

## Potential of Indigenous Enzymatic Activities, Nitric Oxide and Ceruloplasmin in Bovine Milk to Diagnose Subclinical Mastitis

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### Abstract

The objective of this present study was to evaluate some indigenous enzymatic activities, nitric oxide production in milk cells and ceruloplasmin ( $C_p$ ) level in milk as diagnostic indicators of bovine subclinical mastitis (SCM). The crossbred cows were screened with California mastitis test (CMT) to determine the status of SCM in mammary glands. Based on CMT point score and somatic cell count (SCC) of milk, the mammary quarters were classified as healthy (negative CMT reaction and  $SCC < 3$  lakhs/ml) and SCM (CMT reaction + or more and  $SCC > 10$  lakhs/ml). The results of the study revealed that SCC, cyclooxygenase (COX), myeloperoxidase (MPO), lactoperoxidase (LP), nitric oxide (NO) and ceruloplasmin ( $C_p$ ) levels were significantly ( $p < 0.05$ ) higher in milk collected from SCM quarters compared to healthy quarters. Pearson's correlation ( $r$ ) of paired data of individual SCM infected quarters revealed the existence of a significant positive ( $P < 0.01$ ) correlation among SCC and COX activity, LP activity, MPO activity and NO production. However, no significant correlation between SCC and  $C_p$  level was observed in milk samples of SCM infected quarters. Therefore, alteration of these enzyme activities, NO production and  $C_p$  activity in milk could be used as an alternative diagnostic tool to screen for SCM.

**Keywords:** Ceruloplasmin; Cyclooxygenase; Lactoperoxidase; Milk; Myeloperoxidase; Nitric oxide; Subclinical mastitis

### Introduction

Bovine mastitis is an important animal health disease leading to significant economic losses to the dairy industry due to higher treatment cost and culling rate. Massive influx of somatic cells and secretion of inflammatory mediators into udder are the common features in intramammary infection [1]. During inflammation, lipid derived mediators such as prostaglandins are involved in mammary immunity which exerts potent chemokinetic and chemotactic activity on leukocytes [2]. Cyclooxygenase (COX), a key enzyme, plays a vital role in prostaglandin synthesis during inflammation. An increased synthesis of arachidonic acid metabolites and high concentration of COX are observed during mastitis [3].

Nitric oxide (NO), produced from L-arginine by nitric oxide synthase, controls various vital physiological functions of body and it is important in the host defense by destroying microbes. The antimicrobial effect of NO on bacteria is due to peroxynitrite, reactive nitrogen metabolite, derived from oxidation of NO. The macrophage and epithelial cells of mammary gland produce significant amount of nitric oxide that takes part in the inflammatory process [4]. Many workers reported that level of NO in mammary gland secretion increases significantly in subclinical and clinical mastitis [5-7].

The acute phase response is the reaction of animals to disturbance in its homeostasis caused by infection, tissue injury or immunological disorders [8,9]. High concentration of acute phase protein in milk from infected quarters has been detected due to high permeability of blood milk barrier during episodes of mastitis [10]. Ceruloplasmin ( $C_p$ ) is recognized as an important acute phase reactant in the cows and its high concentration has been observed in mastitis [11,12].

Mastitis generally increases the enzymatic and biochemical activities in milk [13]. The myeloperoxidase (MPO), a lysosomal enzyme of neutrophilic granules, is a constituent of oxygen dependent antimicrobial activity of leukocytes [14]. MPO, along with hydrogen peroxide and halide creates potent oxygen dependent antimicrobial agent of the cell against pathogen [15]. Researcher observed increased

level of MPO during mastitis where leukocyte function is activated [16,17]. Similarly, lactoperoxidase (LP) is the predominant enzyme in bovine milk responsible for its antimicrobial properties, which requires sufficient concentrations of hydrogen peroxide and thiocyanate ion. It is the second most abundant enzyme in bovine milk after xanthine oxidoreductase [18]. Andrei et al. [19] observed increased LP activity in mastitis milk.

