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Alteration in oxidative-stress biomarkers in bovine sub-clinical mastitis

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Abstract
Present study was aim to evaluate alteration in oxidative stress biomarkers in erythrocytes and milk of subclinical mastitis cow (SCM) and to compare with healthy dairy cows. Twelve cows were selected and divided in two groups each having six dairy cows. Animals of group- I have no clinical signs of subclinical mastitis served as healthy control group while cows of group-II having positive for subclinical mastitis tests and clinical signs. There was an increased somatic cell count (SCC; $3.5x10^5$ -$4x10^5$ /ml) in milk SCM group cow was observed as compared to control group (SCC; $2.5x10^4$ -$3x10^5$/ml) cows. Significantly increase lipid peroxidation (LPO) level and superoxide dismutase (SOD) activity was observed in erythrocyte of SCM group while catalase (CAT) activity and total protein content was significantly (p<0.05) decline in SCM group cows when compared with healthy control group. Milk of SCM cows showed significantly higher (p<0.05) LPO level while SOD value and catalase activities in milk samples of subclinical mastitis were non-significantly decreased in comparison to healthy animal. Reduced glutathione level (GSH) was significantly lower (p<0.05) in milk of SCM cows but it was not differing significantly in erythrocytes of SCM cows as compared to healthy control animals' milk. The total protein content was significantly higher (p<0.05) inmilk of SCM cows. Result of present study indicated that positive correlation existing between SCC and LPO. Present study concluded that subclinical mastitis may induce oxidative stress and altered the balance between antioxidant-oxidant system of mammary tissue and blood in animals suffering from SCM.

Keywords: Subclinical mastitis, lipid peroxidation, catalase, superoxide dismutase, cow

Introduction
Mastitis is an inflammatory condition of the mammary gland, caused by various factors including infectious agents (bacteria, fungi or viruses etc.) and poor managemental condition of dairy farms (Yuan *et al*., 2017) [23]. In India, annual economic losses due to bovine mastitis are approx. Rs.7165.51 crores, out of which 57.93% (Rs. 4151.16 crores loss) has been contributed by subclinical mastitis (Reshi *et al*., 2015) [18]. During mastitis, granulocytes produce oxygen derived free radicals which destroy intracellular pathogens and damage healthy cells if they are not eliminated. During infection, mammary tissue produces reactive oxygen species (ROS) such as superoxide anion (O_2^-), perhydroxy (HOO^-) and hydroxyl radical (HO^-), as a by-product of electron transport in mitochondria. During mastitis, increase in ROS or decreased antioxidant availability can result in a net increase in intracellular ROS levels in mammary tissue and hence oxidative stress can cause extensive tissue damage. Oxidative stress is a disturbance in balance between oxidants and antioxidants at the cellular level. It is one of the primary factors responsible for damage in biological macromolecules, dysfunction of the immune system, and impaired response during inflammation (Celi *et al*. (2011) [5], which ultimately lead to inflammation of mammary tissues. Oxidative stress management has major role in fighting bovine mastitis and its recurrence and incidence. During lactation period, mammary cells have high metabolic rate and thus produce large amounts of reactive oxygen species and lipid peroxides (Jin *et al*. (2014) [7]; Ganguly *et al*. (2016) [6]. Research works on oxidative stress profiles during subclinical mastitis of dairy animals were done by few researchers. Therefore, the present study was carried out to determine oxidative stress profile in blood and milk of mastitic cows for early detection of subclinical mastitis in bovine.

Materials and Methods
Study design: Cows with subclinical mastitis (SMC) and healthy cows (HC) were selected

and identified by the California Mastitis Test (CMT) and the Somatic Cell Count (SCC) in milk. A total of twelve sahiwal cows were maintained in Livestock Farm Complex, Sardar Vallabhbhai Patel University of Agriculture & Technology, Modipuram, Meerut. The cows were housed in free stall barns under the standard managemental conditions and nutritionally adequate feed and drinking water ad libitum. Cows were separated into two groups' i.e. first group (control), consisted of 6 apparently healthy cows and free from any kind of mastitis. The second group (mastitic cows), consisted of 6 cows with symptoms of subclinical mastitis. The experimental protocol was approved by CPCSEA/IAEC (2058/GO/Re/SL/19/CPCSEA S. No 1) (dated 23.09.2021) and all the procedure performed according to CPCSEA guidelines.

