

Original Research Article

<https://doi.org/10.20546/ijcmas.2018.701.027>

A Study on Bovine Mastitis Related Oxidative Stress along with Therapeutic Regimen

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ABSTRACT

Dairy industry is gradually emerging as a major player in the field of agricultural economy. Consequently the management scene and concomitant disease control regimen have become more important for production and economic reasons. Despite innovations in various therapeutic regimens and improved management practices, mastitis unfortunately has remained ever green. Mastitis is a multi-etiological complex disease, which is defined as inflammation of parenchyma of mammary glands and is characterized by physical, chemical and usually bacteriological changes in milk and pathological changes in glandular tissues (Radostits *et al.*, 2010). Mastitis may be classified as clinical and subclinical. In contrast to visible changes in the clinical form of mastitis, there is absence of gross abnormalities in the milk or udder in case of subclinical mastitis. The average incidence of clinical mastitis varies from 10 to 20 % in most of the herds and the prevalence of intra mammary infection is around 50 % of quarters (Radostits *et al.*, 2007). Further, the subclinical mastitis (SCM) is 3– 4 times more prevalent than clinical mastitis (Bhandari and Garg, 2012). Therefore, milk production loss is more in subclinical mastitis as compared to clinical form because of increase in undesirable milk components like proteolytic enzymes (Pyorala, 2003), salts and also increase in somatic cell count, thereby resulting in decrease in the desirable components such as protein, milk fat and lactose. The present study was conducted for investigating the alterations in the activities of erythrocyte glutathione peroxidase (GSH-Px) and its functional components in cows with subclinical mastitis and normal cows of Odisha along with the therapeutic efficacy for antioxidants along with antibiotics on restoration of changes in oxidative stress indices.

Keywords

Dairy cattle,
Mastitis, Oxidative
stress, Therapy

Article Info

Accepted:
04 December 2017
Available Online:
10 January 2018

Introduction

Both clinical and subclinical, remains a problem in dairy herds and prevalence rates in many countries. MCMT, somatic cell count and electrical conductivity of milk are most frequently used tests in dairy cattle to diagnose clinical and subclinical mastitis. The specificity of the oxidative stress and its relation to mastitis and other diseases is a complex phenomenon. One of the symptoms of mastitis is an increased NO level and a reduced content of ascorbic acid in the blood serum of animals (Sordillo *et al.*, 2009; Jozwik *et al.*, 2012b). Oxidative stress is commonly defined as an imbalance between oxidants and reductants (antioxidants) at the cellular or individual level. The involvement of oxidative stress in mastitis is further substantiated by data from experimental mastitis induced either by intramammary infusion of *E. coli* bacteria (Blum *et al.*, 2000; Weiss *et al.*, 2004; Wakchaure *et al.*, 2015) or *E. coli* or staphylococcal endotoxin (Blum *et al.*, 2000; Bouchard *et al.*, 1999; Komine *et al.*, 2004). Antibacterial activity of neutrophils is interceded, in part, through reactive oxygen species (ROS) (Rinaldi *et al.*, 2008). A surplus of ROS and the absence of optimal amounts of antioxidants are leading to oxidative stress (Lykkesfeldt and Svendsen, 2007). Progressive development of oxidative stress in periparturient dairy cows is a significant underlying factor leading to dysfunctional inflammatory responses (Sordillo and Aitken 2009; Osorio *et al.*, 2014). Alterations in haemato-biochemical parameters have been correlated to aid diagnosis as well as treatment. Recent pathophysiological studies suggest that management of oxidative stress has a significant role in combating bovine mastitis and its recurrence and incidence. During lactation, mammary epithelial cells exhibit a high metabolic rate and thus produce large amounts of reactive oxygen species and

