Lactoperoxidase activity in milk is correlated with somatic cell count in dairy cows

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ABSTRACT

Lactoperoxidase (LPO) is a milk protein with antimicrobial function. The present study was undertaken to examine the correlation between LPO activity and somatic cell count (SCC) in milk to use LPO activity as an indicator of mastitis. Composite milk of 36 cows and quarter milk of 3 cows were collected once per week from 0 to 300 d postpartum and twice per day for 1 wk, respectively. For the measurement of LPO activity, milk was mixed with tetramethylbenzidine solution and incubated at 37°C for 30 min, followed by the measurement of optical density. When only milk with low SCC ($132 \pm 12 \times 10^3$ cells/mL) was used, a significant decrease in LPO activity was detected in primiparous cows from 0 to 4 mo postpartum. Lactoperoxidase activities of primiparous cows in mo 1, 2, and 3 postpartum were significantly higher than those in multiparous cows. When composite milk was divided based on LPO activity, the SCC was significantly higher in the groups with LPO activity >5 and from 3 to 3.9 U/mL in the second- and fourth-parity cows, respectively, compared with the group with LPO activity <2 U/mL. Extremely high SCC were found in the $\geq$ fifth-parity cows, even in low-LPO activity groups. In the case of quarter milk, higher LPO activity was associated with increased SCC in all 3 cows. The percentage of quarter milk samples with high SCC ($4,062 \pm 415 \times 10^3$ cells/mL) increased with an increase in the LPO activity. The percentage of quarter milk samples with high SCC was 50.0 to 100% in the milk with LPO activity ≥ 5 U/mL. These results indicate that the correlation of LPO activity to the SCC in bovine milk may point to the potential use of the former as an indicator of SCC.

Key words: lactoperoxidase, milk, bovine, somatic cell count

INTRODUCTION

Mastitis, the most significant infectious disease of dairy cows, results from the inflammation of the mammary gland and causes huge economic losses due to decreases in milk production, decreases in milk quality, and high treatment costs. The SCC in the milk is the most reliable and useful indicator of mastitis worldwide (Sheldrake and Hoare, 1981). Because SCC consists primarily of neutrophil and mammary epithelium and other leucocytes such as T and B lymphocytes and macrophages, a high SCC usually indicates inflammation of the mammary gland. The California mastitis test is an alternative method that indicates mastitis by detecting elevated SCC (Barnum and Newbould, 1961). This is simple and usable even in the field but yields somewhat subjective results.

Lactoperoxidase (LPO) is an innate immune factor, which is synthesized and released from alveolar epithelial cells (Harada et al., 1973) and neutrophils (Moldoveanu et al., 1982). Lactoperoxidase activity in milk was upregulated by LPS inoculation into the mammary gland of cows (Isobe et al., 2009b). Peroxidase in rat tracheal glands was reported to be increased as a result of exposing the rats to microorganisms (Kinbara et al., 1992).

In contrast, LPO appears to be constitutively present in milk (Marshall et al., 1986; Fonteh et al., 2002; Isobe et al., 2009b). However, little is known about factors regulating its expression and activity (Fragoso et al., 2009). Milk LPO catalyzes, in the presence of hydrogen peroxide, the oxidation of thiocyanate (SCN^-) to yield hypothiocyanite (OSCN^-) and hypothiocyanous acid (HOSCN ; Shin et al., 2001) and the oxidation of I^- to yield hypoiodite (OI^-) and hypoiodous acid (HOI ; Bosch et al., 2000). These compounds react with microbial sulfhydryl groups to inhibit various cellular functions (Shin et al., 2001). According to Marshall et al. (1986), LPO activity was increased from 1.44 U/mL at 14 d before drying-off to 3.28 U/mL at 7 d after drying-off but decreased to 0.87 U/mL within 18 d after calving. Fonteh et al. (2002) reported that LPO activity exhibited a cyclic pattern with alternating peaks and troughs throughout lactation and that

Received December 27, 2010.

