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ANALYSIS OF OXIDATIVE STRESS MARKERS IN CROSSBRED COWS WITH SUBCLINICAL MASTITIS AND CONCURRENT METABOLIC AND INFECTIOUS DISEASES

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ABSTRACT

The aim of this study was to evaluate the oxidative status in crossbred cows with subclinical mastitis and concurrent metabolic and infectious diseases.The crossbred cows were groped asGp-C (n=6) as controls,Gp-M (n=6) with clinical mastitis,Gp-TS consists cows affected withstenosis of teat,Gp-RA with ruminal acidosis,Gp-ND with diarrhea along with dehydration and loss of appetite, Gp-RTIwith coughing, fever, decreased appetite, varying degrees of dyspnea, Gp-RB with a history of failure to conceive after at least two successive inseminations.oxidative stress parameters likecatalase, SOD, GPx, GSH and malondialdehyde were estimatedspectrophotometrically. The antioxidant enzymes like catalase,super oxide dismutase (SOD), Glutathione Peroxidase (GPx) levels were significantly declined and the nonenzymatic parameters reduced glutathione (GSH) and malondialdehyde (MDA) aresignificantly elevated in affected animals than in healthy animals. The results of the oxidative stress parameters indicate imbalance ofantioxidant profile and oxidative stress in the animals with various disorders andprevious illness along with subclinical mastitis.

Keywords: Subclinicalmastitis, oxidativestress, metabolicdisease, markers.

INTRODUCTION

Bovine mastitis is an economic burden for dairy farmers and its management is crucial for thesustainability of any dairy business.Mastitis is defined as the inflammation of udder tissue because of infection. Mastitis is manifested as clinical and subclinical form.Subclinical mastitis is characterized by changes in the milk, such as an increased pH value, chloride content, or leucocyte count, in the absence of obvious swelling of the udder or clots in the milk. It was observed that, for every clinical case of mastitis in the herd, there are 15–40 subclinical cases contributing to an elevated somatic cell count (Bailey,2009).

Many of the research efforts are directed at understanding the nature of pathogenicbacteria that are responsible for the most intra-mammaryinfections.The susceptibility of the animal to develop mastitis may be affected by factors beyond the mammary gland. Therefore, occurrences of other diseases are important determinants of clinical mastitis. Most metabolic diseases like milk fever, ketosis, retained placenta, and displacement of abomasumin dairy cows occur during lactation.Majority of the infectious diseases including mastitisand metritis also occur at the same time. Therefore, there is a close relationship between metabolic and infectious diseasesand both of these types of disease may lead to inflammation and oxidative stress. Investigation of oxidative stress status in cows with subclinical mastitis and co-infections or disorders may pave the way for understanding the disease and aid in decision making in livestock health management.

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The role of free radical biology and antioxidant systems in health and disease is gaining considerable interest currently in human medicine as well in veterinary medicine. An oxidative stress is the excess of reactive oxygen species and the absence of optimal amounts of antioxidants in the body. Various infectious diseases of farm animals, such as pneumonia, enteritis and mastitis are associated with oxidative stress. Different enzymes can prevent the formation of radicals or scavenge radicals or hydrogen peroxide and other peroxides. Among antioxidant enzymes, superoxide dismutase and catalase have been demonstrated in milk (Swaisgood,1995).Lipid peroxidationis a process triggered by a number of reactive oxygenspecies and it results in production of various products like lipid hydroperoxides and malondialdehyde (MDA). Increased plasma MDA concentration is considered as a marker of lipid peroxidation.MDA concentrations were found to be increased in the serum, milk and tissue of rats with mastitis. (HadiEslami, *et.al*., 2015).During peripartum period antioxidantive status of dairy cows is seriously impaired and consequently both the oxidative stress and inflammatory response may present the predisposing factors to their higher susceptibility to intramammary infections (IMI) and mastitis (Turk *et al.*,2017).

