Profile of Some Metabolic Immune Parameters in Dairy Cows mastitis

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SUMMARY

Immune status of the udder during the course of mastitis caused by different organisms in cows aimed in current study. Milk whey and serum samples were collected from mastitic cows and the cause of infection was identified bacteriologically. Concentrations of total antioxidants (TAO), lysozyme, and nitric oxide (NO) in milk whey and blood serum were quantified. Three main organisms were found in more than 100 record of mastitis; Mycobacterium tuberculosis, Staphylococcus aureus, and Mycoplasma sp. Total antioxidant sharply decreased in sera and milk of all cows having mastitis compared with control healthy cows (control group; n=17 cows). Comparing TAO in sera of cows suffered from S. aureus mastitis was significantly greater in concentration than those infected with Mycoplasma spp., and T.B. In the mean time, cows infected with Mycoplasma spp. had greater concentrations of TAO than those infected with T.B. On the other hand, TAO concentrations in milk whey of cows infected with T.B. was greater (insignificantly) than those infected with Mycoplasma spp. but significantly greater than those suffered from S. aureus mastitis. Lysozyme concentrations in sera and milk whey of non infected control cows were lower than that in infected cows. In sera of cows infected with S. aureus, lysozyme concentration was significantly higher than those infected with T.B. or Mycoplasma spp. Also Cows infected with T.B. mastitis had greater lysozyme concentration than those infected with Mycoplasma spp. On the contrary, cows infected with Mycoplasma spp. mastitis had significantly greater concentration of lysozyme in their milk whey than those infected with T.B. or S. aureus mastitis. Nitric oxide concentration in sera and milk whey of normal control cows was significantly lower than that in mastitic cows. In sera of cows there was no significant difference between concentration of NO in the three groups suffered from T.B., Mycoplasma spp. or S. aureus mastitis. Meanwhile, NO concentration in milk whey of cows infected with T.B. and S. aureus mastitis was greater than those infected with Mycoplasma spp. Apparently, the kind of the organism and the type of sample (milk whey and serum) controlled the magnitude of the immune response.
INTRODUCTION

Mastitis is one of the major causes of serious economic losses, animal suffering, negative effects on milk quality, and reduced product hygiene. It is an inflammatory reaction that usually occurs following intra-mammary infections. The inflammatory response involves the massive transmigration of polymorphonuclear neutrophils (PMN) from the blood into the mammary gland (Paape et al., 2000). The presence of functional neutrophils is known to be crucial to host defense against bacterial pathogens (Kehrli et al., 1990). Schalm et al. (1976) demonstrated that treating cows with an antibovine leukocyte serum could turn chronic Staphylococcus aureus mastitis into a gangrenous disease. The main functions of PMN are to engulf pathogens and destroy them via a variety of bactericidal mechanisms such as intracellular granules that contain bactericidal peptides, proteins, and enzymes such as elastase, lysozyme and other proteinases and myeloperoxidase that are released into phagocytic vacuoles or the extracellular environment (Borregaard et al., 1993). Additionally, activated PMN have recently been found to release granule proteins and chromatin that together form extracellular fibers. These extracellular traps bind microorganisms and ensure a high local concentration of antimicrobial agents to degrade virulence factors and kill bacteria (Brinkmann et al., 2004). The other mechanism by which PMN eliminates bacteria is oxygen dependent and produces toxic reactive oxygen species (ROS). The cornerstone of this process is the generation of superoxide (O·-) via the enzyme NADPH-oxidase and nitric oxide (NO) via synthase enzyme that is expressed in the phagocytic cells (Antoine et al. 2004).

In several studies on inflammation, the oxidants and proteases released by PMN have been associated with tissue damage (Weiss, 1989; van Asbeck, 1990; Mehrzad et al., 2005). Oxidative stress can cause damage to all types of biomolecules (DNA, proteins, lipids, and carbohydrates) and therefore induce tissue injury. In cases of acute mastitis, the amount of ROS released by PMN may overwhelm the cow’s endogenous antioxidant protection mechanisms and therefore add to the inflammation, causing extensive tissue damage that invariably causes losses in milk production and may
lead to complete loss of the quarter. Antioxidants are a group of molecules (vitamins, minerals, and enzymes) that act to maintain low concentrations of ROS and lipid hydroperoxides (Bettger et al., 1979; Diplock, 1981; Chew, 1987 & 1993; Putnam and Comben, 1987; Harris, 1992). For that reason, antioxidants could be used as therapeutic agents to neutralize the effect of an overproduction of ROS.