Accepted April 13, 2011.

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extremely large variations were observed between and within cows. However no precise investigation has been done on the possibility of milk LPO as an indicator of mastitis. Cooray (1994) reported bovine myeloperoxidase as an indicator of mastitis in dairy cows. However, his method involved ELISA, which is complicated and time consuming.

The present study was undertaken to examine the relationship between LPO and SCC to evaluate its utility as an indicator of mastitis.

MATERIALS AND METHODS

Animals and Milk Sampling

Holstein-Friesian dairy cows ($n = 36$, 11 primiparous and 25 multiparous) were used. The ages of these cows were from 2 to 7 yr and parities were from 1 to 6. The cows were held at the experimental farm of Hiroshima University in accordance with the regulations of Hiroshima University for animal experiments. Cows were milked twice per day (at 0900 and 1600 h). Composite milk samples were collected once per week from 36 cows, 0 to 300 d postpartum. Quarter foremilk samples were collected by hand twice per day for 1 wk from 3 cows that each had at least one mastitic quarter. After collecting foremilk samples, the remaining milk was removed by milking machine. Milk samples collected were immediately spread on a glass slide for SCC determination and the remaining milk stored at -20°C until measurement of LPO activity.

Measurement of Lactoperoxidase Activity and SCC

A preliminary experiment was carried out to confirm whether the majority of the peroxidase in milk is LPO, using the method described by Wijkstrom-Frei et al. (2003). Dimethyl sulfoxide (23.4 μL) with and without 0.05 *M* dapsone, which is a specific inhibitor of LPO, was added to 10 μL of milk (SCC ranged from 2×10^4 to 6×10^5 cells/mL) or LPO standard solutions (5 and 2.5 U/mL). One hundred microliters of tetramethylbenzidine (TMB) was further added, followed by incubation at 37°C for 30 min. After centrifuging (6,000 $\times g$, 1 min), absorbance was measured at 655 nm. The peroxidase activity of milk and LPO standards with dapsone decreased 72 to 91% compared with those without dapsone, indicating that the majority of the peroxidase in milk is identical to lactoperoxidase.

Milk LPO activity was determined as described by Isobe et al. (2009b). Ten microliter of defatted milk was mixed with 100 μL of TMB solution and incubated at 37°C for 30 min. The optical density was measured as described above. Lactoperoxidase (Sigma, St. Louis,

MO) at a concentration of 0 to 10 U/mL was used as a standard (1 unit formed 1 mg of purpurogallin from pyrogallol in 20 s at pH 6.0 at 20°C). The SCC was measured using Breed's method (Prescott and Breed, 1910). Briefly, 2.5 μL of milk was spread in the square (1 cm^2) on the slide glass and dried. After treatment with xylene and methanol, Giemsa staining was conducted. Cells on the slide were counted under a microscope to calculate the SCC. A $\text{SCC} \geq 500 \times 10^3$ cells/mL was considered to be high because this value is widely used for the criterion of mastitis.

Statistical Analysis

One-way ANOVA, followed by the Tukey multiplex analysis was used to compare LPO activities among different months postpartum, different parity, and SCC among different LPO activity groups. The chi-squared test was used to compare the percentage of high-SCC milk in each LPO activity group. Correlation analysis was carried out using Spearman correlation analysis between LPO activity and SCC. Probability <0.05 was considered to be significant.

RESULTS

Changes in LPO activity in the milk of dairy cows with different parities are shown in Figure 1. Because LPO activity depends on SCC, only milk with SCC of $<500 \times 10^3$ cells/mL ($132 \pm 12 \times 10^3$ cells/mL) were used. A significant decrease in LPO activity was detected in primiparous cows, from 0 to 4 mo postpartum. Activities of milk LPO at 0 to 2 mo were also significantly higher than those at 6 to 7 and 9 to 10 mo in the second parity and $>$ third parity, respectively. Primiparous cows had significantly higher LPO activities in mo 1, 2, and 3 postpartum than those in other parities. No significant difference among parities was detected after the sixth month postpartum.

