CONCENTRATION OF SERUM AMYLOID A AND ACTIVITY OF CERULOPLASMIN IN MILK FROM COWS WITH CLINICAL AND SUBCLINICAL MASTITIS

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Abstract

The aim of the study was to determine the concentration of serum amyloid A (SAA) and the activity of ceruloplasmin (Cp) in milk from cows with clinical mastitis of various severities and with subclinical mastitis in aspect of their usefulness for the detection of mastitis in cows. The concentration of SAA was determined using the commercial ELISA kit. The activity of Cp was determined according to the Rice et al., method. The mean concentration of SAA in milk from cows with mastitis ranged from 4.47 to 322.26 µg/mL. The mean Cp concentration in milk from healthy cows was 11.67 (±7.40) µg/mL and was significantly lower (P<0.01) compared to that in milk from cows with the particular forms of mastitis. The activity of Cp in milk from cows with mastitis ranged from 3.00 to 18.83 U/g of protein. Both in clinical and subclinical mastitis the activity of Cp was significantly higher (P<0.001) compared to that of milk from cows with healthy mammary glands (1.20 ±0.42 U/g of protein). The findings revealed that both the SAA concentration and Cp activity were sensitive indicators of inflammatory processes in the udder, even those graded as mild. Their determination in milk may be a reliable and non-invasive diagnostic method to detect mastitis, particularly its subclinical form.

Key words: cows, mastitis, amyloid A, ceruloplasmin, milk.

Mastitis is the most common and most costly disease of dairy cows (40). Substantial financial losses caused by mastitis are associated with a reduced milk yield, lower milk quality, increased costs of veterinary care, health disorders in calves, decreased fertility, and premature culling of cows (26).

Proper diagnosis is essential for the elimination of mastitis. At present, the routine diagnostic methods used, e.g. somatic cell count (SCC) or bacteriological tests, are not sufficiently effective, particularly for subclinical mastitis cases (24, 32). Therefore, alternative, more reliable markers of mammary inflammatory processes are being explored.

In the recent studies concerning new indicators of mastitis, acute phase proteins are strongly implicated (1, 9, 10, 12, 13, 20, 28, 29). Acute phase proteins are the group of blood proteins, whose concentration is affected by such factors as inflammation, tissue injury, or stress (27, 31). Their blood concentration is related to the severity of disease and the extent of tissue injury; therefore, the determination of their concentration is of diagnostic and prognostic value (11, 27). Acute phase proteins are often determined in serum of animals to monitor their health (19, 27, 31).

Serum amyloid A (SAA) belongs to the most important acute phase proteins in cattle (19, 27, 31). During the inflammatory process, its serum concentration can rapidly increase even over 100-fold (8). Although during the acute phase reaction SAA is mainly produced in the liver, its extrahepatic production is also possible (19, 27). Many studies confirmed the production of SAA in the mammary gland (21, 25, 39). The biological role of SAA has not been fully elucidated, but many findings indicate its involvement in the modulation of immune responses during infections (31).

Ceruloplasmin (Cp) belongs to acute phase proteins, whose serum level may increase several dozen times due to infections or other tissue-damaging factors (6). Hepatocytes are the main source of Cp; however, it may also be synthesised at extrahepatic sites, among others in the mammary gland (3, 7, 16) and at sites where tissues are injured (17). Cp, as an extracellular antioxidant, present also in the colostrum and milk, is essential for antioxidative protection of the organism (7, 18).

Numerous studies described the serum levels of acute phase proteins in animals with various diseases, but only a few were devoted to their levels in milk from cows with spontaneous mastitis. Therefore, the present study was undertaken to determine the concentration of
SAA and activity of Cp in milk from cows with clinical mastitis of various severities as well as with subclinical mastitis and to assess their usefulness for the detection of mastitis in cows.

Material and Methods

The study material included milk samples from single udder quarters from 74 cows with mastitis detected on the basis of clinical examination of the udder, TOK\textsuperscript{1} test estimating the SCC, and bacteriological examinations. Clinical mastitis was diagnosed in 49 cows and 26 cows were affected with subclinical form of mastitis. In the group with clinical mastitis, acute mastitis was observed in 14 cows (elevated internal temperature, oedema, reddening, sore udder, changes in milk, positive bacteriological culture). In 18 cows, the inflammation was mild (slight oedema of the quarter, changes in milk, positive bacteriological culture). In 16 cows, clinical mastitis was chronic (long-term ineffective treatment, persistent hardening of the gland tissue, changes in milk, positive bacteriological culture). Subclinical mastitis was diagnosed on the basis of the increase of SCC (TOK positive). The age of cows with mastitis ranged from 2 to 10 years. They were between 15 and 200 postpartum day. Additionally, the study included 14 samples of milk from healthy cows aged 2-6 years. They were in lactation days 20 to 180. All cows belonged to three breeds: Holstein-Friesian (HF), Polish black-white (PBW), and PBW x HF.

Cysternal milk, about 10 ml, was aseptically sampled to sterile tubes. Having been used for bacteriological tests, milk samples were frozen to –76°C and stored for further examinations.

