

Cytokine response and oxidative stress status in dairy cows with acute clinical mastitis

Abstract

In the friction process of two materials and in the presence of some proper lubricants, the wear process manifest itself as a material transfer from an element of the friction couple on the other, process specific to the selective transfer mechanism. The selective transfer can be sure achieved in a friction couple if there is a favorable energy, the relative movement and if in the friction area is a material made by copper and the lubricant is adequate (glycerin). The selective transfer mechanism is characterized by the physicochemical processes, which take place in the contact zones of the friction couples, and which to allow the selective transfer of some elements of the materials from a surface to the other, forming a thin superficial layer with the superior properties at wear and friction. This is a condition for any friction couple of high efficiency and a normal self-adjusting phenomena. The forming of this layer on the contact surfaces makes as friction force to be reduced. It is in closely related to the structure formed by the selective transfer, between the metallic friction surfaces and with the properties these surface layers metallic.

The most important parameters concerning the physical state of the superficial layers are micro-tensions, the structure and its modification on friction surfaces, the structure defects as well as the way of distribution of the additions and of the alloying elements from alloy. The purpose of the present paper is research these parameters by the structural analysis with X-rays, as the research method of the thin superficial layers.

Keywords: oxidative stress, pro-inflammatory cytokines, antioxidants, dairy cow, acute clinical mastitis

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Introduction

Bovine mastitis is considered a major disease that causes economic losses in dairy industry ranging from decrease in milk production to reproductive and metabolic disorders in dairy cows.^{1,2} During mastitis, immune cells in the body recognize invading pathogens and become activated which release inflammatory mediators including nitric oxide, prostaglandins, and cytokines. These mediators promote local inflammation and increased blood flow to the infected tissue, inflammatory cytokines play a key role in stimulating systemic inflammatory responses, including increased body temperature, increased heart rate, and decreased feed intake.³ Cytokines, including tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and interleukin-12 (IL-12) are able to alter many physiological systems acting through many of the same signaling cascades and often produce similar responses in cells. In addition, phagocytes produce reactive oxygen species that are needed for killing bacteria during inflammatory process.⁴

Cytokines are crucial in driving the acute-phase response operating at every stage in the crucial early events that promote acute inflammation. Cells that make up the innate immune response, including neutrophils, natural killer cells, macrophages, mast cells, and eosinophils, all produce and respond to cytokines generated within seconds of tissue insult. Cytokines prime leukocytes for response to microbial stimuli; and amplify the release of reactive oxygen intermediates, nitric oxide, vasoactive amines, and neuropeptides, and the activation of kinins and arachidonic acid derivatives, prostaglandins, and leukotrienes, which regulate cytokine release.^{5,6}

During lactation, mammary epithelial cells exhibit a high metabolic rate and thus produce large amounts of reactive oxygen species and lipid peroxides *in vivo*.⁷ Increased reactive oxygen species level or decreased antioxidants can disrupt the balance between oxidants and antioxidants which is referred to as oxidative stress.⁸ 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.⁹ Reactive oxygen species can oxidize macromolecules such as lipids, proteins and DNA and cause direct oxidative cell injury or indirectly can modify metabolic pathways.¹⁰ Similarly, the creation of a markedly reduced environment by addition of antioxidants blunts all of the above primary responses of the innate immune system.

Lipid peroxides, mediators linking plasma lipids to inflammation, are produced when intracellular lipids encounter reactive oxygen species such as hydrogen peroxide (H₂O₂) activating inflammatory cascades, which in turn alter nutrient metabolism. Reactive oxygen species are especially harmful to immune cells and can decrease the ability of the immune system to respond to infection.¹¹ Previous studies conducted by Weiss et al.,¹² revealed that the increased lipid peroxidation in clinical mastitis reduces the levels of some antioxidant molecules leading to an increase in the oxidative stress state.¹²

Vitamins and minerals have long been recognized as antioxidants in the animal udder health and production. However, they also have specific roles in mastitis of dairy cows, such as vitamin A, β -carotene, vitamin C, vitamin E, selenium, zinc and copper.^{13,14} In the antioxidant system, zinc is a component of copper-zinc superoxide dismutase. Zinc also induces synthesis of metallothionein, a metal binding protein

that may scavenge hydroxide radicals.¹⁵ In addition to an antioxidant role, zinc may affect immunity via its important role in cell replication and proliferation.¹⁶ The aim of the present study is to evaluate the role of oxidative stress biomarkers, antioxidant enzymes and metabolites, and pro-inflammatory cytokines as possible biomarkers for acute clinical mastitis in dairy cow.

