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Full Length Research Paper

Malondialdehyde level and some enzymatic activities in subclinical mastitis milk

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The purpose of this study was to evaluate the changes occurring in milk malondialdehyde (MDA) level and some enzymatic activities as a result of subclinical mastitis (SCM) in dairy cows. A total of 124 milk samples were collected from 124 lactating cows from the same herd in the period between the 2nd week after calving and the 10th week postpartum. They were classified by bacterial culture and the California mastitis test (CMT) as positive were deemed to have glands with SCM, and the periodic incidence rate of SCM was 26.6%. The most common bacterial isolates from SCM cases were *Staphylococcus aureus* (47%) and coagulase negative Staphylococci (CNS) (27%). The mean level of MDA and activities of lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) were significantly higher in SCM milk than in normal milk, while the mean activity of glutathione peroxidase (GPx) was significantly lower in SCM milk than in normal milk. There were no differences in the activities of superoxide dismutase (SOD) and aspartate aminotransferase (AST) between normal milk and SCM milk. Therefore, the measurement of milk MDA level and GPx, LDH and ALP activities, appears to be a suitable diagnostic method for identifying SCM in dairy cows.

Key words: Subclinical mastitis, mastitis diagnostic, etiology, malonaldehyde (MDA), enzyme.

INTRODUCTION

Mastitis, an inflammatory reaction of the mammary gland is the most dreaded disease of dairy farmers because of reduced milk production, increased treatment costs, labour, milk discarding following treatment, death and premature culling. It is very important to determine efficient techniques that are able to identify the presence of mastitis early in the disease syndrome.

A positive diagnosis of mastitis should fulfill two criteria: a positive bacteriological test and an inflammatory change (Mattila et al., 1985). Cell counts have generally been used for the latter purpose (Babaei et al., 2007). The quantification of cells in milk or somatic cell count (SCC), is estimated using direct microscopic analysis or by an indirect method of estimating SCC using the California mastitis test (CMT) (Babaei et al., 2007). In milk-testing laboratories, the most commonly used method of enumerating somatic cell count is the fluoro-optical electronic or fossomatic counter method (Sierra et al., 2006). However, fossomatic counting is considered as a costly and sophisticated equipment which is not available everywhere (Perrin et al., 1997). The CMT is already used on-farm to diagnose indirectly subclinical mastitis (SCM) for lactating cows. The sensitivity and specificity of the CMT reported in the literature is variable (Pyörlä, 2003).

Malondialdehyde (MDA), the lipid peroxidation end product is one of the most reliable and widely used indexes of oxidative stress (Esterbauer et al., 1991). Milk

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Abbreviations: MDA, Malondialdehyde; **SCM,** subclinical mastitis; **CMT,** California mastitis test; ***S. aureus*,** staphylococcus aureus; **CNS,** coagulase negative staphylococci; LDH, lactate dehydrogenase; **ALP,** alkaline phosphatase; **GPx,** glutathione peroxidase; **SOD,** superoxide dismutase; **AST,** aspartate aminotransferase; **SCC,** somatic cell count; **PPV,** positive predictive values; **NPV,** negative predictive value.

with higher somatic cell count has been shown to have more infiltrated polymorphonuclear cells, and this caused an increase of oxidative reactions (Su et al., 2002). In cow's milk, MDA levels were measured to evaluate the peroxidation status when milk was kept under different circumstances (Cesa, 2004; Miranda et al., 2004), and milk SCC is positively associated with malondialdehyde level in milk (Suriyasathaporn et al., 2006). Glutathione peroxidase (GPx) has no known enzymatic function in milk, in which it binds 30% of the total selenium (Se), an important trace element in the diet. The level of GPx in milk varies with the species (human > caprine > bovine) and diet (Fox and Kelly, 2006). In cows with mastitis, serum lipid peroxidation levels were increased, and the level of blood glutathione peroxidase was decreased when compared to the levels in healthy cows (Atroshi et al., 1996). Several workers have observed superoxide dismutase (SOD) in bovine milk and suggested that the enzyme may play an important role in the oxidative stability of milk (Holbrook and Hicks, 1978). However, no study has determined the malondialdehyde level and glutathione peroxidase and superoxide dismutase activities in milk in relation to subclinical mastitis. The activities of milk lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) changes have been used as an indicator of SCM in dairy cows (Babaei et al., 2007).

