	1124.248 ± 12.121 [a]
ALP (IU/L)	60.816 ± 2.071	84.427 ± 2.032 [a]	82.550 ± 1.996 [a]
BUN (IU/L)	23.302 ± 0.555	24.170 ± 0.537	23.492 ± 0.481

a=Significant ($P \leq 0.05$) difference as compared to healthy buffaloes within same row.

Table 3: Mean ± SEM (mg/dl) concentration of microminerals in blood serum from healthy, subclinical mastitis affected buffaloes

Parameter	Healthy control	*S. aureus* Infection	Infection other than *S.aureus*
Zinc (mg/dl)	71.584 ± 2.212	117.100 ± 2.700[a]	122.794 ± 2.477 [a]
Copper (mg/dl)	144.905 ± 3.202	149.194 ± 2.528	153.962 ± 2.712
Iron (mg/dl)	66.592 ±1.496	83.203 ± 1.884 [a]	81.918 ± 2.675 [a]

a=Significant ($P \leq 0.05$) difference as compared to healthy buffaloes within same row

Conclusion

The present investigation revealed that there was a major compromise in antioxidant defense system in buffaloes suffering from subclinical mastitis due to the colossal rise in concentration of ROS and other stress markers and it is crucial to supplement antioxidants along with the traditional antimicrobial therapy for managing subclinical mastitis in dairy animals.

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