Blood Sampling and separation of erythrocytes

Under aseptic conditions blood samples were collected with EDTA coated vials through the jugular vein puncture and keeping them at room temperature. After collection, the tubes with EDTA were transported to the laboratory; 5 ml blood sample was centrifuged at 2000 rpm for 10 min to obtained erythrocytes pellet. The pellet was washed thrice with 0.15 M NaCl solution. Packed erythrocytes (33% dilution) were made in PBS pH 7.4 by the method of Yagi *et al*. (1989) [22]. The pellet was stored at 4 °C until further analysis of oxidative stress-related parameters.

Milk Sampling

Milk sampling was performed in the early morning. The udder was thoroughly washed with the potassium permanganate solution (1:1000) and wiped with clean cloth to allow dry and the teats were mopped with 70% ethyl alcohol. 10 mL of milk were collected in a sterile tube from each udder quarter of mastitic and healthy group's cows.

Oxidative stress parameters analysis

The concentration of lipid peroxidation in erythrocytes pellet and milk was determined in terms of malondialdehyde (MDA) production by the method of Shafiq-U-Rehman (1984) [21], reduced glutathione (GSH) level determined by the method of Prins and Loos (1969) [17]. Catalase activity in erythrocytes and milk was determined by the method of Bergmeyer (1983) [3]. Superoxide dismutase (SOD) activity was determined by Madesh and Balasubramanian, 1998 [11] and total proteins in erythrocytes and milk was estimated by the method of Lowry *et al*. (1951).

Statistical Analysis

Data were analysed by the t-test using statistical software package SPSS 20.The results were expressed as Mean ± SE, mean varying different were considered significant when p value is $p<0.05$.

Results and Discussion

Mastitis causes great economical loss in dairy industries, India. In present study, the subclinical mastitis cows showed no visible abnormalities in udder tissues and physical appearance, milk was normal except that there is a sudden rise in somatic cell count (SCC) in normal milk from normal udders may indicate the presence of subclinical mastitis (SCM). SCC is an important marker of udder health and quality of milk, and it is done in present study by quantifying the somatic cells present in the milk. In this study, we observed an increased somatic cell count (SCC) in milk of subclinical mastitic animals when compared with healthy animals (Table 1 and Fig. 1A & B). In the present study, healthy cows showed SCC count in the range of $2.5x10^4$ - $3x10^5$ /ml of milk, while the milk samples of subclinical mastitis cows have elevated SCC i.e. $3.5x10^5$ -$4x10^5$ /ml. Normally, milk collected from a healthy mammary gland, has the SCC is lower than $1×10^5$ cells/ml, while bacterial infection of mammary gland may cause increased into to SCC counts. Result of present study may indicated that milk with high SCC is an important indicator of oxidative stress (Zigo *et al*., 2019) [24] and subclinical mastitits (Khan *et al*., 2019) [25]. Milk with a high SCC has an important consideration of generation of reactive oxygen species (ROS) related compounds (Tao, 2015; Khan *et al*., 2019) [26, 25] from polymorphnuclear lymphocytes (PMNLs) during the infection and inflammatory condition of udder (Ellah, 2013) [13]. The increased population of polymorphonuclear leukocytes (PMNLs) in blood produces several effects including increased microbicidal activity, generation of reactive nitrogen intermediates and production of proteolytic enzymes and ROS, which cause damage in the mammary tissue (Ellah, 2013) [13]. Oxidative stress could be one of the main causes in reduction in quality and quantity of milk in dairy animals.

It has been observed that milk has antioxidant activity due to presence of enzymatic (catalase, glutathione peroxidise, superoxide dismutase and lactoperoxidase) and non-enzymatic (vitamin, glutathione polypeptides and protein) defence mechanism (Fox and Kelly 2006; Silanikove *et al*. 2014) [25, 28]. Oxidative stress occurs due to increased production of ROS and decreased antioxidant protection mechanism, leads to imbalance between reactive oxygen species (ROS) production and antioxidant defence system. Further, there is a failure of repair or replacement systems and rise in levels of 'biomarkers' of oxidative stress responsible for disease.