The present research seeks: (i) To investigate the incidence and etiology of SCM in a commercial subtropical dairy farm and (ii) to study the changes occurring in the level of MDA and activities of GPx, SOD, LDH, ALP and aspartate aminotransferase (AST) in cow's milk as a result of subclinical mastitis.

MATERIALS AND METHODS

Animals

A total of 124 milk samples were collected from 124 multiparous lactating dairy (Holstein) cows at the same time, and they were all in the same herd of a subtropical commercial farm in Nanning, China. Every milk sample was collected from four quarters of each cow randomly. All milk samples were collected in the period between the 2nd week after calving and the 10th week postpartum. Cows about 4 to 5 years old selected for this study did not show clinical signs of mastitis or other illnesses.

Collection of samples

After a quarter had been washed with tap water and dried, the teat end was swabbed with cotton wool soaked in 70% ethyl alcohol. Milk samples were collected prior to the morning milking in sterile test tubes (10 ml) after discarding the first three squirts of milk, and then placed in an icebox and transported to the laboratory for examination within two hours after collection.

California mastitis test (CMT)

The California mastitis test was used on all milk samples, using the method by Schalm et al. (1971). The CMT score is based on the number of leukocytes in milk (Csapó et al., 1995). The reaction involved in the CMT is the disintegration of leukocytes when milk is mixed with the reagent (Babaei et al., 2007). According to the visible reactions, the results were classified in four scores: 0 = negative or trace, 1 = weak positive, 2 = distinct positive and 3 = strong positive.

Bacteriological examination of milk samples

Milk samples were examined following standard procedures (Sears et al., 1993; Quinn et al., 1994; Batavani et al., 2003; Yang et al., 2011).

Definition of subclinical mastitis

Mammary glands without clinical abnormalities and with apparently normal milk that were bacteriologically negative and negative on the California mastitis test were considered to be normal milk, while those that were bacteriologically positive and with the CMT positive were considered to have subclinical mastitis.

Measurement of milk MDA level and enzyme activity

Normal and subclinical mastitic milk samples were skimmed by centrifugation at 10,000 g for 20 min at 4°C. Defatted milk was used for MDA level and enzyme activity estimations. Milk MDA level and enzyme activity were measured by spectrophotometric techniques.

Malondialdehyde level was determined by the thiobarbituric acid (TBA) method, which was modified from methods of Satoh (1978) and Yagi (1984). Total (Cu-Zn and Mn) superoxide dismutase (EC 1.15.1.1) activity was measured according to the method described by Sun et al. (1988) and glutathione peroxidase (EC 1.11.1.9) using the method described by Paglia and Valentine (1967) and Goldberg and Spooner (1983). Lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) activity were determined by the method of Bergmeyer (1974) and Goldberg and Spooner (1983), respectively, while aspartate aminotransferase (AST) was carried out by the method of Reitman and Frankel (1957).

Statistical analysis

The sensitivity and specificity of the CMT results were calculated by using standard 2-by-2 contingency tables. A 95% confidence interval was calculated for the sensitivity and specificity of the CMT (Petrie and Watson, 1999). Data for milk MDA level and enzymatic activities were expressed as mean ± standard error of mean (S.E.M) and Student's t-test was used to evaluate differences between subclinical mastitic and healthy milk. Differences with $P < 0.01$ were considered to be significant.

RESULTS

A total of 124 milk samples were collected from lactating glands in the period between the second week after calving and the tenth week postpartum. Positive CMT were recorded from 65 (52.4%) glands. Bacteria were isolated from 45 (36.3%) milk samples. According to the definition of subclinical mastitis described earlier, the incidence of SCM was 26.6% (n = 33). The specificity, sensitivity, positive predictive value (PPV) and negative predictive value (NPV) of California mastitis test in

Table 1. Two-way contingency table to investigate agreement between bacteriological and the CMT results for 124 milk samples.

Parameter	Culture +	Culture –	Total
CMT $^{+}$	33	32	65
CMT $^{-}$	12	47	59
Total	45	79	124

κ Value: 0.3; specificity: 59.49%; sensitivity: 73.33%; proportion positive by the CMT: 52.4%; proportion positive by culture: 36.3%; positive predictive value (PPV): 50.8%; negative predictive value (NPV): 79.7%.

Table 2. Frequency distribution of microorganisms isolates from milk samples positive by culture.

Microorganism	Frequency (%)
S. aureus	21 (47)
CNS	12 (27)
E. coli	4 (9)
S. agalactiae	4 (9)
S. uberis	2 (4)
C. neoformans	2(4)
Total	45 (100)

detecting subclinical mastitis were 59.49, 73.33, 50.8 and 79.7%, respectively (Table 1). The Kappa value (κ = 0.3) demonstrated weak (poor) agreement between the CMT results and culture test.