A significant correlation was found between LPO activity and SCC in all parities (Table 1). Significantly high correlation coefficients were observed in mo 10 to 12 postpartum in primiparous cows, and mo 4 to 6 and 10 to 12 postpartum in $>$ third-parity cows.

When composite milk was divided based on the LPO activity, mean SCC were compared among different LPO activity groups (Figure 2). In the second-parity cows, SCC was significantly greater in the highest LPO activity group (≥ 5 U/mL) than in other groups. Similarly, fourth-parity cows had higher SCC in the group with LPO activity from 3 to 3.9 U/mL than the lowest LPO activity group (<2 U/mL). Extremely high SCC were found in the $>$ fifth-parity cows, even among low-LPO activity groups (<2 , 2 to 2.9, and 3 to 3.9

Table 1. Correlation between lactoperoxidase activity and SCC of bovine composite milk¹

Postpartum month	Parity (no. of cows)		
	1 (11)	2 (11)	≥3 (15)
0-2	-0.06	0.41**	0.45**
2-4	-0.01	0.12	0.04
4-6	-0.10	-0.02	0.54**
6-8	0.06	0.17	0.19
8-10	-0.04	0.30*	0.26*
10-12	0.66**	0.23	0.55**
Total	0.11*	0.22**	0.23**

¹Values are correlation coefficients.* $P < 0.05$; ** $P < 0.01$.

U/mL). Composite milk may consist of mixed-quarter milk with high and low SCC. To clarify the relationship between LPO activity and SCC, quarter milk was used and results are shown in Figure 3. The higher LPO activity was associated with increased SCC levels in

all cows, with significant levels in cow no. 9 and in the total.

The percentage of composite milk with high SCC ($\geq 500 \times 10^3$ cells/mL) was calculated in each LPO activity group and the results are shown in Table 2. In second- to fourth-parity cows, milk samples with higher LPO activity tended to have a higher percentage with high SCC. In first-parity cows, milk samples with LPO activity ≥ 5 U/mL had a significantly higher percentage with high SCC than did milk samples with LPO activity of 3 to 4.9 U/mL. A dramatically high percentage of milk with high SCC was observed even with low LPO activity (< 2.9 U/mL) in $\geq$ fifth-parity cows.

The same calculation was conducted using quarter milk samples and the results are shown in Table 3. In all cows, the percentage of high-SCC quarter milk increased with an increase in LPO activity. When milk LPO activity was ≥ 5 U/mL, percentages ranged from 50.0 to 100% and were significantly higher than those at LPO activity < 3.9 U/mL.

Figure 1. Changes in lactoperoxidase activity in postpartum dairy cows with different parities (first parity: circle; second parity: square; third or greater parity: triangle). One milk sample was used from one cow in each month. Values are expressed as mean $\pm$ standard error of the means ($n = 11, 11,$ and 15 for first, second, and third or greater parity, respectively). Differences between values with different letters within parity (a-e) and within month (X,Y) are significant ($P < 0.05$).

Figure 2. Somatic cell count ($\times 10^3$ cells/mL) in composite milk samples of dairy cows having different lactoperoxidase activities. Values are expressed as mean $\pm$ standard error of the means ($n = 11, 11, 7, 4$, and 4 for first, second, third, fourth, and fifth parity, respectively). Differences between values with different letters within parity (a,b) are significant ($P < 0.05$).