lipid peroxides in vivo (Jin *et al.*, 2014; Ganguly *et al.*, 2016). Increased reactive oxygen species level or decreased antioxidants can disrupt the balance between oxidants and antioxidants which is referred to as oxidative stress (Sordillo *et al.*, 2009). This create a more oxidizing environment that facilitate the binding of pathogens or antigens to effector cells leading to a hyper-responsive innate immune system and enhanced production of cytokines (Maddox *et al.*, 1999). Reactive oxygen species can oxidize macromolecules such as lipids, proteins and DNA and cause direct oxidative cell injury or indirectly can modify metabolic pathways (Miller *et al.*, 1993). Lipid peroxidation in clinical mastitis reduces the levels of some antioxidant molecules leading to an increase in the oxidative stress state (Weiss *et al.*, 2004). Mastitis could induce the increase of free radicals formation in milk and leading to oxidative stress (Gu *et al.*, 2009), especially during the early lactation period of dairy cows (Sordillo *et al.*, 2007). Both CM and SCM are associated with release of free radicals, increased total oxidant capacity and decreased total antioxidants capacity in milk (Atakisi *et al.*, 2010; Patnaik *et al.*, 2014). It has been reported that significant decrease in blood superoxide dismutase (SOD) and catalase activities, reduced glutathione (GSH) concentration and an increase in erythrocytic lipid peroxides was observed in cows with clinical mastitis (Jhambh *et al.*, 2013). The literature on oxidative stress in buffalo mastitis is scanty. Therefore, the present study was carried out to study the oxidative stress aspects of mastitis in cattle. This study has been undertaken to study the changes in the activities of erythrocyte glutathione peroxidase (GSH-Px) with its functional component and also to compare the activities of erythrocyte superoxide dismutase (SOD) with functional components in cows with subclinical mastitis and normal cows of

Odisha. Also, the study was undertaken on the therapeutic efficacy for antioxidants along with the antibiotics on restoration of changes in oxidative stress indices.

Materials and Methods

Study design

60 mastitis cattle were selected for experimental study and divided into three groups of 20 animals in each, namely Group I, II and III. Another 20 cattle reared in the locality of Bhubaneswar, without any overt clinical signs of any disease, apparently healthy and negative for mastitis were taken as healthy control group (Group IV). The therapeutic regimen of the Group I and II were followed as per the following experimental protocol as stated in the Table 1.

In groups I, II and III, twenty cattle clinically affected with clinical mastitis (CM) were treated with Marbofloxacin (Marbodac) as parenteral antibiotic through intra muscular route, repeated after 3 days for two doses at the dose rate of 8 mg/kg body weight along with intramammary infusion of Cefoperazone (Mammicef) at the dose rate of 1 syringe per affected quarter. In group II, animals were also administered with anti-oxidants namely Vitamin-E and Selenium (Repronol) as supportive therapy through intra muscular route, at weekly interval for four doses at the dose rate of 1 ml/30 kg body weight. However, in group-III animals were administered with anti-oxidants namely Vitamin-C (Livocef 25%) and Selenium (Repronol) as supportive therapy through intra muscular route, at weekly interval for four doses at the dose rate of 1 ml/30 kg body weight. In group-IV twenty cattle reared in the locality of Bhubaneswar, sharing a similar environment and climatic conditions as mentioned above were taken healthy control. These animals were without clinical signs of

any disease and negative for bovine mastitis through PH, SCC, EC and MCMT examination. They were monitored for clinical sign of any disease during the study period and thus given no treatment. All the animal procedures were performed according to the guidelines of the Animal Ethics Committee of College of Veterinary Science and Animal Husbandry, OUAT, Bhubaneswar.

Collection of blood samples

The blood samples of the selected 20 cattle for the present experiment was collected in heparinised vials on day 0, day 14 and day 28 i.e. before, during and after treatment for estimation of oxidative indices.

Measurement of therapeutic efficacy

The response to treatment was evaluated on the basis of milk characteristics (SCC, EC, pH) and the time taken for recovery. EC and pH was determined by digital pH meter.

Estimation of erythrocytic oxidative indices

Estimation of lipid peroxidation

It was determined as per placer *et al.*, (1966). Briefly, to 0.2 ml haemolysate in a test tube, 1.3 ml Tris-KCl buffer was added and incubated for 30 minute at 37⁰ C temperature. Then to this 1.5 ml TBA reagent was added and heated in boiling water bath for 10 minute with marble as a condenser. The tube was cooled under tap water and 3 ml pyridine-butanol mixture was added followed by 1 ml IN NaOH and mixed well. A blank was run containing 0.2 ml distill water in place of haemolysate. Then absorbance was measured @ 548 nm against blank.

Taking E548 Molar extinction coefficient of MDA-TBA complex @ 548 nm is used, it is 1.56 X 10⁸ /M/cm of path.

MDA (m/g Hb) = (OD of test/E₅₄₈) x (7/0.2) x 5 x 100 x 10⁶/ Hb (g%) in haemolysate or (mmol/mg Hb)

(7 – Final volume of mixture, 0.2 – Volume of hemolysate taken, 100 – To convert per ml volume to 100 ml volume to bring it into same unit as Hb.)