Materials and methods

Animals

A total of 100 dairy cows at 3-10 years of age were studied. Of all, 50 were exhibiting the clinical signs of acute clinical mastitis. In addition, 50 apparently healthy dairy cows within the same age and under the same environmental condition were randomly selected as a control group. The present study was carried out between January, 2014 and March, 2015 at Damietta governorate, Egypt.

Clinical examination

Data concerned with the case history, clinical findings, and medical record for each cow under investigation were recorded. A detailed clinical examination of the diseased dairy cows, including examination of the mammary gland and its secretion was carried out, and the clinical findings were recorded and scored (Table 1) according to Jánosiet al.¹⁷

Table 1 Clinical signs of acute clinical mastitis dairy cows

	Score 1	Score 2	Score 3
Systemic signs	None	Rectal temperature: ≤40.5 and/or in appetite and mild to moderate depression.	Rectal temperature: > 40.5°C and/or anorexia, severe depression, decreased milk production and recumbency.
Local signs	None	Hotness, moderate swelling and tenderness of the affected quarter(s).	Hotness, severe swelling, firmness and the affected quarter(s) very sore to touch.
Milk appearance	Normal	Slightly watery, discolored, and/or clots and flakes.	Consistency serum-like, pus-like and/or bloody stained.

There are three categories of acute clinical mastitis: abnormal milk, abnormal gland and an abnormal cow (systemic disease). Abnormal milk is visibly abnormal (i.e. is not 'drinkable') such as a watery appearance, flakes, clots, and/or pus. An abnormal gland characterized by sudden onset of redness, hotness, swelling, hardness, and tenderness when compared with other normal quarters. An abnormal cow is pyrexic, depressed or has decreased appetite and/or milk production, reduced rumen function, rapid pulse, dehydration, weakness, and reduced mobility, due to the pain of a swollen udder or simply due to feeling unwell.

Blood samples collection

Two venous blood samples (10mL each) were collected from each

cow via jugular vein puncture. The first blood sample was collected into a clean centrifuge glass tube (Digisystem Laboratory Instrument, His Chih city, Taiwan) containing 5mg of sodium ethylene diaminetetraacetic acid (EMD Chemicals Inc.) as anticoagulant to separate plasma for evaluation of the activity of reduced glutathione (GSH), glutathione reductase (GR), catalase (CAT), superoxide dismutase (SOD) and level of malondialdehyde (MDA), nitric oxide (NO), IL-6 and TNF- α . Meanwhile, the second blood sample was collected into a clean centrifuge glass tube without anticoagulant to separate serum for evaluation of iron and zinc. The separated serum and plasma were kept frozen at -80°C for further biochemical analysis.

Biochemical analysis

Total antioxidant capacity (TAC) and activity of GSH, GR, CAT, SOD as well as level of MDA, NO, iron and zinc were measured spectrophotometrically (Robonik biochemistry automatic analyzer, Robonik India PVT LTD, India) following standard methods using commercially available test kits (Biodiagnostic, Cairo, Egypt). The ratio of the total peroxide levels to the TAC gave the oxidative stress index (OSI), an indicator of the degree of oxidative stress.¹⁸

The OSI value was calculated as follows:

$$OSI = \frac{(total\ peroxide\ mmol/l)}{(TAC\ mmol/l)} \times 100$$

However, plasma TNF- α and IL-6 level was ELISA assayed (Robonik Elisa Plate Reader, Robonik India PVT LTD, India) following standard method using commercially available ELISA test kits (Boster Biological Technology, Pleasanton, Alameda County, California).

Statistical analysis

Data analysis was performed using a statistical software program (Graph pad Prism for Windows version 5.0, GraphPad Software, Inc., San diego, CA, USA). D'Agostino and Pearson omnibus normality test was used to assess normality. Data were normally distributed; therefore, mean and standard deviation were statistically analyzed and presented. *Paired-sample T-test* was used to assess statistical differences between the groups. Correlations between biochemical parameters were assessed by using Spearman correlation analysis. For all statistical examinations, results were considered significant at $p < 0.05$.

Results

Clinically, in dairy cows with acute clinical mastitis, there was a significant ($p < 0.05$) increase in the clinical index score compared with control group. The recorded clinical index score expressed as (Mean \pm SD) for dairy cows with clinical mastitis was 2.44 \pm 0.50. Meanwhile, the index score for control group was 1.00 \pm 0.00.

Biochemically, in dairy cows with clinical mastitis, there were a significant ($p < 0.05$) decrease in the TAC, activity of GSH and CAT as well as in the level of zinc and iron (Table 2) (Table 3). However, there was a significant ($p < 0.05$) increase in the levels of MDA, NO, IL-6 and TNF- α , activity of SOD and the OSI (Table 2) (Table 3). In dairy cows with acute clinical mastitis, there was a negative correlation between MDA and SOD ($r = -0.653$), MDA and IL-6 ($r = -0.860$), and GR and IL-6 ($r = -0.928$).