DISCUSSION

Lactoperoxidase appears to be constitutively present in milk (Marshall et al., 1986; Fonteh et al., 2002; Isobe et al., 2009b), although LPO activity in milk was up-regulated by the LPS inoculation into mammary glands of cows (Isobe et al., 2009b). However, little is known about factors regulating its expression and activity (Fragoso et al., 2009). In the present study, the activity of LPO was decreased significantly in primiparous cows, from 0 to 4 mo postpartum. Activities of milk LPO in 0 to 2 mo were significantly higher than those in mo 6 to 7 and 9 to 10 in the second parity and $\geq$ third parity, respectively. This result indicates that LPO is produced in large amounts in milk in the early stages of lactation, especially in primiparous cows. Fonteh et al. (2002) also reported that some cows showed high LPO activity postpartum, followed by a decrease during lactation. Lingual antimicrobial peptide, secreted from the alveolar endothelium (Isobe et al., 2009a) and found in milk (Isobe et al., 2009c), also showed high concentration for 1 wk postpartum (our unpublished data). Thus, milk at the early postpartum stage seems to contain a large amount of many antimicrobial components, including LPO. These components may be important

for the maternal passive immunity transfer to neonates through the milk, which leads to the establishment of an appropriate immune system in the calf.

Milk LPO activities of primiparous cows at 1, 2, and 3 mo postpartum were significantly higher than those in any other parities. Neutrophils and macrophages in the milk have the potential to produce reactive oxygen species, such as hydrogen peroxide, to eventually kill bacteria (Weber et al., 1983). Hydrogen peroxide is essential for the production of antibacterial components such as OSCN⁻ and HOSCN by LPO. Neutrophils in primiparous cows are more viable than those of multiparous cows (Mehrzaad et al., 2002). Therefore, higher LPO activity in primiparous cows in the present study may be associated primarily with the production of hydrogen peroxide from neutrophils.

The relationship between LPO activity and SCC in composite milk was examined. During 0 to 2 mo postpartum, significantly positive correlations were shown in the second- and $\geq$ third-parity cows but not in primiparous cows. The reason why correlation was poor at 0 to 2 mo in primiparous cows is possibly attributable to the extremely high LPO at this stage.

To examine the relationship between LPO activity and SCC in a different way, composite milk was divided

Table 2. Percentage of cows with high SCC in each lactoperoxidase activity group of composite milk¹

Parity	Lactoperoxidase activity (U/mL)				
	<2	2–2.9	3–3.9	4–4.9	≥5
First	33.3 ^{ab} 1/3	22.2 ^{ab} 2/9	11.5 ^a 3/26	14.7 ^a 5/34	36.7 ^b 11/30
Second	4.3 ^a 1/23	10.9 ^{ac} 5/46	25.0 ^{bc} 15/60	22.9 ^{ab} 8/35	46.2 ^b 12/26
Third	0.0 ^a 0/6	26.3 ^{ac} 10/38	17.8 ^a 8/45	20.0 ^{ac} 2/10	100 ^{bc} 1/1
Fourth	25.8 ^a 8/31	25.0 ^{ac} 3/12	63.6 ^{bc} 7/11	40.0 ^{ab} 4/10	80.0 ^b 4/5
≥Fifth	76.5 ^a 13/17	70.0 ^a 14/20	33.3 ^{ab} 2/6	21.4 ^b 3/14	50.0 ^{ab} 1/2

^{a-c}Values with different superscripts within a row are significantly different ($P < 0.05$).
¹Upper values represent the percentage of composite milk samples with high SCC ($\geq 500 \times 10^3$ cells/mL). Lower values represent the number of composite milk samples with high SCC/total number of composite milk samples.

based on the LPO activity and averages of SCC were compared among different LPO activity groups. In the second-parity cows, SCC was significantly higher in the highest LPO activity group than in other groups. Fourth-parity cows had a higher SCC in the group with LPO activity of 3 to 3.9 U/mL than the lowest LPO activity group. Excluding those cows of $\geq$ fifth parity, high LPO activity in the milk seems to be reflected

in the high SCC values. When using quarter milk, a higher LPO activity resulted in higher SCC in all cows. These results strongly suggest a correlation between LPO activity and SCC in bovine milk. Furthermore, the percentage of quarter milk with high SCC ($\geq 500 \times 10^3$ cells/mL) in each group of LPO activity was assessed to confirm the possibility that indicative SCC can be expected from LPO activity. In all cows, the percentage