In most of the studies on udder immune response during mastitis, researchers concentrated on blood immune parameters only, whereas data of milk immune parameters in comparison with blood parameters are few. Recently, many researchers focused on milk immune parameters against mastitis (Paape et al. 2002, Paape et al. 2003, Sordillo and Streicher, 2002, and Rainard, 2003). Therefore, we aimed in current study to compare between immune parameters in milk whey and serum in cows suffering from mastitis caused by different types of microorganisms. Also we aimed to test whether or not the type of the microorganism can affect the immune response of the udder.

MATERIAL AND METHODS

1- Animals: A total of 100 mastitic cows from different localities in Egypt (Sharkia, Gharbia, and Alexandria Provinces) were examined for mastitis. Clear clinical signs of mastitis were apparent such as enlarged, inflamed, hot, and painful udder. Some cases showed fibrosis in one or more quarter of the udder due to chronic mastitis.

2- Milk and serum samples: Milk samples were collected from cows in sterilized tissue culture tubes and kept on ice. Blood samples were collected from same cows in v accutainers and kept also on ice and sent to the lab. All milk samples were subjected to bacteriological examination. Blood samples were centrifuged to separate serum and milk samples were centrifuged to obtain whey. Both serum and milk whey kept at -40° C until assayed for total antioxidants (TAO), lysozyme, and nitric oxide (NO) concentrations.

3- Bacteriological isolation and identification from milk: All milk samples were subjected to bacteriological culture using specific media (blood agar, MacConky agar, Manitol salt agar, Edward's medium and nutrient agar). Culture and biochemical identification of bacterial isolates were done according to Toply and Wel son (1998). Suspected milk samples from cows suffering from T.B. and mycolpasma were cultured on specific media for each organism (Lowenstein Jensen and PPLO
agar media respectively).

A) *Mycobacterium tuberculosis*: It was done according to Guindi *et al.* (1980) as follow: 100 ml of well mixed milk samples were transferred into two sterile centrifuge tubes and spun for 30 minutes at 3000 rpm. The cream and milk whey were poured off and the remaining sediment was subjected for T.B. examination, where this sediment was mixed thoroughly with an equal volume of 6% HCl and incubated at 37º C for 30 minutes. The mixture was centrifuged for 30 minutes at 3000 rpm. The supernatant fluid was poured off and the sediment was neutralized with 4% sterile sodium hydroxide solution using phenol red as indicator (the change in color from orange to pink indicated correct neutralization). The neutralized sediment was thoroughly mixed using sterile Pasteur pipette before being inoculated onto slop of modified Lowenstein Jensen media with 0.5% glycerol and incubated at 37º C and examined daily for 7 days then periodically once a week up to 6 weeks. Direct smears were made from isolated colonies, fixed by gentle heating, stained by Ziehl–Neilsen method and examined microscopically for acid fast organisms. Suspected colonies were purified and streaked onto Lowenstein slants and incubated at 37ºC for further identification. The growth of mycobacterium colonies were examined at different temperature degrees according to Jones *et al.*, (1966). Three slants of Lowenstein Jensen media were inoculated with each isolate, one of them was incubated at 28º C, while the other two slants were incubated at 37º C and 45ºC.

B) *Mycoplasma spp.*: Bacto-PPLO agar or broth (Difico) supplemented with 20% horse serum, 10% yeast extract, 0.0002 % DNA, penicillin G-sodium and thallium acetate was used for isolation of *Mycoplasma* organisms (Sabry and Ahmed, 1975). For biochemical characterization media for *Mycoplasma*, a glucose medium (pH 7.6) and modified arginine medium were used to differentiate between *Mycoplasma* and achloplasma (Sabry, 1968).

C) *Staphylococcus aureus*: Mannitol salt agar medium was used for the selective isolation of staphylococci due to its ability to grow in the presence of 7.5% sodium chloride as well as test the aerobic fermentation of mannitol (Toply and Welson, 1998).

Bacteriological examination revealed that 43 cows were infected with T.B., 38 were infected with *S. aureus*, and 19 cows were infected with *Mycoplasma spp*. Mixed infections were eliminated from the study to avoid interactions between the different microorganisms and to study the effect
of single organism on the immune parameters assayed. A total of 17 blood and milk samples were taken from health normal dairy cows free from mastitis and used as control group.