There is therefore a close relationship between oxidative stress that may lead to inflammation of various tissues including mammary gland, which can be responsible for causing mastitis.The present study is undertaken to assess the oxidative stress that could affect the origin of inflammation of the mammary gland. The present study would help to take appropriate preventive measures for avoiding mastitis which is a very costly disease worldwide.

MATERIALS AND METHODS

Animal Selection

The cross bred lactating cows (n=42) from various organized farms in central Karnataka were selected based on the history of previous illness and grouped as detailed below for the study. The cows (Gp-C, n=6) which were consideredas controls,had history of no co-infections or previous illness for a period of 3 months. The cows (Gp-M,n=6) with clinical mastitis were considered as positive controls. The experimental groups (n=6 in each group) were considered as following. Gp-TS consists cows affected with stenosis of teat,Gp-RA with ruminal acidosis,Gp-ND with diarrhea along with dehydration and loss of appetite, Gp-RTIwith coughing, fever, decreased appetite, varying degrees of dyspnea, Gp-RB with a history of failure to conceive after at least two successive inseminations.

California Mastitis Test (CMT)

The California Mastitis Test (CMT) as per Schalm and Noorlander (1957) was performed by pouring each sample of well-mixed milk to a level of 3 ml in one quarter of a partitioned plastic paddle, then adding 3 ml of the commercial CMT reagent by means of an automatic pipette. The paddle, which held four samples, was rotated quickly by hand 10 times.

Interpretation and scoring of the CMT test: Depending upon the degree of gel formation, the grades were assigned according to Sastry (1978) as follows: Negative-no change in consistency. T Trace -no visible change in consistency, but when paddle is tipped a slime is momentarily seen on the bottom.1+ -a gel or thick slime forms, but when the paddle is swirled the solution does not move into the centre. 2+ -a thick lumpy gel forms, which, when swirled, quickly moves toward the centre. 3+ -a distinct gel forms which tends to adhere to the bottom of the paddle, and during swirling a distinct central peak form.

Estimation of enzymatic antioxidants

Superoxide dismutase (SOD) activity

Total SOD activity of blood plasma is determined according to the method of McCord and Fridovich (1969) as modified by by Holland et al (1982). Briefly, the analysis was carried out as following. The assay reaction mixture was composed of 0.1 mM EDTA, 50 µM acetylated ferricytochrome C (freshly prepared), 200 µM of xanthine, 20 mU/ml of xanthine oxidase and 50 µl of samples in 0.05 M potassium phosphate buffer, pH 7.8 in a total reaction volume of 1 mL.910 µL of assay pre-mixand 50 µL sample weretaken and mixed thoroughly. Subsequently, the enzymatic reaction is initiated by adding 20 mU of xanthine oxidase (in 40 µL of assay buffer) to the reaction mixture and the decrease in absorption at 550 nm is observed for a period of 0 to 5 min.The SOD activity of blood plasma is expressed as units/ml.

Catalase activity:

Catalase (CAT) activity of a sample is determined

according to the method of Aebi (1984). This assay is carried out using sample's ability to decompose hydrogen peroxide (H_2O_2) into H2O and O_2 and the rate of decomposition of H_2O_2 can be followed directly by the decrease of its absorbance at 240 mm over a period of time (usually 30 seconds).Blood sample (50 µL) was mixed with 450 µL of RBC lysis buffer and kept for 5 minutes for efficient erythrocyte lysis. Then the resultant blood lysate was used for evaluation of catalase enzymes.

Total glutathione peroxide (GPx) activity

Total glutathione peroxidase (GPx) activity is determined according to the method of Pagila and Valentine (1967) as modified by Lawrence and Burk (1976). The assay mixture is consisted of 1 mM EDTA, 0.2 mM NADPH, 1 mM GSH, 1 mM NaN., 1 U/mL glutathione reductase, 1.5 mM cumene hydroperoxide and the enzyme source or sample in 0.1M potassium phosphate buffer, pH 7.0. In a total volume of 1 mL,On the day of the experiment, an assay pre-mix is prepared taking all the above reagents except cumene hydroperoxide and sample/standard. Sample (0.1mL) is added to 0.8 mL of the above assay mixture and allowed to incubate for 5 min at room temperature before initiation of the enzymatic reaction by the addition of 0.1 mL of pre-warmed cumene hydroperoxide solution. The decrease in absorbance of NADPH at 340 nm is monitored for 5 min.