Estimation of catalase

It is based on degradation of H₂O₂ by catalase as per the method described by Cohen, *et al.*, 1970. Here 2.9 ml of H₂O₂ solution was taken into cuvette directly to which 0.1 ml of haemolysate solution was added and mixed immediately by inversion /plastic paddle. O.D. was recorded at 240 nm by using double beam spectrophotometer. Time required for the Abs₂₄₀ nm to decrease from 0.45 to 0.40 was noted. Reading is taken against phosphate buffer as blank.

Units/g Hb in hemolysate = 3.45 x 100/ T x 0.1 x Hb (g % of haemolysate)

T = Time in minute required for the Abs₂₄₀, to decrease from 0.45- 0.40 Absorbance unit

Estimation of superoxide dismutase (SOD)

SOD was estimated as per Marklund and Marklund (1974) with little modification as per Masayasu Hiroshi, (1979). SOD activity of RBC haemolysate samples can be measured using nitro blue tetrazolium as a substrate.

The reaction was carried out in a total volume of 3ml consisted of 50 mM of Tris-cacodylic acid buffer Ph 8.2, Enzyme preparation after suitable dilution of sample and 0.2 mM of pyrogallol. In the blank enzyme was substituted by equal quantity of distilled water. The increase in absorbance due to auto oxidation of pyrogallol was recorded at 420nm using spectrophotometer.

Calculation

One unit of SOD activity was defined as the amount of enzyme which inhibited the pyrogallol auto oxidation by 50% under the given experimental condition. The values were expressed as units/ mg of haemoglobin or protein.

SOD (units/mg) = (OD of Blank-OD of Test) / OD OF Blank 100/ 50 Dilution factor Mg of Hb in Haemolysate.

Statistical analysis

All the data generated in the above experiments were statistically analyzed using SPSS (1996) computer package. For comparison of groups, Generalized Linear Model, ANOVA procedure and were used (Steel and Torrie, 1980).

Results and Discussion

Comparative efficacy of different therapeutic regimes in the treatment of acute bovine clinical mastitis

20 animals each in 3 groups evaluated for recovery rate after 3 days and 5 days based on milk pH, SCC and EC. The present study revealed there is significant difference of group 2 (p=0.05) than group 1, group 3 in recovery rate after 3 days. Similarly group 2 is significantly differs from group 1 in term of recovery rate after 5 days. The mean recovery± S.E. time (days) of group 1, group 2, group 3 are 7, 6, 5 respectively. From the present study it revealed that group 2 which was supplemented with vit-E and selenium along with antibiotics showed better recovery rate in day 3 and day 5 and also recorded a very less mean recovery time. The comparative efficacy of each treatment regime in the recovery of clinical mastitis is given in Table 1. The recovery was established on the

basis of clinical presentation and changes in milk characteristics (Table 2).

Erythrocytic oxidative indices

The erythrocytic oxidative enzymes of all the 60 animals of the three groups were studied by collection of blood samples on day 0, day 15 and day 30 of the experiment. The different oxidative enzymes studied were lipid peroxidase (LPO), super oxide dismutase (SOD) and catalase.

Mean erythrocytic lipid peroxidase (LPO)

The mean erythrocytic lipid peroxidase (LPO) in cattle of different experimental groups at different observation periods is given in Table

3. On day 0, the mean erythrocytic lipid peroxidase concentration in cattle in group I (4.77 ± 0.15), group II (4.76 ± 0.19) and in group III (4.78 ± 0.13) were at significantly higher level ($p < 0.05$) as compared to healthy control group IV (1.13 ± 0.04). Significant ($p < 0.05$) decrease in the mean erythrocytic lipid peroxidase level in group I (3.24 ± 0.07), group II (1.91 ± 0.08) and group III (2.93 ± 0.11) were found on day 15 as compared to their day 0. Significant ($p < 0.05$) decrease in the mean erythrocytic LPO level of both group I (2.04 ± 0.06), group II (1.01 ± 0.04) and group III (1.74 ± 0.08) were found on day 30 as compared to their day 15. On day 30 only group II has non significantly differ with healthy control group suggesting better relief from oxidative stress (Table 3).