Although, diagnosis of clinical mastitis by visual inspection and palpation is relatively easy but, diagnostic problems arise when dealing with subclinical mastitis where an increased somatic cell count (SCC) is the only finding. Further, SCM possess a potential health threat to other cows for new infection. Therefore, efficient detection of SCM is very much required to reduce the incidence of mastitis in a well-managed dairy herd. As per the recommendation of International Dairy Federation (IDF), the diagnosis of mastitis is based on the SCC and microbiological status of the quarter [20]. Bacteriological sampling is not always feasible as a routine test to identify subclinical mastitis as the procedure is both time consuming and uneconomical in large dairy herds. Presently, SCM is generally diagnosed by cow-side tests like the California Mastitis Test (CMT) or by SCC in milk samples. These tests are less efficient in detecting chronic subclinical mastitis than for acute clinical cases [21]. Therefore, it is important to investigate alternative parameters/methods for detection of SCM. Hence, the present study was conducted to evaluate certain indigenous enzymatic activities and NO production in milk cells and  $C_p$  activity in milk as diagnostic indicators of bovine SCM.

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## Materials and Methods

### Selection of animals and collection of milk samples

The present study was conducted in a livestock research farm (Cattle & Buffalo) of the institute. These crossbred cows were maintained in the animal shed of the institute under identical environmental conditions. Thirty SCM infected cows with single quarter infection (Group I) and thirty healthy cows (Group II) were included for the present investigation after screening with CMT. Approximately, 80ml milk from each quarter was collected in sterile vials after cleaning the teat orifice with 70% ethyl alcohol and discarding few streams of foremilk. The milk samples with negative CMT reaction, SCC < 3 lakhs/ml and negative for bacterial growth were classified as healthy quarters. Whereas, milk samples with positive CMT reaction (+ or more), SCC > 11 lakhs/ml and positive for bacterial growth were classified as SCM infected quarters. The SCC per ml of milk was determined as per standard method [22]. The isolation of causative organism in collected milk samples was done by spreading 10 µl of milk over 5% bovine blood agar plate and identified on the basis of colony morphology, characteristic hemolytic pattern and Gram's staining [23].

### Isolation of milk leukocytes

The milk leukocytes were isolated from the milk samples as per the standard method [24]. Viability of the cells was checked by trypan blue exclusion technique and the cell suspension was adjusted to  $1 \times 10^8$  for COX assay and  $1 \times 10^6$  cells/ml in phosphate buffer saline (PBS) for NO production and MPO assay. The whole milk was used for estimation of LP and  $C_p$ .

### COX assay

The cell pellets ( $\approx 1 \times 10^8$ ) were sonicated in cold PBS for COX measurement. Total COX activity in the milk cell lysate was estimated by COX activity assay kit as per manufacturer's instruction (Cayman Chemical Company, Ann Arbor, MI).

### MPO assay

The MPO was assayed by using o-dianisidine (Sigma, St. Louis, MO, USA) as electron donor [25] and enzyme concentration was calculated by using molar extinction coefficient for oxidized o-dianisidine. In brief, the test was initiated with adding 0.2 ml of milk leukocyte suspension ( $1 \times 10^6$  cells/ml) to 2.0 ml of substrate solution (0.08 M  $H_2O_2$ , 0.32 mM o-dianisidine and 0.05% Triton X-100 in 0.1M citric acid sodium citrate buffer (pH 5.5 ± 0.1)). The mixture was incubated at room temperature for 1.0 minute and reaction was terminated by adding 2.0 ml of 35% perchloric acid. Absorbance was read at 560nm in spectrophotometer. Since no standard is available, the molar extinction coefficient for oxidized o-dianisidine was assessed with known amounts of glucose oxidase-peroxidase assay and found to be  $20,040 \pm 400 \text{ mol}^{-1} \text{dm}^{-1} \text{cm}^{-1}$ .

### NO production assay

NO production in milk leukocytes was measured by nitrate reduction on copper-cadmium alloy (Cu-Cd alloy) followed by colour development with Griess reagent as per the method described earlier [26]. In brief, 100 µl of  $1 \times 10^6$  cells/ml stimulated with 25 µg of lipopolysaccharide (LPS) was incubated at 37 °C for 24 hours. After incubation 100 µl of pre stimulated cells were suspended in 400 µl of carbonate buffer with 150 mg of Cu-Cd fillings and again incubated for 1 hr at room temperature with frequent vortexing. The reaction was stopped by adding sodium hydroxide (NaOH, 0.35 M) and Zinc sulfate

( $ZnSO_4$ , 120 mM). Further the mixture was vortexed and centrifuged at 400 g for 15 minutes. Finally, the Griess reagent was added to the clear supernatant in equal volume. After 10 minute OD was measured at 545 nm in micro plate reader. The value of NO production was calculated from a standard curve using various concentrations of potassium nitrite.