Figure 3. Somatic cell count ($\times 10^3$ cells/mL) in quarter milk samples of 3 dairy cows having different lactoperoxidase activities. Parities of cow no. 4, 9, and 12 are 4, 6, and 3, respectively. Values are expressed as mean $\pm$ standard error of the means. Differences between values with different letters within cow (a–c) are significant ($P < 0.05$).

Table 3. Percentage of quarter milk with high SCC in each lactoperoxidase activity group¹

Cow number	Parity	Lactoperoxidase activity (U/mL)			
		<3	3–3.9	4–4.9	≥5
No. 4	Fourth	0.0 ^a 0/4	0.0 ^a 0/8	16.7 ^a 4/24	50.0 ^b 10/20
No. 9	Sixth	19.4 ^a 6/31	50.0 ^b 7/14	100 ^{bc} 4/4	100 ^c 7/7
No. 12	Third	0.0 ^a 0/5	25.0 ^a 4/16	47.1 ^{ab} 8/17	66.7 ^b 12/18
Total		15.0 ^a 6/40	28.9 ^{ab} 11/38	35.6 ^b 16/45	64.4 ^c 29/45

^{a–c}Values with different superscripts within a row are significantly different ($P < 0.05$).

¹Upper values represent the percentage of quarter milk samples with high SCC ($\geq 500 \times 10^3$ cells/mL). Lower values represent the number of quarter milk samples with high SCC/total number of quarter milk samples.

of quarter milk samples with high SCC increased with an increase in the LPO activity. These results validate the possibility of LPO activity as an indicator of SCC. Lactate dehydrogenase (Wellnitz et al., 2010), L-lactate (Davis et al., 2004), and N-acetyl- β -D-glucosaminidase (NAGase; Obara and Komatsu, 1984) are also known as alternative ways to diagnose mastitis.

To compare the time lag from bacterial invasion to increase of SCC and LPO activity in milk, these changes were examined after infusion of LPS by Isobe et al. (2009b). At 2 h after LPS treatment, SCC increased to $\geq 500 \times 10^3$ cells/mL, although LPO activity was increased significantly at 7 h after LPS treatment compared to before infusion. This indicates that neutrophil chemotaxis preceded the production of LPO. If LPO is used as an indicator of SCC, this time lag between them must be considered.

When the percentages of composite milk with high SCC ($\geq 500 \times 10^3$ cells/mL) in each LPO activity group were compared, milk with LPO activity ≥ 5 U/mL showed a significantly higher percentage of high SCC than did milk with LPO activity < 2 U/mL in second- to fourth-parity cows. Therefore, LPO activity may be used as an indicator of high SCC. When calculation was conducted using quarter milk, a clearer tendency was detected. This result indicates that quarter milk is more suitable for LPO analysis. Moreover, exceptionally high LPO activity was detected in primiparous cows at the early postpartum stage and in high-parity cows, even with LPO activity. This should be considered when using LPO activity as an indicator of SCC. The present method of detecting LPO activity can be completed within 1 hr and is very simple compared with general ELISA, which detects LPO protein. Color intensity determination with the naked eye makes it possible to eliminate the use of special equipment such as spectrophotometers and to perform such tests on farm. Therefore, such significant advantage of the present method may enable better control and monitoring of

the problem of mastitis in the dairy industry. However, further study on bacteriology is required to validate the LPO activity assay as an indicator of mastitis.

ACKNOWLEDGMENTS

We thank Lawrence M. Liao, Graduate School of Biosphere Science, Hiroshima University (Higashi-Hiroshima, Japan) for his critical reading of our manuscript.

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