4- Biochemical assays: Total antioxidants (TAO) in serum and milk whey were assayed spectrophotometrically using standard diagnostic kit (Bio-Diagnostic) according to Koracevic et al. (2001). Lysozyme concentration in serum and milk whey samples was assayed as described by Peeters and Vantrapen (1977). Nitric oxide concentration in serum and milk whey samples was assayed as described by Rajaraman et al., (1998).

5-Data analysis: Statistical Analysis System (SAS) was used to find out if there are any significant differences between concentrations of TAO, lysozyme, and NO, in milk whey and serum samples and between different organisms isolated from milk.

RESULTS

As shown in Figure (1), concentration of TAO in sera of cows infected with S. aureus mastitis is significantly (P > 0.05) greater than those infected with Mycoplasma mastitis which have greater concentrations than those infected with T.B. mastitis.

![Figure (1): The concentration of total antioxidants in sera of mastitic cows infected with Tuberculosis, Mycoplasma, or Staphylococcus aureus (P > 0.05).](image)
Figure (2): The concentration of total antioxidants in mastitic cows' milk infected with *Tuberculosis*, *Mycoplasma*, or *Staphylococcus aureus* (P > 0.05).

Concentration of TAO in milk of cows infected with *T.B.* mastitis is significantly (P > 0.05) greater than those infected with *S. aureus*.

Figure (3): The concentration of nitric oxide in sera of mastitic cows infected with *Tuberculosis*, *Mycoplasma*, or *Staphylococcus aureus* (P > 0.05).

As shown in the figure, there is no significant difference between the concentrations of NO in sera of cows infected with *T.B.*, *Mycoplasma*, or *S. aureus* mastitis.
Figure (4): The concentration of nitric oxide in mastitic cows' milk infected with *Tuberculosis*, *Mycoplasma*, or *Staphylococcus aureus* (P > 0.05).

As shown in the figure, concentration of NO in milk of cows suffering from *Mycoplasma* mastitis is significantly less than cows infected with *T.B.* or *S. aureus* mastitis. No significant difference between cows infected with *T.B.* or *S. aureus* in NO concentration.

Figure (5): The concentration of lysozyme in sera of mastitic cows infected with *Tuberculosis*, *Mycoplasma*, or *Staphylococcus aureus* mastitis (P > 0.05).
Concentration of lysozyme in sera of cows infected with *S. aureus* mastitis is significantly (P > 0.05) greater than those infected with *T.B.* or *Mycoplasma* mastitis. Also lysozyme was significantly greater in sera of cows infected with *T.B.* mastitis than cows infected with *Mycoplasma* mastitis.

Figure (6): The concentration of lysozyme in mastitic cows' milk infected with *Tuberculosis, Mycoplasma,* or *Staphylococcus aureus* mastitis (P > 0.05).

*Mycoplasma* mastitis in cows elicited a significant (P > 0.05) greater concentration of lysozyme in milk than those infected with *T.B.* or *S. aureus* mastitis.
Table (1): Concentrations of total antioxidants, nitric oxide, and lysozyme in serum and milk of cows infected with *T.B.*, *Mycoplasma*, or *Staphylococcus aureus*.