### Estimation of $C_p$ in milk

For  $C_p$  estimation, milk serum (whey) was prepared at a two-step centrifugation procedure. At first milk samples were centrifuged at 3000 rpm for 10 min to remove their creams and cells. Samples were then treated with 0.1 M hydrochloric acid at controlled pH for 20 min for casein precipitation. Treated samples were recentrifuged and the supernatants (Whey) were collected. The  $C_p$  level in milk serum was determined by measuring paraphenylene diamine (PPD) oxidase activity as per method described earlier [27].

### Estimation of LP in milk

The measurement of lactoperoxidase (LP) in milk was performed as per method described earlier [28]. In brief, 1.0 ml milk was taken and diluted to five times in 0.1M- acetate buffer (pH 4.5). From diluted milk samples, 30µl was rapidly added to 2.95 ml of 1.0 mM-2, 2'-azinodi-3-ethylbenzthiazoline-sulphonic acid (ABTS) in acetic buffer in a cuvette. The baseline absorbance at 412 nm was adjusted to zero before addition of 30µl 10 mM hydrogen peroxide solution in acetate buffer. The increase in extinction was followed for 5 minutes and units were expressed as the amount of enzyme required to oxidize 1 µmol ABTS/minute. The molar extinction coefficient of ABTS is  $32400 \times 10^3 \text{ mol}^{-1} \text{dm}^{-1} \text{cm}^{-1}$ .

### Statistical analysis

Data were analyzed using statistical software package (SPSS Version 10.1, South Asia, and Bangalore, India). Paired sample 't' test was employed to note the differences of various parameters between milk samples of healthy and SCM infected animals.  $P < 0.05$  was considered statistically significant. Correlation (r) analysis was performed to determine the relationship among SCC and COX, SCC and MPO, SCC and NO, SCC and LP as well as SCC and  $C_p$ .

## Results

The mean values of SCC, LP activity,  $C_p$  level of milk, COX, MPO activity, NO production in milk cells between SCM infected (group I) and healthy (group II) quarters were compared and presented in Table 1. The SCC of milk was significantly ( $P < 0.05$ ) high ( $11.19 \pm 0.28 \times 10^5$ /ml) in SCM infected quarters as compared to healthy quarters ( $2.79 \pm 0.12 \times 10^5$ /ml). The organism isolated from 15 milk samples of SCM infected cows were *Staphylococcus aureus* (33.33%), *Streptococcus agalactiae* (13.33%), other streptococci (40.00%) and Coliform bacilli

Mean ± standard error (SE)

Parameters (n=30)	Group I (SCM)	Group II (healthy)
SCC ( $\times 10^5$ /ml)	11.19 ± 0.28 <sup>a</sup>	2.79 ± 0.12 <sup>b</sup>
COX (nmol/min/ml)	15.08 ± 0.61 <sup>a</sup>	2.41 ± 0.25 <sup>b</sup>
NO (µmoles/ $1 \times 10^6$ cells/24hrs)	21.55 ± 1.24 <sup>a</sup>	9.13 ± 0.49 <sup>b</sup>
$C_p$ (g/Liter)	0.93 ± 0.03 <sup>a</sup>	0.31 ± 0.01 <sup>b</sup>
MPO (µmoles/ $10^6$ cells)	6.42 ± 0.27 <sup>a</sup>	3.15 ± 0.21 <sup>b</sup>
LP (u/ml)	1.59 ± 0.09 <sup>a</sup>	0.27 ± 0.01 <sup>b</sup>

\*Superscripts in each row (a, b) differ significantly ( $p < 0.05$ )