Result of oxidative stress biomarkers in blood and milk of sub-clinical mastitis animals are presented in Table 2 & 3. Perusal of result indicated that formation of malondialdehyde was significantly higher ($p<0.05$) in blood and milk samples of mastitis group of animals as compared with the healthy control group animals. Lipid peroxidation is an important indicator of oxidative stress which is caused by reactive oxygen species generated during oxidative stress. Lipid peroxidation is measured by formation of malondialdehyde (MDA). In the present study, significant ($p<0.05$) increase in malondialdehyde levels in erythrocytes and milk of subclinical mastitis animals may be due to high metabolic rate in mammary epithelium during lactation which is responsible to produce large amounts of reactive oxygen species, lipid peroxides in mastitis animal (Neelam and Anand, 2019) [14], and increased protein-carbonyl levels and increased oxidative degradation of proteins in milk (Andrei *et al*., 2016) [29]. Zigo *et al*. (2019) [24] observed positive correlation with lipid peroxidation level (malondialdehyde; MDA) and somatic cell counts (SSC) and considered as an indicator of oxidative stress associated mastitis.

In the present study, significantly increased malondialdehyde level in milk and blood with increase in number of SCC is further associated with more free radicals generation during oxidative stress due to subclinical mastitis. Result of present study was in agreement of findings of Zigo *et al*. (2019) [24] who have also reported significant increase in LPO in subclinical mastitis cow's blood and milk and it was due to

release of free radicals and decreased total antioxidants capacity (Atakisi *et al*., 2010) [2] and increased total oxidant activity (Patnaik *et al*., 2014) [15]. The alterations in the lipid peroxidation and glutathione in the biological fluids are the first and most important markers of oxidative stress (Jozwik *et al*., 2012) [8].

Superoxide dismutase (SOD) and catalase (CAT) are main enzymes that act as defence against free radicals-induced oxidative stress and act in concert with non-enzymatic reduced glutathione (GSH) antioxidant to protect against the adverse effects of ROS. SOD is responsible for catalytic dismutation of highly reactive and potentially toxic superoxide radicals to hydrogen peroxide (Buettner, 2011) [4]. SOD value was significantly higher (p<0.05) in blood of subclinical mastitis animals while its value was nonsignificantly declined in milk of subclinical mastitis animals as compared with healthy control animals. It indicated that superoxide dismutase (SOD) was initially involved in the first line defence against free radical induced oxidative damage and to protect erythrocytes from lipid peroxidation during subclinical mastitis infection in cow. Lindmark-Mansson and Akesson (2000) [30] observed that SOD activity in cow's milk is not affected by stage of lactation, animal age and SCC in milk (Andrei *et al*. 2010) [31].

CAT is responsible for catalytic decomposition of hydrogen peroxide to molecular oxygen and water (Nandi et. al., 2019) and plays important role in protection against deleterious effects of lipid peroxidation. Significant (p<0.5) decrease in catalase activity in blood and non-significant decline in milk of subclinical mastitis animal in the present study may indicated that subclinical mastitis induced-oxidative stress in cow. Decreased CAT activity in the present study may be due to depletion or inactivation of these enzymes or due to feedback inhibition by production of free radicals (Ajuwon *et al*., 2011) [1] such as superoxide and H_2O_2 which in turn generate hydroxyl radical resulting in initiation and propagation of lipid peroxidation by ROS or over-utilization of the antioxidant enzymes due to persistent oxidation (Poljsak *et al*., 2013) [16]. In contrast, Silanikove *et al*. (2012) [32] reported that milk catalase-antioxidant defence system regulated the redox-system of milk during subclinical mastitis by increasing catalase activity during bacterial infection (Hamed *et al*. 2008) [33].

Glutathione is an essential intracellular reducing agent for maintenance of thiol groups on intracellular proteins and for antioxidant molecules. GSH serves many functions including detoxification of electrophiles, scavenging of free radicals and it is also involved in phase II conjugation and other reactions (Ribas et.al. 2011) [19]. In the present study, GSH level was non-significantly altered in erythrocytes of subclinical mastitis animals when compared with healthy ones while it was significantly (p<0.05) declined in milk of subclinical mastitis cow indicative of oxidative stress. Jhambh *et al* (2013) [20] reported that significant decrease in GSH concentration in blood of mastitic cows due to conversion of reduced form to oxidized form (GSSH) by excessive production of reactive oxygen species from inflamed mammary gland.