Total Gpx activity (units/mL of sample)= ΔA_{340}/min (Sample) - ΔA_{340}/min (Blank)] x DF/6.22 x V, Where,ΔA_{340}/min = Change in absorbance at 340 nm/min of sample or blank, DF = Dilution factor of the original sample, if any, before adding to the reaction mix,V = Sample volume in mL, 6.22 = Milliomolar extinction coefficient of B-NADPH at 340 nm

Determination of Blood Glutathione (GSH) level:

Glutathione (GSH) in whole blood was determined by method of Butler et.al.0.5 ml of the sample was added to 2 ml of phosphate solution, followed by theaddition of 0.25 ml of DTNB reagent. The absorbance was measured at 412 nm within 5 minutes of the addition of DTNB reagent against blank (prepared using 0.5ml of 1% metaphosphoric acid. GSH concentration = Absorbance of sample X concentration of standard in 0.5 ml. GSH concentration expressed as µg/ml

Malondialdehyde (MDA) Assay

The peroxidative damage in plasma was evaluated in terms of lipid peroxidation (LPO). Lipid peroxidation in tissue samples was measured as thiobarbituric acid-reactive substance called malondialdehyde (MDA) formed *per* ml of plasma according to Yagi *et al*,(1984).Taking the Spectrophotometric measurement of the standard solution, which is obtained by reacting 0.5 nmol of tetramethoxypropane with TBA by steps 4-6, as F and that of the sample as f, the lipid peroxide level *(Lp)* can be expressed in terms of malondialdehyde

Plasma *Lp*= 0.5 x f/F x 1.05/0.05 x 1.0/0.5=f/ F x 21 (nmol/ml of blood)

Statistical analysis

The values obtained from the various experiments were expressed as Mean $\pm$ S.E with 'n' unequal to number of animals or samples. Data obtained were statistically subjected to one-way analysis of variance (ANOVA) followed by Bonferroni's post *hoc* Multiple Comparison Test using Graph Pad Prism software programme (GraphPad® software Inc., Version 5.0; San Digo, CA, USA). difference was considered significant at $p<0.05$ or lower.

RESULTS

California. Mastitis Test (CMT)

Out of 170 lactating animals screened by CMT 35.2% of the cows were found positive, The mean CMT scores among the groups were enlisted (Table 1)

Table 1: Scores of California Mastitis Test

Group	CMT Score
Gp-C	0.00
Gp-M	3.00
Gp-TS	2.75
Gp-RA	2.75
Gp-ND	3.00
Gp-RTI	2.25
Gp-RB	2.75

Note: Values are mean, Means with different superscripts differ significantly within the Column ($p < 0.05$)

Estimation of Oxidative Stress Parameters

Superoxide Dismutase (SOD) activity was significantly ($p < 0.05$) different from group C with all the groups of affected animals and are significantly lower in groups compared to Group C. Catalase activity values are significantly lower ($p < 0.05$) in groups compared to control. Glutathione peroxidase (GPx) activity showed significant difference in the GSHPx activity observed between Groups C with all the Groups and GSHPx levels are significantly lower in groups M, TS, RA, ND, RTI, RB, compared to C ($p < 0.05$). There is significant ($p<0.05$).difference between Group M and Group C. (Table 2).