Bacteriological testing. The bacteriological examinations of milk samples were carried out according to commonly accepted methods (23).

Determination of SAA. The concentration of SAA was determined using the commercial ELISA kit (Tridelta Development Ltd., Ireland). Prior to testing, milk samples were diluted 1:50. The sensitivity of the assay was 0.10 µg/mL. The results were expressed in µg/mL.

Determination of Cp. The activity of Cp was determined according to the Rice et al. (34) method. The reaction mixture contained 1 ml of acetate buffer (300 mmol/L, pH 5.7) and 1 ml of p-phenylenediamine (0.2%). After 5 min of incubation at 37°C, 100 µl of milk was added and the mixture was incubated for 30 min at 37°C. The enzymatic reaction was stopped by adding 2 ml of sodium azide (0.04%). After 10 min of incubation at 20°C, the absorbance was measured at 540 nm. The control sample contained the same components but the first 2 ml of sodium azide was added following 100 µl of milk after 30 min incubation. The calculations were made according to the formula: U/L = (absorbance of the examined sample – absorbance of the control sample) x 137. The results were expressed in units per gram protein of milk (U/g protein).

Protein determination. The protein content in milk was determined using the commercial kit (Total Protein Kit, Cormay).

Statistical analysis. The results were statistically analysed defining the arithmetic mean and standard deviation (±SD). The inter-group differences were analysed using the Student’s t-test at P<0.01 and P<0.001.

Results

The microorganisms were found in all samples of milk from cows with mastitis (Table 1). The bacteria isolated from milk of cows with acute mastitis included

\textit{Str. agalactiae} (1), \textit{S. aureus} (10), and \textit{E. coli} (3). In mild clinical cases, \textit{Str. dysgalactiae} (8), \textit{Str. uberis} (5), \textit{S. aureus} (2), coagulase-negative staphylococci (CNS) (1), and \textit{E. coli} (2) were identified. In cows with chronic clinical mastitis, \textit{Str. dysgalactiae} (6), \textit{Str. uberis} (2), and \textit{Candida sp.} (8) were detected whereas in those with subclinical mastitis, \textit{Str. agalactiae} (9), \textit{Str. dysgalactiae} (4), \textit{Str. uberis} (8), and CNS (5) were isolated. No microorganisms were demonstrated in milk from healthy cows.

The concentration of SAA in milk from cows with mastitis ranged from 4.47 to 322.26 µg/mL (Table 2). The comparison of mean values of SAA concentration in milk from mastitis affected cows, revealed differences depending on the severity of the inflammatory process. The highest mean SAA concentration was found in milk from cows with chronic mastitis (97.37 ±90.0 µg/mL), whereas the lowest one in milk from cows with subclinical mastitis (74.05 ±73.94 µg/mL). The differences in mean SAA concentrations between groups of cows with various forms of mastitis were not statistically significant.

\begin{table}[h]
\centering
\caption{Microorganisms isolated from milk of cows with mastitis}
\begin{tabular}{|l|c|c|c|c|}
\hline
Microorganisms & Acute mastitis & Mild mastitis & Chronic mastitis & Subclinical mastitis \\
\hline
\textit{Str. agalactiae} & 1 & - & - & 9 \\
\textit{Str. dysgalactiae} & - & 8 & 6 & 4 \\
\textit{Str. uberis} & - & 5 & 2 & 8 \\
\textit{S. aureus} & 10 & 2 & - & - \\
CNS & - & 1 & - & 5 \\
\textit{E. coli} & 3 & 2 & - & - \\
\textit{Candida sp.} & - & - & - & 8 \\
\hline
\end{tabular}
\end{table}

\textsuperscript{1}TOK test – the equivalent of California Mastitis Test
The mean SAA concentration in milk from healthy cows was 11.67 (±7.40) µg/mL and was significantly lower (P<0.01) compared to that in milk from cows with the different forms of mastitis.

The activity of Cp in milk from cows with mastitis ranged from 3.00 to 18.83 U/g of protein. No statistically significant mastitis severity-related differences in the Cp activity were found. The highest mean activity was observed in chronic mastitis cases (6.00 ±3.65 U/g of protein). Both in clinical and subclinical cases, the activity of Cp was significantly higher (P<0.001) compared to that in milk from cows with healthy mammary glands (1.20 ±0.42 U/g of protein).

### Discussion

Acute phase proteins are considered biological markers of the inflammatory reaction in mammals, including cattle (19, 27, 31). During inflammation, they are mainly synthesised in hepatocytes and their production is activated by the complex cascade of inflammation mediators, including cytokines (14). The present study demonstrated low values of SAA and ceruloplasmin in milk from healthy cows and their substantially increased levels in milk from mastitis cows. The results obtained indicate the activation of acute phase reaction in cows with mastitis and are consistent with the findings reported by other authors (1, 10, 13, 20, 29, 35).