Present study revealed that there was significant decrease in value of total protein content in erythrocytes of subclinical cows as compared to healthy control which was in contrast to value of total protein concentration in milk of SCM cows. The decrease in total protein concentration in erythrocytes indicative of oxidative stress while increase values of total protein in milk was due to increase in the concentration of whey proteins; a change in the proportion of caseins; and decrease in lipid concentration and an increase albumin concentration in the infected mammary gland, which could explain its high concentration (Le Maréchal *et al*., 2011) [9].

Table 1: Effect of subclinical mastitis on Somatic Cell Count (SCC) on milk

S.N.	Groups	SCC(per ml) (Mean± SE)	CMT Score
1	Control (Healthy cows) (N=6)	$2.5x10^4$ -$3x10^5$	+
2	Subclinical mastitis cows (N=6)	$3.5x10^5$ -$4x10^5$	++

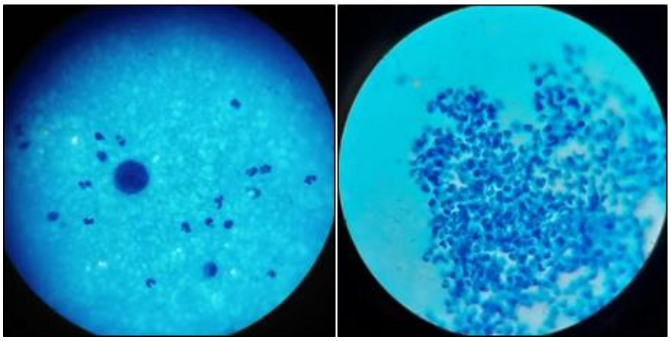

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Fig 1: Somatic cell in subclinical mastitis cases

Table 2: Effect of Subclinical mastitis on oxidative stress biomarkers in blood

Groups	LPO (nM MDA/ml blood)	SOD (U/ mg of protein)	GSH (mM GSH/ml blood)	Catalase (mM H_2O_2 utilized/min/ mg of protein)	Total Protein (mg/ml)
Control (Healthy cows)	13.78±1.49	179.24±5.62	0.082±0.005	97.34±4.60	15.44±0.45
Subclinical mastitis cows	53.39±12.14*	243.09±21.66*	0.097±0.012	39.99±17.98*	9.70±0.29*

Values are different group differs significantly. p<0.05 is considered significant (n=6).

Table 3: Effect of Subclinical mastitis (SCM) on oxidative stress biomarkers inmilkof cow

Groups	LPO (nMMDA/mlmilk)	SOD (U/mg of protein)	GSH (mMGSH/mlmilk)	Catalase (mMH_2O_2 utilized/min/mg of protein)	Total Protein (mg/ml)
Control (Healthy cows)	8.33±0.81	477.96±30.35	0.13±0.009	6.21±1.24	15.22±0.16
Subclinial mastitis cows	37.5±6.50*	439.84±48.60	0.065*±0.005	3.71±1.08	17.33±0.51*

Values are different group differs significantly. p<0.05isconsidered significant.(n=6).

Conclusion

Mastitis is one of the most serious disease of dairy animals and cause huge economic loss of dairy farmer due to decrease milk quantity and quality, rejection of milk, early culling of animal and economic involvement in treatment of mastitis animals. Oxidative stress- could be the causes of subclinical mastitis in dairy animals. Result of present study concluded that subclinical mastitis (SCM) cause significant increase in somatic cell count (SCC), Malondialdehyde level and SOD activity and significant decrease in erythrocytic protein content and catalase activity in blood of subclinical mastitis dairy cow. Similarly, significant increase in LPO and total protein levels, and GSH level significantly decline in milk of subclinical mastitis cow as compared with the healthy group of cows may suggestive of oxidative stress in cows. In addition, oxidative stress measurement could be used as a potential index for monitoring the health status of udder. Further, incorporation of antioxidants in the ration of dairy animals could be beneficial in prevention of this disease.

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