Table 2 Antioxidant enzymes activity and oxidative stress markers (Mean±SD) in clinically healthy cows and in those with acute clinical mastitis

Groups	TAC(mmol/L)	GSH(mg/dL)	GR(U/L)	CAT(U/L)	SOD(U/mL)	MDA(nmol/mL)	NO (µmol/L)	OSI
Control (n=50)	0.82±0.07	1.80±0.07	0.87±0.38	454±28	1658±23	4.47±2.90	34.70±1.92	0.55±0.38
Mastitis (n=50)	0.40±0.08	3.09±0.25	1.23±0.17	156±20	1815±27	19.86±7.83	66.06±3.02	5.36±2.65
P value	P=0.000	P=0.000	P=0.000	P=0.000	P=0.000	P=0.000	P=0.000	P=0.000

TAC, total antioxidant capacity; GSH, reduced glutathione; GR, glutathione reductase; CAT, catalase; SOD, superoxide dismutase; MDA, malondialdehyde; NO, nitric oxide; OSI, oxidative stress index

Table 3 Interleukin-6, TNF-α, Zn and Fe levels (Mean±SD) in clinically healthy cows and in those with acute clinical mastitis

Groups	IL-6 (ng/mL)	TNF-α (ng/mL)	Zn (µM/L)	Fe (µM/L)
Control (n=50)	69.80±3.83	7.06±0.71	7.97±0.92	24.46±1.39
Mastitis (n=50)	97.7 ±7.0	12.53±1.55	3.76±1.22	7.18±2.43
P value	P=0.147	P=0.000	P=0.000	P=0.000

IL-6, interleukin-6, tumor necrosis-α, zinc and iron levels; Zn, zinc; Fe, iron

Discussion

Free radicals are natural end products of the intensive metabolism in cells of the living organism, including high-yielding dairy cows. When the homeostasis is disturbed principally by generation and accumulation of these free radicals, oxidative processes lead to oxidative stress causing mastitis in dairy cows. The inflammation of mammary glands can cause reduction of milk yield and unfavorable changes in the milk composition, e.g. reduction in fat, casein proteins and calcium content with a simultaneous increase in the concentration of whey proteins, sodium and chlorine.¹⁹ Clinically, in dairy cows with acute clinical mastitis, the clinical index score was significantly ($p<0.05$) increased in comparison with control group. This could be attributed to inflammation of the mammary glands following their infection which represents the stage at which clinical mastitis occurs with varying degrees of clinical abnormalities of the udder and variable systemic effects with appearance of gross abnormalities of the milk. These findings were in agreement with those previously reported by Jánosí et al.¹⁷ In the current study, the antioxidant defense system is compromised in dairy cows with acute clinical mastitis, which is evidenced by decreased plasma TAC and increased MDA level, which may indirectly indicate increased whole free radical activity with a resultant increased OSI reflecting the state of oxidative stress in such cases.²⁰

Reduced glutathione is present in living cells at high concentrations. It becomes oxidized upon reaction with reactive oxygen species to glutathione radical which can be regenerated to its reduced form by glutathione reductase.²¹ In this study, the plasma GSH concentration in dairy cows with acute clinical mastitis was significantly increased ($p<0.01$) compared to control group. The increased GSH concentration could be explained by the enhancement of activities of both types of enzymes glutathione peroxidase (GPx) and GR, leading to intense regeneration of GSH from the oxidized form (GSSH) obtained after reduction of peroxides into alcohols.²² In contrast, some studies recorded a decrease in GSH in dairy cows with

acute clinical mastitis.²³ This variation could be attributed to different GPx activity associated with different bacterial pathogens.²⁴

There was a significant increase in the level of GR and SOD in diseased cow compared to healthy ones which was probably a response to the higher superoxide radicals ($\bullet\text{O}_2^-$) generation as a result of inflammatory reactions in the mammary gland tissue. SOD catalyzes the dismutation of $\bullet\text{O}_2^-$ into oxygen and H_2O_2 , and it is an important antioxidant defense mechanism in aerobic organisms, although too much SOD may sometimes be deleterious. These findings were in accordance with the findings of other authors.²⁵⁻²⁷ The protective effect of CAT has been demonstrated in bovine neutrophil-induced model of mammary cell damage.²⁸ In the present study, there was a significant decrease in CAT activity in dairy cows with acute clinical mastitis. Such decrease might be attributed to its increased consumption to counteract reactive oxygen species produced from inflamed mammary glands, suggesting a compromise in antioxidant defense of the body.²³