Experimental protocol

S.No.	Group	No of animals	Clinical condition	Treatment
1	I	20	Clinical mastitis	1. Inj. Marbofloxacin (Marbodac ^R) @ 8mg/kg b.wt. (I/m 2 doses repeated after 3 days) 2. Inf. Cefoperazone (Mammicef ^R) @ one syringe per affected quarter.
2	II	20	Clinical mastitis	Inj. Marbofloxacin (Marbodac ^R) @ 8mg/kg bwt (I/M 2 doses repeated after 3 days) Inf. Cefoperazone (Mammicef ^R) @ one syringe per affected quarter. Vitamin-E and Selenium (Repronol) @ 1ml/50 kg b.wt. weekly once
3	III	20	Clinical mastitis	1. Inj. Marbofloxacin (Marbodac ^R) @ 8mg/kg bwt (I/M 2 doses repeated after 3 days) 2. Inf. Cefoperazone (Mammicef ^R) @ one syringe per affected quarter. 3. Vitamin-C(Livocef ^R) @ 25 mg/kg b.wt. weekly once
4	IV	20	Healthy control	No treatment

Table.1 Comparative efficacy of different therapeutic regimes in the treatment of acute bovine clinical mastitis

Treatment group	Recovery rate after 3 day	Recovery rate after 5 day	Mean recovery± S.E. time(days)
GROUP I	40%(8/20) ^a	60%(12/20) ^a	7
GROUP II	75%(15/20) ^b	90%(18/20) ^b	6
GROUP III	50%(10/20) ^a	75%(15/20) ^{ab}	5

Table.2 Effect of treatment on milk chemistry in bovine mastitis (mean± S.E.)

Parameter	Group I		Group II		Group III	
	Pre treated	Post treated	Pre treated	Post treated	Pre treated	Post treated
pH	7.2±0.024	6.7±0.031	7.2±0.032	6.7±0.025	7.2±0.027	6.7±0.031
EC(ms/cm)	6.5±0.32	4.2±0.032	6.6±0.19	4.2±0.041	6.5±0.44	4.2±0.029
SCC x 10⁵	8.64±0.65	2.75±0.21	8.72±0.53	2.17±0.21	8.69±0.41	1.68±0.16

Table.3 Mean erythrocytic lipid peroxide (LPO) (nmol/mg Hb)

S.No.	Group	Day 0	Day 15	Day 30
1	I	4.77±0.15 ^{bC}	3.24±0.07 ^{cB}	2.04±0.06 ^{bA}
2	II	4.76±0.19 ^{bC}	1.91±0.08 ^{bB}	1.01±0.04 ^{aA}
3	III	4.78±0.13 ^{bC}	2.93±0.11 ^{cB}	1.74±0.08 ^{bA}
4	IV	1.13±0.04 ^a	1.14±0.06 ^a	1.14±0.03 ^a

*Values are expressed as Mean± S.E. values. Parenthesis denotes range. Mean values with different superscripts (A, B & C) differs in a row significantly within a group at P≤0.05 and superscripts (a, b & c) differs in a column between the groups significantly at P≤0.05.

Table.4 Mean erythrocytic super oxide dismutase (SOD) (units/mg Hb)

S.No.	Group	Day 0	Day 15	Day 30
1	I	1.86±0.13 ^{aa}	2.56±0.17 ^{ab}	2.91±0.16 ^{ab}
2	II	1.85±0.11 ^{aa}	2.94±0.21 ^{ab}	4.14±0.29 ^{bc}
3	III	1.84±0.17 ^{aa}	2.66±0.19 ^{ab}	3.12±0.11 ^{ab}
4	IV	4.04±0.14 ^b	4.06±0.17 ^b	4.01±0.16 ^b

Table.5 Mean erythrocytic catalase (units/mg Hb)

S.No.	Group	Day 0	Day 15	Day 30
1	I	1.47±0.13 ^{aa}	1.82±0.14 ^{ab}	2.02±0.16 ^{ab}
2	II	1.49±0.11 ^{aa}	2.08±0.11 ^b	2.70±0.18 ^{bc}
3	III	1.50±0.08 ^{aa}	1.96±0.17 ^b	2.09±0.11 ^{ab}
4	IV	2.13±0.06 ^b	2.10±0.08	2.07±0.12 ^a

Mean erythrocytic super oxide dismutase (SOD)

