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Editorial

Role of Free Radicals and Antioxidants in Mastitis

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Mastitis is a worldwide problem and of major economic threat to dairy farmers. Its incidence depends on the microorganisms, the surrounding environment and the defense mechanisms in the udder tissues and blood. In the dairy industry, both clinical and subclinical mastitis produce great economic losses. Subclinical mastitis has no apparent clinical signs but accompanied with elevation of somatic cells count (SCC) in milk. However, clinical mastitis has elevated body temperature, inappetance, a red, swollen and/or painful udder and/or abnormal milk (Huijps *et al*., 2008). Milk with higher SCC is positively associated with malondialdehyde level in milk (Atroshi *et al*., 1996; Cesa, 2004; Miranda *et al*., 2004; Suriyasathaporn *et al*., 2006), consequently more free radicals being released and a stat of oxidative stress arise (Su *et al*., 2002). The inflammatory reaction accompanying mastitis is generally caused by bacterial infection and results in damage to the secretory epithelium of the mammary gland and the release of cytotoxic radicals and pro-inflammatory cytokines by the phagocytic cells (Knaapen *et al*., 1999).

Oxidative stress in mastitis

Oxidative stress is associated with some disorders of dairy cattle as mastitis (Erskine *et al*., 1987; Parantainen *et al*., 1987), retained placenta and udder edema (Miller *et al*., 1993). Mastitis could induce increase formation of free radicals in milk leading to oxidative stress (Gu *et al*., 2009) especially during the early lactation period (Bernabucci *et al*., 2005; Castillo *et al*., 2005; Sordillo *et al*., 2007). Both clinical and subclinical mastitis are associated with release of free radicals, increased total oxidant capacity and decreased total antioxidants capacity in milk (Atakisi *et al*., 2010).

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There are some important factors that contribute to the voluminous production of free radicals in mastitic milk; one of these factors is the increased numbers of cells like neutrophiles, macrophages, lymphocytes, eosinophiles and various epithelial cells of mammary tissue (Smith, 1994; Knaapen *et al*., 1999). Another factor is the increase of some cytokines (TNF-α, IL-1b, IL-6, IL-8) and other molecules such as nitric oxide (NO·) (Blum *et al*., 2000; Riollet *et al*., 2000; Notebaert *et al*., 2008). Cytokines activate reactive nitrogen intermediates (RNI), which play a complex role in the inflammatory process (Moilanen and Vapaatalo, 1995; Goff *et al*., 1996; MacMicking *et al*., 1997).

One of the most important RNI is NO·, which operates in a variety of tissues to regulate a diverse range of physiological processes, including the inflammatory response (Dawson and Dawson, 1995). During inflammation, macrophage and epithelial cells of the mammary gland produce a significant amount of NO; this inducible NO· mediates inflammation during mastitis (Bouchard *et al*., 1999). Another source for NO· is the mammary epithelial cells and/or mononuclear phagocytes, which contribute to NO· production upon stimulation with lipopolysaccharide and cytokines (Boulanger *et al*., 2001).

Nitric oxide has a key role in mediating microbistatic and/or microbicidal activity, as the activated macrophages synthesize NO· (Jungi, 2000), which is considered as a primary defence system that eliminate intracellular pathogens (Huie and Padmaja, 1993; O'Flaherty *et al*., 2003). The antimicrobial properties of NO· is attributed to peroxynitrite, a reactive nitrogen metabolite, derived from oxidation of NO· (Beckman *et al*., 1990). In severe mastitis, peroxynitrite is produced in excess, which may result in alterations in the antioxidant balance (Chaiyotwittayakun *et al*., 2002). This means that excessive release of NO· results in oxidative damage to mammary gland secretions

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(Bouchard *et al.*, 1999; Komine *et al*., 2004; Atakisi *et al*., 2010).

