Subclinical mastitis causes alterations in nitric oxide, total oxidant and antioxidant capacity in cow milk

Onur Atakisi a,∗, Hasan Oral b, Emine Atakisi a, Oguz Merhan a, S. Metin Pancarci b, Ayla Ozcan a, Saban Marasli a, Bulent Polat c, Armagan Colak c, Semra Kaya b

a Department of Biochemistry, Faculty of Veterinary Medicine, Kafkas University, Kars, Turkey
b Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Kafkas University, Kars, Turkey
c Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Ataturk University, Erzurum, Turkey

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ABSTRACT

The aim of this study was to investigate total antioxidant (TAC), and oxidant capacity (TOC) and nitric oxide (NO) levels in milk of cows with subclinical mastitis. Brown Swiss and Holstein breed cows were screened with California Mastitis Test (CMT) to determine mammary glands with subclinical mastitis. Moreover, somatic cell counts (SCC) were determined electronically in all milk samples. Mammary quarters were classified as healthy (n = 25) or subclinical mastitis (n = 35) based on CMT scores and somatic cell count (SCC: ≤200,000/ml or >200,000/ml) in milk. Nitric oxide, TOC and SCC levels were significantly higher (p < 0.005 and p < 0.001, respectively) in milk from mammary quarters with subclinical mastitis compared to those from healthy mammary quarters. In conclusion, subclinical mastitis results in higher NO concentrations, TOC and SCC, and NO and TOC were positively correlated with SCC. Moreover, alterations in NO levels and TOC in milk could be used as an alternative diagnostic tool to screen for subclinical mastitis.

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1. Introduction

Mastitis is a common disease that decreases milk yield and quality in lactating cows (Philpot and Nickerson, 1991a; Christ et al., 1983). Because of higher treatment cost and higher culling rate, mastitis causes economical losses (Moore et al., 1991; Philpot and Nickerson, 1991b). In addition to control of clinical mastitis, earlier diagnose and treatment of subclinical mastitis, which is spread unrecognizably in a herd leading to lower milk production, is also required (Craven, 1987; Egan, 1995; Yalcin et al., 2000). Somatic cell count (SCC) in milk is a well known indicator reflecting mammary health and milk quality. Excessive amounts of neutrophiles, macrophages, lymphocytes, eosinophiles and various epithelial cells of mammary tissue in milk is considered as a response of mammary tissue to microorganisms in part of inflammation of mammary gland (Knnapen et al., 1999; Smith, 1994; Weiss, 1989). The level of cytokines such as TNF-α, IL-1β, IL-6, IL-8 and some other molecules such as nitric oxide (NO) are reported to increase during infections (Molinanen and Vapaatalo, 1995; Notebaerta et al., 2008; Riollet et al., 2000). Nitric oxide is produced from L-arginine by nitric oxide synthase (NOS) (Moncada et al., 1991). Two calcium-binded isoforms of NOS are generally present in endothelial cells and brain (Breedt and Snyder, 1994). The inducible form of NOS (iNOS) is generally present in macrophages, and production of NO during inflammatory diseases is quite higher (MacMicking et al., 1997; Molinanen and Vapaatalo, 1995). NO, produced in higher amount during inflammation by iNOS and activated by cytokines, is accepted as a primary defense system (Huie and Padmaja, 1993; Okamoto et al., 1997). Antimicrobial effect of NO on bacteria is due to peroxynitrite, a reactive nitrogen metabolite, derived from oxidation of NO (Beckman et al., 1990). Substances, such as peroxynitrite, cause alterations in antioxidant balance in the organism (Chaiyotwittayakun et al., 2002). In the presence of inflammation, pro-inflammatory cytokines and cytotoxic radicals released from phagocyte cells (Knnapen et al., 1999) result in inhibition of cellular metabolic pathways and lipid peroxidation (Goff et al., 1996). Previous studies revealed that increase in lipid peroxidation during mastitis causes a decrease in levels of some antioxidant molecules leading to an increase in oxidative stress (Goff et al., 1996; Komine et al., 2004; Weiss et al., 2004). Oxidative stress is generally described as an imbalance between oxidant and antioxidant levels (Richter-Landsberg and Vollgraf, 1998; Lykkesfeldt and Svendsen, 2007). Oxidative stress is commonly
observed in different pathological events such as pneumonia, sepsis and mastitis of farm animals (Basu and Eriksson, 2001; Lauritzen et al., 2003; Lykkesfeldt and Svendsen, 2007). When the cellular oxidant state is overwhelmed by excessive production of reactive oxygen species and the condition may end up with cellular damage due to oxidative stress and lipid peroxidation (Chaiyotwitayakun et al., 2002; Halliwell and Gutteridge, 1999). Therefore, earlier diagnosis and treatment of diseases, such as mastitis, is important to minimize economical losses. Although it is well known that mastitis increases epithelial cells in milk (Philpot and Nickerson, 1991c), alterations in biochemical composition of milk due to mastitis is not well known. Consequently, the objectives of this study were to investigate nitric oxide (NO) levels, total antioxidant (TAC) and oxidant capacity (TOC) in milk of cows with subclinical mastitis in this study.