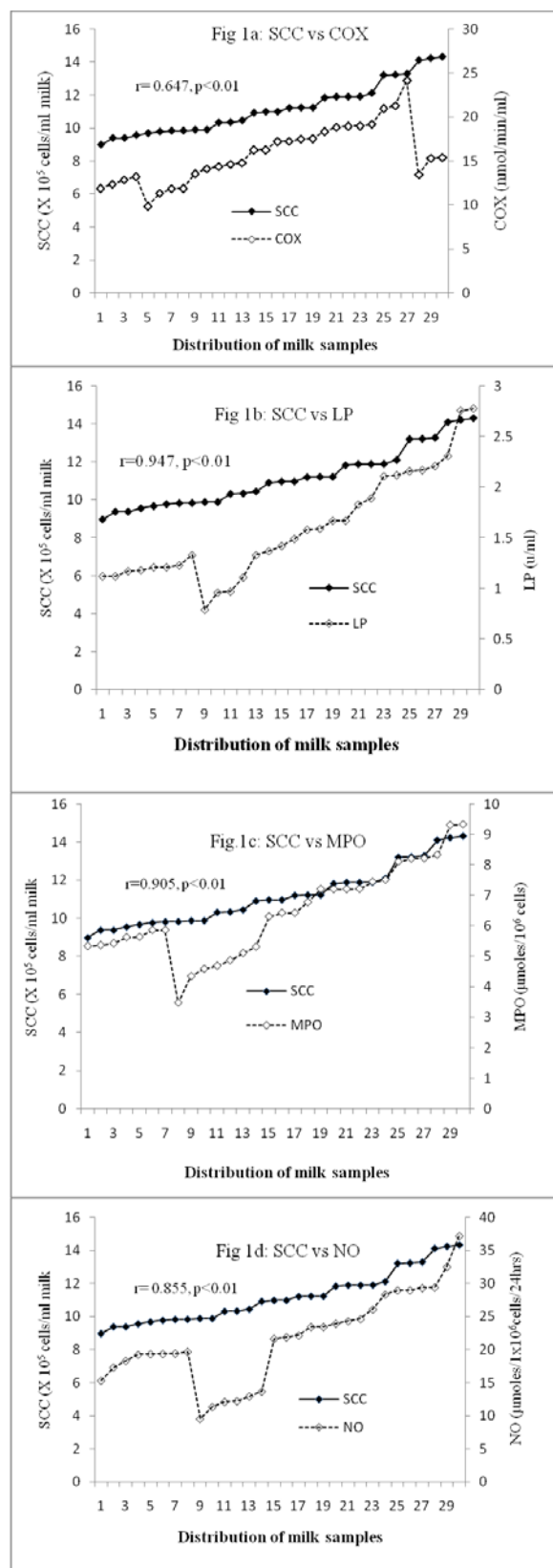
**Table 1:** Comparison of SCC, COX activity, NO production,  $C_p$  level, MPO activity and LP activity in SCM infected (Gr. I) and healthy (Gr. II) quarters.

(13.33%). The average LP activity and  $C_p$  level in milk of healthy quarters (group II) were  $0.27 \pm 0.01$  u/ml and  $0.31 \pm 0.01$  g/liter respectively. The LP and  $C_p$  activities were significantly ( $P < 0.05$ ) increased to 83.01 % and 66.66 % respectively, in milk samples collected from SCM infected quarters (group I). Similarly, mean values of COX activity in milk cell lysate, MPO activity and NO production in milk cells were  $2.41 \pm 0.25$  nmol/min/ml,  $3.15 \pm 0.21$   $\mu$ moles/ $1 \times 10^6$  cells and  $9.13 \pm 0.49$   $\mu$ moles/ $1 \times 10^6$  cells/24hrs respectively, in group II healthy quarters. However, the values of COX activity, MPO activity and NO production were significantly ( $P < 0.05$ ) augmented to 84.01%, 50.93% and 57.63% respectively, in SCM infected quarters (group I). Pearson's correlation ( $r$ ) of paired data of individual SCM infected quarters revealed the existence of a significant positive ( $P < 0.01$ ) correlation between SCC and the values of COX activity ( $r = 0.647$ ), LP activity ( $r = 0.947$ ), MPO activity ( $r = 0.905$ ) and NO production ( $r = 0.855$ ) (Figure 1a,1b,1c & 1d). However, no significant correlation between SCC and  $C_p$  level was observed in milk samples of SCM infected quarters.

## Discussion

The major changes in the udder during mastitis include leaking of ions, proteins and enzymes from the blood into the milk due to an increased permeability and alteration in cellular activity. Elevated SCC in cow milk is a sign of increased immunological activity due to invasion of pathogens, which may result in mastitis. Different factors are responsible for natural defense of mammary gland against invading microorganisms. Inflammatory mediators like prostaglandins and leukotriens exert chemotactic activity of milk leukocytes and enhance the bactericidal activity of phagocytes in dairy cows [2,29]. The key enzymes such as cyclooxygenase play a vital role in prostaglandin synthesis during inflammation. In this present study, COX activity in milk cell lysate was significantly ( $P < 0.05$ ) high in SCM infected quarters as compared to healthy quarters. Although clinical investigation does not result in visible signs of inflammatory process in SCM, however, elevated level of COX activity could be a result of inflammation and increased immunological activity of mammary gland. In this study, a statistically positive correlation between SCC and COX activity ( $r = 0.647$ ;  $P < 0.01$ ) in SCM quarters indicates that the relationship between raised COX activity and inflammation. In this regard, alteration of COX level in milk cells could be used as an alternative diagnostic tool to detect inflammation during SCM. Sarikaya et al. [30] observed increase synthesis of prostaglandin and COX-2 mRNA expression in dairy cows with increased SCC in milk.