Table 2 :Assessment of enzymatic antioxidants in subclinical mastitis affected Animals with metabolic disorders and co-infections

Group (U/mL)****	SOD Activity (U/mL)****	Catalase Activity (U/mL)****	GPx Activity (µg/mL)**	Redused GSH
Gp-C	23.07±1.053[a]	266.1±30.43a	96.37±2.588[a]	25.3±0.6759[a]
Gp-M	15.14±0.6286[b]	107.8±10.21b	64.35±5.075[b]	33.18±2.824[a]
Gp-TS	16.49±1.219[b]	123.4±12.98b	73.61±2.963[b]	36.42±1.41[b]
Gp-RA	15.6±0.8349[b]	108.3±10.44b	69.51±2.975[b]	32.9±1.108[c]
Gp-ND	13.83±0.4097[b]	85.93±11.4b	63.94±3.62[b]	30.68±1.674[a]
Gp-RTI	14.23±0.9375[b]	113.7±11.22b	64.82±5.813[b]	31.43±2.334[a]
Gp-RB	13.47±1.031b	97.33±10.76b	65.27±3.602b	31.27±1.94d

Note: Values are mean ± SE , Means with different superscripts differ significantly within the Column ($p < 0.05$).

Levels of Malondyaldehyde (MDA): There is significant difference between all the Groups with Group C whereMDA levels are significantly higher ($p < 0.05$) in all the groups. Groups M, TS, RA, ND,RTI, RB, as compared to group C. There is significant differencebetween Group M and C with MDA levels significantly higher in Group M comparedto Group C. (Table3).

Table 3: Assessment of malondyaldehydein subclinical mastitis affected animals with metabolic disorders and co-infections.

Group	Malondyaldehyde –MDA (nmol/mL)****
Gp-C	14.43±0.383[a]
Gp-M	20.41±0.65[b]
Gp-TS	19.71±1.209[b]
Gp-RA	20.42±0.690[b]
Gp-ND	22.02±0.589[b]
Gp-RTI	19.97±0.603[b]
Gp-RB	20.75±0.600[b]

Note: Values are mean ± SE , Means with different superscripts differ significantly within the Column ($p < 0.05$).

DISCUSSION

Oxidative stress is associated with many diseases including sepsis, enteritis, pneumonia and arthritis in animals of veterinary importance. In dairy cows, it maycause mastitis and reproductive disorders (Turk *et al.*, 2012). The mean activities of SOD (U/mL), CAT(U/mL) and GPx(mU/mL) were 15.04±0.3224, 110.3±4.079, and 68.38±1.413

respectively. These values are significantly lower in animals with subclinical mastitis and compare to healthy animals. These results are in accordance with previous studies on dairy cows with clinical mastitis and subclinical mastitis (Rehaman *et al*., 2017, Sharma *et al*., 2011). The preventive body antioxidative defense systems can be accomplished by enzymes SOD, GPx and CAT. The decrease in these enzymes in this study can be attributed to excessive production of free radicals and reactive oxygen species. Imbalance in the antioxidant and oxidant system lead to damage of biological macromolecules as evidenced by increased MDA levels (Trevisan *et al*., 2001). The significant increase in blood MDA levels in the current study are in line with the reported involvement of mammary cellular damage due to oxidative stress in both clinical and subclinical mastitis (Rehman *et al*., 2017). It was also noted that an increased level of somatic cells can be correlated with an increase in the concentration of MDA thus with increased lipid peroxidation processes. (Andrei *et. al*., 2011). The mean concentration + SE of GSH (µg/ml) is 32.72±0.6882, $p<0.05$ in affected groups and is significantly increased compared to healthy animals (25.3±0.6759, $p < 0.05$). These results are in accordance with Kizil *et al*. (2007) showed significant increases of plasma GSH concentrations in SCM affected cows ($p<0.01$).

The oxidative stress among the groups is similar indicating the all the disorders or previous illness had led to the oxidative stress. When reactive oxygen species are not effectively and safely removed oxidative stress may impair the health of dairy cows both metabolic and hormonal disease (Celi, 2011). The disorders and previous illness might have influenced the occurrence of mastitis due to oxidative stress. Many studies have shown that multiple diseases, including mastitis, mammary edema, metritis, and retained fetal membranes most commonly occur during the periparturient period when dairy cows are known to experience oxidative stress (Kankofer, 2002; Miller *et al*., 1993). All the lactating animals in the study were under oxidative stress aggravated by the concurrent diseases or disorders. Therefore, the aim of the treatment of mastitis should be coupled with antioxidant therapy.

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