Nitric oxide was reported to increase in milk during clinical mastitis (Lacasse *et al*., 1997, Atakisi *et al*., 2010) and in milk and plasma after intramammary infusion of *E. coli* bacteria or *E. coli* endotoxin (Bouchard *et al*., 1999; Blum et al., 2000; Komine *et al*., 2004). Milk SCC is a well-known indicator reflecting mammary health and milk quality, a positive correlation between SCC and NO· concentration had been reported (Bouchard *et al*., 1999; Atakisi *et al*., 2010). Changes in milk NO· level thus could be proposed as an additional diagnostic tool to detect inflammation during mastitis. It is clear from the above paragraphs that the body uses free radicals to destruct microorganisms at the site of inflammation. However, with chronicity and severity of inflammation more free radicals being released at the site of inflammation, which accompanied with oxidative damage to tissues, lipid peroxidation and oxidative stress.

Protective role of antioxidants in mastitis

The cells contain a variety of antioxidants that play an important role in the protection against excessive release of reactive oxygen species in blood and tissues (Pár and Jávor, 1984; Halliwell, 1991) including the udder tissue and milk in mastitis (Smith *et al.*, 1984; Erskine, 1993; Bowers, 1997; Weiss et al., 1997; Scaletti *et al*., 2003). Antioxidants protect the body from free radicals either by directly scavenging free radicals or by inhibiting the activity of oxidizing enzymes (Abd Ellah *et al*., 2009; Abd Ellah, 2010). The antioxidant system consists of antioxidant enzymes (superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSH-Px), glutathione, ancillary enzymes (glutathione reductase (GR), glutathione S-transferase, and glucose 6-phosphate dehydrogenase, metal-binding proteins (transferrin, ceruloplasmin, and albumin), vitamins (alpha-tocopherol, ascorbate, and beta-carotene), flavonoids, and urate (Halliwell, 1994; Abd Ellah, 2010).

It had been reported that antioxidant supplementation could decrease the duration, incidence, and severity of clinical mastitis (Smith *et al*., 1984). Despite significant evidence supporting the role of antioxidants in enhancing resistance to mastitis, there is no information as to how the expression of specific antioxidant defenses change in mammary tissues during the preparturient period when dairy cattle experience increased oxidative stress.

In dairy cattle, cytosolic GSH-Px is the predominant intracellular form of GSH-Px and it is the most studied selenoprotein (Smith *et al*., 1997). The activity of GSH-Px in milk varies with the species and diet (Fox and Kelly, 2006). Glutathione peroxidase catalyzes the reduction of various peroxides, protecting the cell against oxidative damage (Abd Ellah *et al*., 2007) and protects milk lipids from oxidation (Bhattacharya *et al*., 1988). GSH-Px activity is often used as a diagnostic tool when assessing the selenium status of dairy cows or as an indicator of increased ROS accumulation (Gärtner, 2009; Köhrle and Gärtner, 2009). It has been reported that blood concentration of selenium and GSH-Px activity negatively correlated with the prevalence of intramammary infection (Erskine *et al*., 1987). Low GSH-Px activity in mastitic cows with high SCCs and high level of prostaglandin formation has been recorded (Hogan *et al*., 1993), which may be attributed to the excessive release of free radicals that may result in inhibiting enzymes activity.

Superoxide dismutase was reported first in milk by Hicks *et al*. (1975), and they suggested that SOD may play an important role in keeping the oxidative stability of milk (Holbrook and Hicks, 1978). The activity of milk SOD varies between breeds of cows (Holbrook and Hicks, 1978; Lindmark-Mansson and Akesson, 2000). In cow's milk, it is not affected by stage of lactation or age of cow, and/or high SCC (Przybylska *et al*., 2007). Based on previous studies, Atroshi *et al*. (1986) reported a substantial decrease in blood SOD in cows with mastitis, while Cetin *et al*. (2005) reported an increase in erythrocyte SOD activity in ewes with gangrenous mastitis, which may be attributed to the elevated need of the enzyme to boost the defensive mechanism of the animal against oxidation.

During inflammatory diseases, high levels of NO· react with superoxide anions leading to formation of peroxinitrite radical (Beckman *et al*., 1990), the latter oxidize long chain fatty acids in cell membranes leading to lipid peroxidation (Al-Sa'doni and Ferro, 2000; Wang *et al.*, 2002), which may inhibit the activity of some antioxidant molecules as GSH-Px (Atroshi *et al*., 1996) leading to oxidative stress (Goff *et al*., 1996; Komine *et al*.,

2004; Weiss *et al*., 2004).