The mean erythrocytic super oxide dismutase (SOD) in cattle of different experimental groups at different observation periods is represented in Table 4. Mastitis affected cattle revealed significantly ($p < 0.05$) lower level of mean erythrocytic super oxide dismutase on day 0 in group I (1.86 ± 0.13), group II (1.85 ± 0.11) and group III (1.84 ± 0.17) as compared to healthy control group III (4.04 ± 0.14). Significant ($p < 0.05$) increase in the mean erythrocytic super oxide dismutase level of both group I (2.56 ± 0.17), group II (2.94 ± 0.21) and group III (2.66 ± 0.19) was found on day 15 as compared to their day 0. On day 15 the level of SOD in group I, II & III were at a significantly lower level than group IV. On day 30, significant ($p < 0.05$) increase in the mean erythrocytic SOD level of group II (4.14 ± 0.29), and group III (3.12 ± 0.11) were found with respect to its day 15 whereas in group I (2.91 ± 0.16) there was increase in SOD level at a non-significant higher level.

Mean erythrocytic catalase

The mean erythrocytic catalase level in cattle of different experimental groups at different observation periods is given in Table 5. Mastitis affected cattle revealed significantly ($p < 0.05$) lower level of mean erythrocytic catalase on day 0 in group I (1.47 ± 0.13), group II (1.49 ± 0.11) and in group III (1.50 ± 0.08) as compared to healthy control group IV (2.13 ± 0.06). Significant ($p < 0.05$) increase in the mean erythrocytic catalase level of group II (2.08 ± 0.11) was found on day 15 as compared to their day 0, but the increase in catalase level in group I (1.82 ± 0.14) was at a non-significantly higher level with respect to its day 0. However on day 30, the mean erythrocytic catalase level of group II (2.70 ± 0.18) was found to be at a significantly higher level than in group I

whereas there was increase in catalase value at a statistically non-significant level in group I (2.02 ± 0.16) with respect to its day 15 value.

Mastitis is one of the important disease entities that create hindrance in the development process of dairy industry. Oxidative stress occurs when the production of reactive oxygen metabolites (ROM) exceeds the capacity of the antioxidant system of the cell, tissue or body. Certain nutrients act as antioxidants and are components of antioxidant enzymes and have a direct effect on oxidative stress. Both water and fat soluble antioxidants are needed because free radicals are found in both areas of cells. Vitamin E and selenium has been shown to reduce prevalence and severity of mastitis and reduce SCC (Smith *et al.*, 1997; Weiss *et al.*, 1997). Selenium and vitamin E act in an apparently synergistic way to protect the mammary gland.

There is increased phagocytic activity when vitamin E was provided with antibiotics in clinical mastitis (Mukherjee, 2007). In this study, antioxidant supplementation like Vit-E and Se or Vit-C along with antibiotics increased the recovery rate and decreased the mean recovery time in treatment of bovine mastitis. Ascorbic acid (vitamin C) has multidimensional bioactive role in the body system, the most important being that of an antioxidant, antibacterial activity and maintenance of optimum immune responses. The polymorphonuclear cell (PMN) migration and its lysosomal antioxidant enzyme, myeloperoxidase (MPO) synthesis is largely dependent on ascorbates. The antioxidant role of ascorbates is to neutralize the toxic peroxides and other reactive oxygen species (ROS) released during cellular activation due to bacterial infection, which cause tissue injury. Singh and Pachauri (2003) observed a significant decrease of ascorbic acid in milk and blood of both subclinical and clinical

cases of mastitis and suggested its supplementation during the disease

From the present study, it may be concluded that ascorbic acid therapy is of potential benefits, particularly when used concurrently with antibacterial formulation in order to modulate udder immunity, prevent mammary tissue from noxious insult of toxic lipid peroxidases and also potentiate antimicrobial activity of antibacterial drugs. In conclusion, a therapeutic trial was conducted on cases of acute bovine mastitis. In conclusion, Marbofloxacin @ 8mg/kg body weight (I/M) and cefoperazone intramammary infusion along with vitamin E and Selenium was proved as an effective therapy against acute bovine clinical mastitis.

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How to cite this article:

Anshuman Mahapatra, Sumitra Panigrahi, Ramesh C. Patra, Mangalika Rout and Subha Ganguly. 2018. A Study on Bovine Mastitis Related Oxidative Stress along with Therapeutic Regimen. *Int.J.Curr.Microbiol.App.Sci*. 7(01): 247-256.
doi: <https://doi.org/10.20546/ijcmas.2018.701.027>