Nitric oxide, an important powerful microbial oxidant, plays some role during inflammatory process [31]. Over production of NO has been observed in several inflammatory diseases [32]. The macrophage and epithelial cells of mammary gland produce significant amount of nitric oxide, the inducible NO takes part in the inflammatory process [33]. Moreover, NO stimulates prostaglandin synthesis in macrophages as a result of direct activation of cyclooxygenase activity [34]. Blum et al. [35] indicated a possible clinical relevance of nitric oxide production associated with a rise of intramammary and systemic TNF- $\alpha$  during acute, *E. coli* mastitis. In the current study, over production of NO in milk samples of SCM infected quarters might be due to activation of COX and TNF- $\alpha$  of milk leukocytes. Several workers found that intramammary infection leads to release of significant amount of NO that closely parallels increases in all classical markers of mastitis such as SCC and NAGase [33,36]. In the present investigation, a positive correlation between SCC and NO ( $r = 0.855$ ;  $P < 0.01$ ) in SCM quarters indicates that the relationship between increased NO production and inflammation. Therefore, alteration of NO in milk cells could be used



**Figure 1:** The correlation between SCC and COX activity (1a), SCC and LP activity (2b), SCC and MPO activity (1c) and SCC and NO production (1d) in milk samples with SCM ( $P < 0.01$ ).



as alternative diagnostic indicator to assess the inflammation during SCM of dairy cows.

Acute phase proteins regulate the immune system and have stimulatory effect on immune response. Cp, an acute phase protein, acts as a peroxidase and it oxidizes ferrous to ferric state. It protects tissues from iron-mediated free radical injury and is involved in various antioxidant and cytoprotective activities [37]. In this study, Cp activity in milk was significantly higher ( $p < 0.05$ ) in SCM infected quarters than healthy ones but there was no significant correlation between SCC and Cp activity in milk. Level of acute phase protein in milk from infected quarters increases due to high permeability of blood milk barrier during episodes of mastitis [10,12,38]. It has been reported that determination of Cp in milk rather than plasma is a suitable indicator for early diagnosis of mastitis in dairy cows [39]. Here, elevated level of  $C_p$  in milk samples of SCM cases suggests an indicator of the mammary gland infection. Since, the activity of Cp is mainly linked to changes in vascular permeability, biosynthetic, metabolic and catabolic profile of many organs rather than milk cells [40-42], therefore, it may not be expected that the  $C_p$  activity increases with the enhancement of SCC and thus with the occurrence of subclinical mastitis in lactating cows.

Mammary gland infection induces inflammatory reaction which leads to enhancement of SCC and activation of bacteristatic enzymes in milk. Neutrophils are the predominant cell type found in the mammary gland during inflammation and important for udder defense mechanism. The MPO, a lysosomal enzyme of neutrophilic granules, is a constituent of oxygen dependent antimicrobial activity of leukocytes [15]. Similarly, LP, the predominant antioxidant enzyme synthesized by polymorphonuclear leukocytes, responsible for a potent non-specific bacteristatic or bactericidal activity in milk [43,44]. In the current study, MPO and LP activity were significantly ( $p < 0.05$ ) high in milk samples of SCM infected quarters compared to healthy quarters. A pronounced positive correlation was also observed between SCC with MPO activity ( $r = 0.905$ ;  $P < 0.01$ ) as well as with LP activity ( $r = 0.947$ ;  $P < 0.01$ ) in milk of SCM infected quarters. Several workers demonstrated augmented MPO activity in mammary tissue, milk and blood cells during mastitis in different animal species [45-47]. Similarly, LP is increased in mastitis milk of cows and goat and is being established a positive correlation between enzyme activity and number of SCC [19,48]. Since the number of somatic cells in milk increases in SCM and since these enzymes are synthesized mainly by milk leukocytes, therefore, it is logical to expect that the MPO and LP activity increase with the enhancement of SCC during SCM. Further, the biological role of both the enzymes is to protect the mammary gland from infection, and suggests its involvement in the natural host defence system against invading pathogens.

In conclusion, SCM augments COX, LP, MPO activities and NO production in milk cells and  $C_p$  activity in whole milk. A significant correlation between SCC with COX, LP, MPO activities and NO production during SCM in lactating cows suggests that measurement of these parameters could be used as alternative diagnostic tool for detection of SCM in cows.

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