Macrophages, neutrophils and other phagocytic cells considered as the potent cells of immune response of mammary glands against microbial infection. Those cells generated large amounts of reactive oxygen species and reactive nitrogen species that considered as the main cause of lipid peroxidation which is used as an indicator of oxidative stress in such tissues.²⁹ Lipid peroxidation is a well-established mechanism of oxidative damage caused by reactive oxygen species, and measurement of the MDA provides a convenient index of lipid peroxidation.³⁰ In this study, the mean level of MDA was significantly higher in dairy cows with acute clinical mastitis compared with control group indicating a worse state of oxidative stress as previously recorded by Ranjan et al.³¹ and Jhambh et al.²³ Nitric oxide is one of the most important reactive nitrogen radicals; which operate in a variety of tissues such as epithelial cells and macrophage of mammary gland, producing a significant amount of NO that mediates inflammation during mastitis.³² In this study, there was a significant increase in plasma NO in dairy cows with acute clinical

mastitis in comparison with control group, similar to that reported by Atakisi et al.³³ In animals suffering mastitis, neutrophils produce considerable amount of NO and the myeloperoxidase enzyme, i.e. substances that together may lead to the formation of nitrotyrosine, which has the ability to disintegrate proteins and have a destructive effect on tissues.¹⁹

Enhancement of cytokines production is considered a non-specific defense mechanism of the body against intra-mammary infection playing not only a critical role in protection against bacterial infection, but also involved in the pathogenesis and development of symptoms in infection.^{19,34} Numerous factors promote cytokines expression *in vivo*, including cell-cell contact, immune complexes/ autoantibodies, local complement activation, microbial species and their soluble products, reactive oxygen and nitrogen intermediates, trauma, ischemia, DNA (mammalian or microbial), and cytokines themselves in autocrine loops.³⁵ Depending on this; the IL-6 and TNF- α level was significantly increased in dairy cows with acute clinical mastitis compared with healthy group. The increased IL-6 may be because of increased MDA level as IL-6 level was positively correlated with by-products of oxidative stress as previously stated by Moldoveanu et al.^{19,36}

However, increase of TNF- α level in the present study is due to the binding of the lipopolysaccharide (LPS) to binding protein (LBP) complex to form the cluster of differentiation 14 (CD14) molecules.³⁷

Tumor necrosis- α is a potent activator of leukocytes and enhances the phagocytosis and killing of mastitis pathogens by bovine neutrophils.³⁸ In the current study, chemotactic factors released by infectious bacteria and other components of the immune system are the signals for neutrophil recruitment to sites of infection. However, this influx of neutrophils is a double-edged sword. This may cause not only an inflammatory reaction that results in the elimination of infection, but also tissue damage that leads to fibrosis and impaired mammary function.^{19,39}

Trace elements as copper, iron, zinc, and manganese play important roles in several biochemical processes as they are essential component in the antioxidant enzymes as SOD and catalase.⁴⁰ Therefore, participation of zinc as a component of the oxidant defense mechanism was indicated and its deficiency is associated with decreased leukocyte function, increased susceptibility to bacterial infection⁴¹ with a resultant of a state of oxidative stress.⁴² In this study, there was a significant decrease in zinc and Iron in dairy cows with acute clinical mastitis compared with healthy cows. Decreased serum zinc and iron level are regarded as non-specific host defense mechanisms against bacterial infection, which will reduce the availability of these divalent cations needed for bacterial growth as previously stated by Ranjan et al.³¹ & Failla.⁴³ Moreover, sequestration of iron may, also be a mechanism to reduce generation of oxygen radicals, which are potent mediators of tissue damage during acute inflammation.⁴⁴

Conclusion

The results of the current study showed that the oxidative stress was the principal factor causing dysfunction of the immune system of the organism impairing the response to inflammatory conditions, leading to numerous diseases in dairy cows, above all to mastitis. Under oxidative stress, which leads to a reduced resistance to the invasion of pathogenic micro-organisms, the probability of appearance of inflammatory conditions and especially of mastitis increases. All the results point to dairy cows with acute clinical mastitis as a significant factor in alterations of the oxidant and antioxidant balance in plasma

associated with elevation of pro-inflammatory cytokines, resulting in potent oxidative stress, which could be a potential biomarker for diagnosing of bovine mastitis and monitoring health status of udder. Therefore, mastitis is a disease which not only reduces cows' productivity, but also leads to deterioration of the milk chemical composition and quality.

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Conflict of interest

Author declares that there is no conflict of interest.

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