Role of micronutrients antioxidants in mastitis

Micronutrients that are associated with antioxidant activity including vitamin A, vitamin E, β-carotene, selenium, zinc, and copper had been studied for their effect on mastitis (Erskine, 1993; Bowers, 1997; Mukherjee, 2008). Supplementation of antioxidant vitamins may reduce oxidative stress and the inflammatory response during mastitis. In dairy cows, supplementation with vitamin E (Smith et al., 1984) or a combination of vitamins A, D, and E (Barnouin and Chassagne, 1998) played a significant role in reducing the incidence of clinical mastitis.

Ascorbic acid is the most important water-soluble antioxidant in mammals (Sauberlich, 1994). It has been identified as oxidative stress biomarkers in bovine mastitis. Many studies had been reported that its milk concentration significantly decreased in acute and subclinical forms of mastitis especially when accompanied by an increase in the levels of lipid hydroperoxide in erythrocytes (Weiss *et al*., 2004; Kleczkowski *et al*., 2005, Ranjan *et al*., 2005).

Vitamin C administered to cows by subcutaneous injection may have therapeutic value in mastitis (Chaiyotwittayakun *et al*., 2002; Naresh *et al*., 2002; Ranjan *et al*., 2005). However, its therapeutic effect decreased in the presence of lipid peroxidation with moderate improvement in clinical signs of mastitis (Ranjan et al., 2005).

Vitamin E and selenium (Se) are fundamental components of the antioxidant defence of tissues and cells. Vitamin E is the most important lipid-soluble antioxidant and the biologically most active form is α-tocopherol. Vitamin E is an integral component of all lipid membranes and has a role in protecting lipid membranes from attack by high tissue concentrations of reactive oxygen species (Rice and Kennedy, 1988).

Vitamin E deficiencies are frequently observed in peripartum dairy cows. Most cases of clinical mastitis occur during the first month of lactation (Green *et al*., 2002), and coincide with the lowest vitamin E blood concentration (Goff and Stabel, 1990). It was suggested that maintaining an optimal vitamin E level, together with low levels of oxidative stress is an important factor in dry cow management and improvement of udder health.

Dairy cattle have several known endogenous antioxidant defense mechanisms that can counteract the harmful effects of ROS accumulation. The selenium-dependent selenoproteins had been studied extensively with respect to mammary gland health (Miller *et al*., 1993). Selenium supplementation to periparturient cows reduces the incidence and severity of mastitis (Smith *et al*., 1984). The beneficial effects of selenium supplementation are thought to be due to the actions of certain antioxidant selenium-dependent enzymes (Papp *et al*., 2007). Selenium is an integral component of the enzyme GSH-Px (Diplock, 1981; Erskine, 1993; Smith *et al*., 1997). Several recent studies, documented the presence of other selenoprotein enzymes in the blood and tissues of dairy cattle that may play an important role in controlling oxidative stress, including thioredoxin reductase and phospholipid hydroperoxide glutathione peroxidase (Trigona *et al*., 2006; Balla *et al*., 2007; Bruzelius *et al*., 2007; Sordillo *et al*., 2007).

Pavalata *et al*. (2002) found that about 50% of dairy cows sampled in the Czech Republic had marginal or deficient Se status. Other studies in Slovenia, Germany and Ireland have reported similar findings with the majority of dairy cows being marginal or deficient in Se (Mee *et al*., 1993; Gründer and Auer, 1995; Zust *et al*., 1996). Weiss *et al*. (1990) found that high serum Se concentrations were associated with reduced rates of clinical mastitis and low milk SCC. Erskine *et al*. (1987) found that herds with the low SCC (less than or equal to 150,000 cells/ml) had significantly higher mean blood Se-dependent GSH-Px activity than did the herds with the high SCC (greater than or equal to 700,000 cells/ml). Whole blood concentrations of Se were also significantly higher in the herds with low SCC than in the herds with high SCC.