2. Material and methods

2.1. Animals and clinical examination

This study was conducted in cows (Brown Swiss and Holstein) under the same nutrition and management conditions at Atatürk University, Veterinary Faculty, Research and Training Farm. All mammary quarters were screened with California Mastitis Test (CMT) to investigate healthy mammary quarters and mammary quarters with subclinical mastitis. Following washing and drying the mammary teats, 70% ethanol was sprayed and a few streams of milk were discarded. Afterwards, milk samples (10 ml) were collected into sterile tubes, and immediately transported to the laboratory for SCC and biochemical analyses. Somatic cells were counted electronically (Bentley Somacount, Italy). Milk samples with CMT (–) and SCC less than 200,000/ml were classified as healthy (n = 25). Milk samples with CMT (+) and SCC more than 200,000/ml were classified as having subclinical mastitis (n = 35). All milk samples were stored at −50 °C until analyzed for related biochemical parameters.

2.2. Biochemical analysis

2.2.1. Determination of nitric oxide levels in milk

Nitric oxide concentrations were determined using a spectrophotometer (PowerWave XS, BioTek, Instruments, USA) in milk samples by the method of Miranda et al. (2001). Initially, milk samples were deproteinized with 10% zinc sulphate. Total NO concentrations (nitrate and nitrite) were determined colorimetrically by the acidic Griess reaction via reaction involving reduction of nitrate to nitrite by vanadium (III) chloride (Miranda et al., 2001).

2.2.2. Determination of total antioxidant and oxidant capacity in milk

In order to measure TAC and TOC accurately in milk, the method reported by Erel (2005) was slightly modified, and milk samples were diluted and filtered to obtain transparent milk samples. For this purpose, milk samples were diluted (10-fold) with physiologic saline prior to assay. Afterwards, solid phase in diluted milk samples were removed by using naylon-66 syringe filter (0.45-μm) in two times.

In transparent samples, total antioxidant and oxidant capacities were determined colorimetrically (PowerWave XS, BioTek, Instruments, USA) with a commercial kit (Rel Assay Diagnostic, Turkey). Antioxidants in the sample reduce dark blue-green colored 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical to colorless reduced ABTS form. The change of absorbance at 660 nm is related to total antioxidant level of the sample. Oxidants present in the sample oxidize the ferrous ion–chelator complex to ferric ion. The oxidation reaction is prolonged by enhancer molecules, which are present in the reaction medium. The ferric ion makes a colored complex with chromogen in an acidic medium. The color intensity is related to the total oxidant molecules present in the sample at 530 nm. Trolox and hydrogen peroxide standards were used for total antioxidant and total oxidant capacities (Erel, 2005).

2.3. Statistical analyses

Statistical analysis was performed using the SPSS statistical program (SPSS 10.0 for windows). Normal distribution of the data was determined using Anderson–Darling Normality test. Independent samples t-tests were performed to examine the differences between milk with subclinical mastitis and milk without subclinical mastitis. Correlation analyses were used to determine relationship among SCC and NO, SCC and TOS, SCC and TAS. Values were expressed as mean ± standard error of mean (SEM). Significant level was set at p < 0.05.

3. Results

For milk samples from mammary glands with and without subclinical mastitis, NO concentrations, TAC, TOC and SCC were 8.89 ± 0.89, 3.96 ± 0.44 μmol/L; 0.42 ± 0.047, 0.54 ± 0.051 mmol TroloxEquiv./L; 20.88 ± 0.90, 15.91 ± 0.57 μmol H2O2 Equiv./L; (859.46 ± 173.73) × 1000/ml, (87.41 ± 9.53) × 1000/ml, respectively (Table 1). NO and TOC levels were significantly (p < 0.001, p < 0.005, respectively) higher in milk samples from mammary glands with subclinical mastitis compared to mammary glands without subclinical mastitis. In contrast, TAC levels were numerically lower (p = 0.09) in milk samples from mammary glands with subclinical mastitis compared to those from mammary glands without subclinical mastitis. SCC were significantly (p < 0.001) higher in milk samples from mammary glands with subclinical mastitis compared to those from mammary glands without subclinical mastitis. Statistically significant (r = 0.598; p < 0.05) positive correlation was detected between SCC and milk NO concentrations (Fig. 1). While there was no correlation (r = 0.102) between SCC and TOS, there was a statistically significant positive correlation (r = 0.312; p < 0.05) between SCC and TOS.