Besides haptoglobin, SAA is the acute phase protein most commonly determined in cows to monitor their health (19, 31). Its level was found to increase rapidly both in serum and milk protein during experimental udder infection with *E. coli* (15), *S. aureus* (12), and *Staphylococcus uberis* (29), and intra-mammary infusion of endotoxin (LPS) from the *E. coli* cell wall (22). Increased SAA levels were demonstrated to occur much earlier in milk than in serum and preceded increased somatic cell count (30). According to Nielsen *et al.* (28) the concentration of SAA was higher in infected than uninfected quarters of the same udder, and the inflammatory processes outside the udder, which increased serum levels of SAA, did not cause substantial increases in its concentration in milk. Once the inflammation subsided, the SAA concentration quickly returned to the baseline value (15). The above-mentioned results demonstrate that an increase in SAA concentration in milk may be an objective marker of mastitis.

According to some authors, the concentration of SAA in milk was positively correlated with the severity of mastitis (9, 15), that is inconsistent with the findings presented by Lehtolainen *et al.* (22) who found the highest SAA concentration in milk and serum only once acute symptoms of mastitis induced by intra-mammary LPS infusion subsided. This, according to them, confirms the hypothesis that SAA is involved in the tissue repairing processes and inhibition of the inflammatory reaction. Our study did not show any significant differences in acute phase protein levels in various forms of mastitis. On the other hand, the highest SAA concentration and ceruloplasmin activity were observed in milk from cows with chronic mastitis, which is likely to imply that the long-lasting inflammatory process resulted in most extensive damage to the udder tissues. It should be stressed however, that the parameters examined were measured only once; more objective results are likely to be achieved if their values would be assessed during the entire course of inflammation.

The results reported by Jakobson *et al.* (15) revealed that increased SAA concentration in milk during mastitis resulted from its passage from blood as well as from its local production in the udder. The ability to produce serum amyloid A by mammary epithelial cells was confirmed in numerous studies (21, 25, 39). It was demonstrated that LPS released by *E. coli* and lipotechoic acid from *S. aureus* had stimulating effects on the secretion of SAA by bovine mammary epithelial cells (21, 39). According to some authors, the production of SAA in the udder suggests that SAA plays an important role in local protection of the udder against pathogenic microorganisms (38).

Cp is a ferric oxidase involved in the antioxidative defence of the organism (27). It scavenges free radicals, binds copper ions and oxidises ferric ions to ferrous ions, which enables these ions to participate in the reactions generating the reactive oxygen species (ROS) (18, 37). Cp is mainly synthesised in the liver, but may also be produced extrahepatically (27). Numerous studies demonstrated that Cp was produced in the mammary gland (3, 7, 16). The studies in rats revealed that the bulk of Cp present in milk came from the mammary gland; and only its small amounts pass from blood (7). Cp exerts anti-inflammatory effects by

### Table 2

SAA concentration and ceruloplasmin activity in milk from mastitic and healthy cows

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Acute mastitis</th>
<th>Mild mastitis</th>
<th>Chronic mastitis</th>
<th>Subclinical mastitis</th>
<th>Healthy cows</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SAA</strong> (µg/ml)</td>
<td>82.52 ±72.45²</td>
<td>74.21 ±72.23²</td>
<td>97.37 ±90.0²</td>
<td>74.05 ±73.94²</td>
<td>11.67 ±7.40²</td>
</tr>
<tr>
<td><strong>Cp</strong> (U/g of protein)</td>
<td>4.88 ±1.62³³</td>
<td>5.39 ±2.18³³</td>
<td>6.00 ±3.65³³</td>
<td>5.29 ±1.03³³</td>
<td>1.20 ±0.42³³</td>
</tr>
</tbody>
</table>

² - P<0.01; ³³ - P<0.001.
Reducing the adhesion of neutrophils to the endothelial cells and by the removal of free radicals (27). The activity of Cp is less commonly used as the diagnostic factor in cattle compared to other acute phase proteins; nevertheless, its diagnostic value in detecting various inflammatory conditions in cattle has been confirmed (5, 6, 36). Chasagné et al. (5) indicated that the activity of Cp might be a good marker for early detection of clinical mastitis in cows. An increased activity of Cp in blood was observed in cows with spontaneous (6) as well as experimental mastitis (4). Considering its antioxidative properties, higher levels of Cp in milk from cows with mastitis are likely to reflect the activation of defence mechanisms in response to an increased production of ROS at the inflammation site.

Our results demonstrating significantly higher concentrations of SAA and Cp activity in milk from cows with subclinical mastitis compared to that in healthy cows indicate that both proteins are good markers of inflammatory processes in the udder, even mild ones. The usefulness of acute phase protein determinations for the detection of subclinical mastitis in cows is confirmed by other studies (10, 12, 13, 20). This is particularly important as subclinical mastitis is widespread in herds of dairy cows and is difficult to diagnose due to no visible signs of inflammation (33). At present, the studies are carried out to design a simple method for the determination of levels of acute phase proteins in milk, which could be used routinely (2).

In conclusion, the present study revealed that both proteins are sensitive markers of the inflammatory process in cow udders. Their determination in milk may be a reliable and non-invasive diagnostic method to detect mastitis, particularly its subclinical form.

References