Deficiencies in either vitamin E or Se have been related to increased incidence and severity of mastitis. Reduced neutrophils activity is a known physiological consequence of alpha- tocopherol or selenium deficiency. Vitamin E and the selenium-containing enzyme protect neutrophils from the destructive action of toxic oxygen molecules, which is necessary for intracellular kill of ingested pathogens. Recently, trials have shown that subcutaneous injections of vitamin E approximately 10 days prior to calving successfully raised neutrophil's alpha-tocopherol concentrations during

the preparturient period and negated the suppressed intracellular kill of bacteria by neutrophils that are commonly observed at calving (Hogan *et al*., 1990; Hogan *et al*., 1993). Furthermore, dietary supplementation of cows with Se and Vitamin E resulted in a more rapid PMN influx into milk following intramammary bacterial challenge and increased intracellular killing of ingested bacteria (Smith *et al*., 1997), as well as lowering the frequency and shortening the duration of clinical mastitis (Smith *et al*., 1984).

Substantial research has been carried out on the effect of selenium and vitamin E on the immune function of the mammary gland and subsequent improvement in bovine udder health and mastitis control (Cerri *et al*., 2009; Salman *et al*., 2009). Vitamin E and selenium given prior to calving have been proposed for prophylactic treatment of mastitis (Smith *et al*., 1997). Cows supplemented with vitamin E throughout the dry period had lower incidence of clinical mastitis during the next lactation. However, cows supplemented with both vitamin E and Se had shorter duration of clinical signs than cows supplemented with either micronutrient alone (Smith *et al*., 1984). In a study by Smith *et al.* (1985), diets of heifers supplemented with Se and vitamin E from 60 days pre-partum and continuing throughout lactation had significantly fewer infected quarters at calving, reduced prevalence of infection throughout lactation, fewer cases of clinical mastitis, infections of shorter duration, and lower milk SCC compared with the none supplemented heifers.

Both β-carotene and vitamin A play an important role in protecting udder tissue and milk from the harmful effect of free radicals. Beta-carotene functions independently of vitamin A in mastitis and reproduction (McDowell, 1989). It was reported that, low concentrations of plasma vitamin A (<80μg/100 ml) and β-carotene (<200μg/100 ml) were linked with severity of mastitis (Chew *et al*., 1982; Jukola et al., 1996). Dahlquist and Chew (1985) carried out a study, the results revealed that cows fed vitamin A plus β-carotene had fewer new infections during the early dry period than cows fed the other treatments. Chew and Johnston (1985) carried out another study and found that vitamin A plus β-carotene reduced mean SCC during the 2nd to the 8th weeks of lactation. In another study, supplementation of vitamin A and/or β-carotene for cows did not affect new infections during the dry period, new infections at calving and cases of clinical mastitis (Oldham *et al*., 1991).

Minerals (Zinc, copper, iron, manganese, selenium) are essential for the formation of antioxidants' enzymes. For example, zinc is required for the formation of Mn-Zn SOD, deficiency of zinc affect the activity of SOD in blood and tissues, which results in increased superoxide radicals. It is known that mastitis associated with increased SCC counts in milk, which act as a source for free radicals and hence oxidative stress. Supplementation of zinc helps the animal to recover from increased oxidative stress by reducing the SCC (Kincaid *et al*., 1984; Popovic, 2004)). Experimental studies approved that copper supplementation reduced the severity of clinical signs of *E. coli* mastitis (Scaletti *et al*., 2003). In a separate study, Scaletti *et al.* (2002) attempted to determine the effect of copper source on the response to intra-mammary challenge with *E. coli* at day 32 of lactation. The study revealed that copper and zinc supplementation to mastitic cows enhanced the resistance to bacterial mastitis.

Conclusion

The fact that mastitis paralyze the milk production of dairy animals, make that disease of major importance. Another fact is that the increased free radicals during mastitis are mostly associated with decreased antioxidants defence as established by many researchers. This makes the supplementation of mastitic animals with nutrients antioxidants as vitamin C, E and beta carotene, and minerals very important to help the animal recover early. On the other hand, diary animals must be supplemented with the optimal ration that contains all required nutrients needed for optimal production and reproduction. Antioxidants status of milk could be used as a diagnostic indicator for early detection of mastitis. Another possible use for measuring antioxidants in milk is the evaluation of treatment and also establishing a prognosis.

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