4. Discussion

Nitric oxide is a molecule regulating many biological functions in the body and plays some important roles during inflammatory process (Dawson and Dawson, 1995). It has been reported that mammary gland epithelial cells and macrophages produce NO in significant amounts during inflammation (Bouchard et al., 1999; Cuzzoerea and Caputi, 1999; Goff et al., 1996). Mastitis is caused

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups</th>
<th>Healthy (n = 25)</th>
<th>Subclinical mastitis (n = 35)</th>
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</thead>
<tbody>
<tr>
<td>Nitric oxide (μmol/L)</td>
<td>3.96 ± 0.44&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.89 ± 0.89&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>Total antioxidant capacity (mmol TroloxEquiv./L)</td>
<td>0.54 ± 0.051&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.42 ± 0.047&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>Total oxidant capacity (μmol H2O2 Equiv./L)</td>
<td>15.91 ± 0.57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.88 ± 0.90&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Somatic cell count (∗1000)</td>
<td>87.41 ± 9.53&lt;sup&gt;b&lt;/sup&gt;</td>
<td>859.46 ± 173.73&lt;sup&gt;a&lt;/sup&gt;</td>
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</table>

Values are expressed as mean ± SEM.  
<sup>a,b</sup> Refers to statistical significance between the groups (P < 0.005) in the same row.  
<sup>c,d</sup> Refers to statistical significance between the groups (P < 0.001) in the same row.
by inadequate hygiene, stress, trauma, improper milking techniques and housing conditions leading to inflammation in a single or all mammary glands (Philpot and Nickerson, 1991c; Philpot and Nickerson, 1991d). Positive correlation between intra-mammary or all mammary glands (Philpot and Nickerson, 1991c; Philpot and Nickerson, 1991d). Positive correlation between intra-mammary and systemic TNF-α and NO production has been reported in an acute mastitis model caused by *E. coli* inoculation in early lactation in cows (Blum et al., 2000; Goff et al., 1996). Likewise, increase in NO levels in milk from cows with experimentally induced mastitis with endotoxin were reported, and that elevated NO concentration in milk was speculated as an inflammatory response of mammary gland (Bouchard et al., 1999). In the current study, higher NO concentrations in milk from mammary glands with subclinical mastitis indicate the relationship between elevation of NO levels and inflammation. In this regard, alterations in milk NO levels could be used as an alternative diagnostic tool to detect inflammation during subclinical mastitis. Bouchard et al., 1999 reported positive relationship between SCC and NO concentration (Bouchard et al., 1999). In the present study, a statistically significant positive correlation (*r* = 0.502; *p* < 0.05) between SCC and NO concentration indicates the positive relationship between elevated SCC during inflammation and NO concentrations.

During inflammation, NO increases and reacts with superoxide anions leading to formation of peroxinitrite radical (Beckman et al., 1990), and this peroxinitrite radical, quite reactive, oxidizes long chain fatty acids in cell membranes leading to increase in lipid peroxidation and formation of free radicals (Al-sadoni and Ferro, 2000; Pryor and Squadrato, 1995; Wang et al., 2002). Increase in lipid hydroperoxide level following lipid peroxidation during experimentally induced acute mastitis in cattle (Richter-Landsberg and Vollgraf, 1998; Waller, 2000) clearly indicates that mastitis causes formation of free radicals. In the current study, TOC was higher in milk from mammary glands with subclinical mastitis compared to milk from mammary glands without subclinical mastitis; whereas, total TAC was lower in milk from mammary glands with subclinical mastitis compared to those in from mammary glands without subclinical mastitis. These results could imply that subclinical mastitis could increase TOC leading to increase in formation of free radicals in milk. Similarly, the significant positive correlation between elevated SCC in milk during subclinical mastitis and TOS in this study supports this hypothesis.

**Gu et al. (2009)** reported an increase in reactive oxygen species (ROS) following experimentally induced acute mastitis with lipopolysaccharide injection. However, there was a decrease in ROS with retinoid administration following experimentally induced acute mastitis by lipopolysaccharide injection. It has been postulated that retinoid treatment could be effective against oxidative stress in mammary tissue due to mastitis (Gu et al., 2009). It has been known that oxidative stress due to an increase in oxidant levels could cause changes in vital metabolic and physiological functions (Miller et al., 1993). In a study of experimentally induced acute mastitis, an increase in antioxidant capacity by administering vitamin E and selenium was reported (Mukherjee, 2008). Likewise, improper oxidant/antioxidant balance and decrease in antioxidant property of milk from mammary glands with subclinical mastitis were observed in this study. In conclusion, subclinical mastitis alters oxidant/antioxidant balance leading to a decrease in antioxidant levels of milk and results in higher NO concentrations and TOC in parallel with SCC.

**Fig. 1.** The correlation between SCC and NO concentrations in milk samples with subclinical mastitis (*r* = 0.598; *p* < 0.05).

**References**


Mukherjee, R., 2008. Selenium and vitamin E increases polymorphonuclear cell phagocytosis and antioxidant levels during acute mastitis in riverine buffaloes. Veterinary Research Communications 32, 305–313.