<table>
<thead>
<tr>
<th>Total Antioxidants</th>
<th>Sample</th>
<th>T.B. vs. <em>Mycoplasma</em></th>
<th>T.B. vs. <em>S. aureus</em></th>
<th><em>Mycoplasma</em> vs. <em>S. aureus</em></th>
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<tr>
<td></td>
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<td></td>
<td>Serum</td>
<td>0.1442 ± 0.0169</td>
<td>0.1442 ± 0.0169</td>
<td>0.2587 ± 0.1100</td>
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<td></td>
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<td>0.2587 ± 0.1100</td>
<td>0.4045 ± 0.0462</td>
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<td><strong>0.0280</strong></td>
<td><strong>0.0084</strong></td>
<td><strong>0.1230</strong></td>
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<td>Milk</td>
<td>0.2455 ± 0.0369</td>
<td>0.2455 ± 0.0369</td>
<td>0.2085 ± 0.0460</td>
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<td>0.2085 ± 0.0460</td>
<td>0.1342 ± 0.0192</td>
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<td></td>
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<td><strong>0.6327</strong></td>
<td>0.0616</td>
<td><strong>0.1131</strong></td>
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<td></td>
<td>Nitric Oxide</td>
<td>5.8913 ± 0.8427</td>
<td>5.8913 ± 0.8427</td>
<td>7.5857 ± 2.7514</td>
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<td></td>
<td>Serum</td>
<td>7.5857 ± 2.7514</td>
<td>7.0136 ± 1.1858</td>
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<td><strong>0.4315</strong></td>
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<td>Milk</td>
<td>12.7490 ± 0.7211</td>
<td>12.7490 ± 0.7211</td>
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<td></td>
<td></td>
<td>7.3800 ± 1.0841</td>
<td>10.7227 ± 1.5794</td>
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<td></td>
<td></td>
<td><strong>0.0021</strong></td>
<td><strong>0.5246</strong></td>
<td><strong>0.3347</strong></td>
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<td></td>
<td>Lysozyme</td>
<td>82.6087 ± 8.9772</td>
<td>82.6087 ± 8.9772</td>
<td>47.4286 ± 14.600</td>
</tr>
<tr>
<td></td>
<td>Serum</td>
<td>47.4286 ± 14.600</td>
<td>165.2273 ± 41.7181</td>
<td>165.2273 ± 41.7181</td>
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<td></td>
<td></td>
<td><strong>0.0317</strong></td>
<td><strong>0.041</strong></td>
<td><strong>0.0308</strong></td>
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<td></td>
<td>Milk</td>
<td>63.5000 ± 15.144</td>
<td>63.5000 ± 15.144</td>
<td>336.2000 ± 157.103</td>
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<td></td>
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<td>336.2000 ± 157.103</td>
<td>95.2273 ± 41.347</td>
<td>95.2273 ± 41.347</td>
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<td></td>
<td></td>
<td><strong>0.0023</strong></td>
<td><strong>0.4920</strong></td>
<td><strong>0.0417</strong></td>
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</table>
Concentrations of TAO in sera of cows infected with Mycoplasma mastitis was significantly higher than those infected with T.B. mastitis (P = 0.028). Meanwhile, cows infected with S. aureus mastitis had greater TAO in their sera than those infected with T.B. mastitis (P = 0.0084). On the other hand, there was no significant difference in TAO concentrations in milk of cows infected with T.B., Mycoplasma, or S. aureus mastitis. Concentrations of NO in sera of cows in the three groups showed no significant differences. Only cows infected with T.B. mastitis had greater NO in their milk than those infected with Mycoplasma (P= 0.0021). Lysozyme concentrations in sera of cows infected with T.B. was greater than those infected with Mycoplasma mastitis (P = 0.0317). Meanwhile, Lysozyme was greater in sera of cows infected with S. aureus than those infected with T.B. (P = 0.041) or Mycoplasma (P = 0.0308). On the other hand, lysozyme concentrations in milk of cows infected with Mycoplasma was greater than those infected with T.B. (P = 0.0023) or S. aureus (P= 0.0417).

**DISCUSSION**

During the course of mastitis, accumulation of large number of immune cells (mainly granulocytes) occurs in the udder tissues, as a sequel of infection, to encounter the pathogen(s). As a consequence, the immune cells increase their respiratory burst and produce large quantities of oxygen metabolites named reactive oxygen species (ROS) that are responsible for destruction of pathogens. The increased amounts of ROS is an oxidative stress and their effects are not limited to the invading pathogens but extend to the udder tissues causing their damage. A physiological protective mechanism of the body against the destructive action of ROS on udder tissue is what is called antioxidants which prevent the ROS from destroying the udder cells. Total antioxidants (TAO) are a group of molecules of different chemical structures having the same duty which is removing the deleterious effects of ROS, originating during infections, from the body. Total antioxidants include vitamins (E, C, A, β-carotene), minerals (Se, Zn, Cu) and enzymes (super oxide dismutase, glutathione peroxidase, catalse). They act in many different ways to remove excess ROS from the body. In current study, the concentrations of TOA in sera and milk whey of mastitic cows were nearly close to each other, but the nature of the organism manipulated the concentration. Cows infected with S. aureus mastitis had greater concentration of TAO than those infected with T.B. It is well
known that *S. aureus* is responsible for majority of mastitis in cows and elicit highest SCC in mastitic udder (Attia et al., 2003; Burvenich et al., 2003; Nagahata et al., 2007). It seems that as a defense mechanism of the body, greater TAO was secreted to quench the oxidative effect of excess amount of ROS secreted by somatic cells. Surprisingly, TAO in milk whey was significantly lower in cows infected with *S. aureus* than those infected with *T.B.*. It is possible that because *T.B.* is localized in the udder of these cows and did not generalized yet in the body, the concentration of TAO was greater in milk whey. It is worth mentioning that TAO in milk whey of mastitic cows were lower than those in sera, suggesting that great amount of TAO were consumed by the ROS generated by the udder tissue where the infection took place. Ranjan et al. (2005) noticed that antioxidant concentrations declined in the inflammatory udder conditions in cows. Our data indicated that TAO in sera and milk whey of mastitic cows were too much lower than control healthy ones.