Distributions of microbial isolates responsible for infected milk samples were: *S. aureus* (47%), coagulase-negative staphylococci (CNS) (27%), *Escherichia coli* (9%), *Streptococcus agalactiae* (9%), *Streptococcus uberis* (4%) and *Cryptococcus neoformans* (4%) (Table 2).

The level of malondialdehyde (MDA) and activities of glutathione peroxidase (GPx), superoxide dismutase (SOD), lactate dehydrogenase (LDH), alkaline phosphatase (ALP) and aspartate aminotransferase (AST) in normal and subclinical mastitic milk samples are presented in Table 3. The mean level of MDA and activities of LDH and ALP were significantly higher in SCM milk than in normal milk ($P < 0.01$), while, the mean activity of GPx was significantly lower in SCM milk than in normal milk ($P < 0.01$). There were no differences in the activities of SOD and AST between normal milk and SCM milk ($P > 0.05$).

DISCUSSION

According to the results of this study, the incidence of subclinical mastitis (SCM) in dairy cows in subtropical China is in agreement with the report by Getahun et al. (2008), but it seems to be higher than that reported by Mungube et al. (2004) and Gianneechini et al. (2002). A direct comparison between these earlier results is difficult, because many factors could affect the incidence of SCM in dairy cows, especially management, case definition and the diagnostic criteria used.

The California mastitis test (CMT) has been standardized for cow's milk and only reacts with liberated nuclear DNA (Batavani et al., 2003), while bacteriological culture of milk is a definition of intramammary infections (IMI) in dairy cows. The CMT showed a higher incidence of SCM than bacteriological culture (52.4% versus 36.3%). The sensitivity and specificity of the CMT reported in the literature is variable (Pyörlä, 2003). Sargeant et al. (2001) found that sensitivity and specificity to identify any IMI at a quarter level at 3 days in milk were 57 and 56%, respectively. On the other hand, Vijaya Reddy et al. (1998) reported a sensitivity of 71% and a specificity of 75%. According to our result, the specificity (59.49%) and sensitivity (73.33%) of the CMT in detecting subclinical mastitis was quite high. However, the California mastitis test was not a very good tool to correctly diagnose subclinical mastitic quarters by any pathogen in subtropical dairy farms. Low specificity and a low-to-moderate prevalence yielded low positive predictive values (PPV). False positives, associated with a low specificity, would occur when somatic cells are present in the milk with bacteria not being isolated. False negative reactions, associated with a low sensitivity, would occur when bacteria are indeed present in the gland but somatic cells are not.

The common isolate from subclinical cases were *S. aureus* (47%) and coagulase negative staphylococci (27%). These organisms have been considered to be the major cause of non-clinical IMI in a number of previous investigations (Getahun et al., 2008). The high prevalence of *S. aureus* is mainly attributed to the wide distribution of microorganism inside the mammary gland and on the skin of teat and udder (Workineh et al., 2002). Classically, coagulase negative staphylococci (CNS) were classified as minor pathogens and their importance as an independent cause of subclinical or clinical mastitis was judged to be limited. The impact of CNS IMI on cow level SCC was intermediate when compared to culture-negative animals and cows infected with major pathogens (Schukken et al., 2009). In view of this, the sensitivity of the CMT was lower by the high prevalence of coagulase negative staphylococci IMI. False negative reactions occur when bacteria are indeed present in the gland but somatic cells are not.

Several studies have evaluated milk LDH, ALP and AST activities changes to diagnose udder infections in dairy cows (Bogin et al., 1977; Kitchen, 1981; Babaei et al., 2007), but little information was available in relation to changes in milk malondialdehyde level and GPx and SOD activities for cows SCM. IMI increases the permeability of microcirculatory vessels by secretion of various chemical mediators such as histamine, prostaglandin, kinins and free oxygen radicals from inflammatory cells (Honkanen-Buzalski and Sandhom, 1981).

Table 3. Mean level of MDA and activities of GPx, SOD, LDH, ALP and AST in normal and subclinical mastitic milk samples of lactation cows.