Nitric Oxide (NO) is a small molecular weight (30 DA) short-lived molecule which mostly secreted and acts locally Boulanger et al. (2001). It is one of the important ROS secreted by the immune cells, non-specifically, in response to infections. It can easily diffuse through the cell membrane of microorganisms and after a long series of chemical alterations; it eventually ends with destruction of the invading organism. Recently, many studies documented the important role played by NO in mastitis (Bourchard et al., 1999; Boulanger et al., 2001; Kenichi Kominet al., 2004 and Boulanger et al., 2007). In current study, NO concentration did not significantly differ in sera of cows infected with *T.B.*, *Mycoplasma*, or *S. aureus* mastitis. On the other hand, it was sharply increased in milk whey of cows than in their blood sera. Our data support the idea that NO acts locally in the site of infection, i.e. in the udder. Moreover, its concentration in milk whey of cows infected with *T.B.* and *S. aureus* mastitis showed significant increase than those infected with *Mycoplasma* mastitis may be due to the fact that *Mycoplasma* is an intracellular pathogen that may reduce host immune defenses (Kaklamanis and Pavlotos, 1972; Bradbury, 1984; Thomas et al., 1990). Concentration of NO in healthy control cows (either in serum or milk whey) was significantly less than cows suffering from mastitis. This finding may support the role of NO in case of infections.

Lysozyme is a naturally occurring antimicrobial component
of milk (Schmedt Auf Der Gunne et al., 2002). It is secreted from polymorphonuclear leukocytic cells in the milk and acts on the cell wall of bacteria causing, ultimately, destruction of the invading organism. Approximately 70 to 80% of the total body lysozyme is either within or is released from leucocytes (Finch et al., 1974). Reiter and Oram (1967) described that lactoferrin, together with the enzymes lactoperoxidase, xanthine oxidase, and lysozyme constitute a nonspecific antimicrobial defense system in the mammary gland. Gary and Manford (1978) stated that if lysozyme concentrations increase during abnormal cell behavior, lysozyme measurement would indeed be a valuable diagnostic tool. Moreover, if lysozyme appears relatively early during the development of cellular disorder because of some defense mechanism, lysozyme assay even would be suitable as a predictive tool. In current study mastitis caused by Mycoplasma elicited greater lysozyme concentration in milk whey of cows than did T.B. or S. aureus mastitis. It is not clear that why Mycoplasma infection increased lysozyme concentration in milk whey, but it is worth mentioning that lysozyme is the only parameter among the 2 immunological parameters measured (NO and lysozyme) which increased in case of Mycoplasma. We assume that lysozyme synthesis and secretion from the somatic cells in milk whey escaped the suppressive effects of Mycoplasma on immune cells. Lysozyme concentration in milk whey of control healthy cows was close to that concentration in infected cows except in case of Mycoplasma mastitis where sharp increase in lysozyme occurred in mastitic cows than other infected and control ones.

In conclusion, data of this study endorsed the idea that TAO play a role in case of udder infection and we recommend the addition of antioxidants in ration of animals during mastitis to substitute the exhausted body reserve of antioxidants during infection. Nitric oxide is important effector oxidative molecule acts locally during infections and plays a key role in elimination of pathogens from the body. Lysozyme may be important lytic enzyme in case of intracellular immunosuppressive organisms such as Mycoplasma mastitis.

REFERENCES:


Schmedt, Auf Der Gunne H.;