Biochemical parameter	Normal milk (47)	Subclinical mastitic milk (33)
MDA (nmol/ml)	24.37±0.9^{A}	28.45±0.96^{B}
GPx (IU/ml)	32.81±1.41^{A}	26.41±2.03^{B}
SOD (IU/ml)	1.82±0.11	1.6±0.12
LDH (IU/L)	177.94±12.55^{A}	724.49±34.91^{B}
ALP (IU/L)	71.85±3.71^{A}	116.58±6.18^{B}
AST (IU/L)	151.99±11.56	140.98±11.76

^{A,B} Different superscript capital letters within the same row means $P < 0.01$.

Malondialdehyde (MDA) is the final product of lipid peroxidation and therefore is used as index of this process. In our study, the mean level of MDA was significantly higher in SCM milk at 28.45 ± 0.96 nmol/ml when compared to 24.37 ± 0.9 nmol/ml in normal milk ($P < 0.01$), and MDA would be considered as an indicator of subclinical mastitis udders. The higher malondialdehyde levels in subclinical mastitis milk reported in this study demonstrated that the auto-oxidative activity of SCM milk is higher than normal milk. Similar results have been reported by Suriyasathaporn et al. (2006). Milk from mastitic udders is of low quality and less suitable for consumption. In addition, the increased malondialdehyde due to high somatic cell count further reduces milk quality (Suriyasathaporn et al., 2006). In fact, malondialdehyde is known to be a mutagen and a suspected carcinogen (Aubourg, 1993); it can react with DNA to generate mutations.

Glutathione peroxidase (GPx) has been established as a selenium-containing enzyme catalyzing the reduction of various peroxides and protecting the cell against oxidative damage, and sufficient GPx could protect milk lipids from oxidation (Bhattacharya et al., 1988). Glutathione peroxidase activity has been detected in bovine milk at levels between 12 and 32 IU/m, and its activity correlated significantly with selenium concentration (Przybylska et al., 2007). In cows with mastitis, serum lipid peroxidation levels were increased, and the level of blood glutathione peroxidase was decreased when compared to the levels in healthy cows (Atroshi et al., 1996). In this study, the mean activity of GPx was significantly higher in normal milk at 32.8 ± 1.41 IU/ml when compared to 26.41 ± 2.03 IU/ml in subclinical mastitic milk ($P < 0.01$). Thus, normal milk with higher glutathione peroxidase activity may be better as a source of milk products than subclinical mastitic milk with lower GPx activity, because glutathione peroxidase exhibits a cellular defense function through decomposition of hydroperoxides. Atroshi et al. (1986) found that the decline in GPx in mastitic cows may be related to the changes in lipid peroxidation and prostaglandin formation. Human milk glutathione peroxidase activity have been shown to decrease within the time of lactation (Hojo, 1986), but there is no published literatures pertaining to bovine milk.

Superoxide dismutase (SOD) was reported first in milk by Hicks et al. (1975), and they suggested that the enzyme may play an important role in the oxidative stability of milk. The average activity of milk SOD was 1.82 ± 0.11 IU/ml with a range of 0.67 to 3.23 IU/ml in normal udders, while in subclinical mastitic udders, it was 1.6 ± 0.12 IU/ml with a range of 0.39 to 3.02 IU/ml. The activity of milk SOD varies between cows and breeds (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 high SCC (Przybylska et al., 2007). In present study, we also demonstrated that the pattern of distribution of superoxide dismutase in the milk of normal and subclinical mastitis udder showed no significant difference.

Changes in enzyme activities in blood or other biological fluids such as milk can be a consequence of cell structural damage (Bogin et al., 1977). Our findings showed that the mean activities of lactate dehydrogenase (LDH) and alkaline phhosphatase (ALP) were significantly higher in subclinical mastitic milk than in normal milk ($P < 0.01$), but there was no significant difference in the activity of aspartate aminotransferase ($P > 0.05$). Similar results have been reported previously (Bogin and Ziv, 1973; Bogin et al., 1977; Batavani et al., 2003; Babaei et al., 2007). LDH activity in milk has been considered as a sensitive indicator of changing mammary gland function due to disease and the ALP activities test was reliable in the early diagnosis of subclinical mastitis (Symons and Wright, 1974; Babaei et al., 2007).

In conclusion, the present study indicated that: (1) CMT has a certain value for diagnosing subclinical mastitis in subtropical dairy cows; (2) *S. aureus* and coagulase negative staphylococci are predominant causes of SCM in the commercial dairy farm and; (3) the measurement of milk MDA level and GPx, LDH and ALP activities appear to be suitable diagnostic methods for identifying subclinical mastitis in dairy cows.

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