TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2022) 46: 304-313

doi:10.3906/vet-2107-25

A comparative study of blood and milk chemiluminescence in healthy cows and with subclinical and clinical *mastitis*

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Received: 09.07.2021 • **Accepted/Published Online:** 24.01.2022 • **Final Version:** 25.04.2022

Abstract: The aim of the study was to evaluate the luminol-dependent spontaneous and stimulated chemiluminescence (CL) of whole blood and milk in cows with subclinical and clinical *mastitis*. The research was carried out on 108 Holstein-Friesian dairy cows aged 3–5 years. Based on the assessment of somatic cell count (SCC) in quarter milk, the animals were divided into 3 groups: I – clinically healthy cows (n = 35), II – animals with subclinical *mastitis* (n = 30) and III – cows with clinical *mastitis* (n = 43). The material for the research was whole blood and quarter milk. The samples were examined for spontaneous CL and stimulated by a Fc receptor binding antibody and complement (zymosan-Z), opsonized zymosan (OZ) and a chemotactic receptor (N-formyl-methionyl-leucyl-phenylalanine - fMLP) as well as nonreceptor stimuli (phorbol myristate acetate - PMA). The conducted studies showed that inflammation of the mammary gland of cows results in an increase in the spontaneous and stimulated chemiluminescence of not only quarter milk but also peripheral blood. It was found that there is a greater correlation between SCC and milk CL indices than there is a correlation between SCC and blood CL. Thus, milk CL determinations better reflect the intensity of the inflammation of the udder, which suggests their usefulness in the early detection of the disease. On the other hand, blood CL tests, especially with the use of PMA as a PMN cell stimulator, can be a useful tool, especially in the diagnosis of subclinical *mastitis*.

Key words: Chemiluminescence, blood, milk, *mastitis*, cows

1. Introduction

Inflammation of the mammary gland (*mastitis*) is one of the most common and costly diseases for dairy farmers [1]. The direct effect of this disease is a decrease in milk yield, a deterioration in milk quality and a potential threat to healthy cows in the herd [2]. Depending on the etiological factor and the efficiency of immune mechanisms, the inflammation may proceed with visible clinical symptoms (*mastitis clinica*) or without these symptoms (*mastitis subclinica*). In properly managed dairy herds, it is extremely important to detect both forms of this inflammation quickly. For this purpose, microbiological tests and the measurement of somatic cell count (SCC) in milk are mainly used. To a lesser extent, mainly in scientific research, other indicators of inflammation are also used, such as N-acetyl-β-D-glucosaminidase (NAG) activity in milk, concentration of plasmin, ATP, lactose in milk, the content of acute phase proteins (haptoglobin, serum amyloid A) in the serum and the electrical conductivity (conductance) of milk [2]. The inflammatory response that occurs during the disease involves a number of specific and nonspecific reactions of the organism, including the increase in the activity of polymorphonuclear cells (PMN). It manifests itself in the so-called "respiratory burst", i.e. increased production of reactive oxygen species (ROS), combined with the emission of light [3–5]. It should be noted that the main purpose of the respiratory burst is not to generate energy, but to generate (already at the stage of phagosome formation) strong bactericidal agents (ROS), resulting from partial oxygen reduction [6]. Since the magnitude of the respiratory burst is proportional to the intensity of the light emission by the activated phagocytes, the chemiluminescence measurements allow an indirect assessment of the oxygen-dependent bactericidal properties of these cells [7,8].

The chemiluminescence (CL) method is increasingly used in research and clinical diagnostics. This is justified, because this relatively cheap method allows one to obtain a number of repeatable results from a small volume of the tested material. The usefulness of CL measurements

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is confirmed by numerous studies that have shown changes in the oxygen metabolism of neutrophils, e.g., in acute bacterial and viral infections, neoplastic diseases and inflammations, regardless of their etiology [9–12]. The discussed method also enables the study of the pathomechanisms of congenital or acquired defects of surface receptors and intracellular enzymes, as well as the assessment of the effect of drugs and preparations supporting the activity of PMN cells [13,14].

Although the use of the CL method to assess the activity of neutrophils via the oxygen-dependent pathway is widely used in the study of many human diseases, the extent of its use in veterinary medicine is relatively limited. It has been used to assess the influence of local inflammation (*endometritis, mastitis*) on the oxygen metabolism of PMN cells in mares [15] and dairy cows [16–19]. However, most studies have either been limited to the spontaneous CL measurements or used only one way to activate PMN cells. The observations were mostly carried out on isolated cells. However, it is believed that the CL determination in whole blood (and milk) better reflects the body's homeostasis and, therefore, is more useful in clinical trials [20]. They do not require any laboratory procedures to isolate PMN cells, which may affect their survival, activity and receptor expression [3,21].

The aim of the study was to evaluate the spontaneous and stimulated chemiluminescence (CL) of whole blood and milk in cows with subclinical and clinical *mastitis*. An interesting issue seems to be the assessment of the practical application of the CL method as a supplementary test for *mastitis* diagnostics.

2. Materials and methods

All animal procedures described were conducted under ethical approval from the Local Ethics Committee for Animal Experimentation at the Faculty of Animal Breeding and Biology, UTP University of Science and Technology in Bydgoszcz (Poland) gave consent to the research (license no. 59/2012). Laboratory procedures were carried out in accordance with the standards of good laboratory practice developed in the National Veterinary Research Institute in Pulawy (Poland).

2.1. Animals

The research was conducted on 108 multiparous Holstein-Friesian dairy cows, aged 3–5 years, coming from 3 free-stand barns. The cows were between 46th and 242nd day of lactation. The animals were divided into 3 groups depending on the intensity of udder inflammation (*mastitis*), estimated on the basis of somatic cell count (SCC) and the presence of pathogens in milk:

- Group I (control): 35 clinically healthy cows without *mastitis* symptoms, SCC in quarter milk up to 100,000/mL, negative microbiological test result (in all quarter milk samples), hematology – reference/normal values,

- Group II: 30 cows with subclinical *mastitis*, SCC in milk 100,000/mL – 400,000/mL (in at least one quarter) and positive microbiological test result in this quarter milk sample,

- Group III: 43 diseased animals with clinical symptoms of *mastitis* (swelling, hardness, redness, udder pain, elevated udder temperature, macroscopic changes in milk, decreased milk yield, lack of appetite, symptoms of diarrhea and dehydration, decreased mobility due to swelling and udder pain, elevated body temperature), SCC >400,000/mL of milk (in at least one quarter) and positive microbiological test in this quarter milk sample, milk without macroscopic changes (e.g., watery appearance or flakes, clots, blood, pus in milk).

The feeding of cows was based on total mixed ration (TMR) doses administered to cows twice a day (8:00 and 16:00) and the basic forage of the ration was corn silage. The animals' health status was confirmed by routine clinical tests performed by a veterinarian as well as field tests (California mastitis test - CMT) and laboratory tests (hematological determinations, SCC measurement in quarter milk, microbiological tests of milk).

2.2. Collection and storage of quarter milk samples

Immediately before collecting the quarter milk, the udder and teats were rinsed with warm water and dried with a disposable towel. The secretion of each quarter was then visually assessed on the premilker. All quarters were dipped in an effective premilking teat disinfectant for at least 30 s and dried thoroughly with an individual towel. Subsequently the teat end was disinfected rubbing with cotton swabs soaked with antiseptic 70% alcohol as often as necessary, until the last swab keeps clean. After disinfecting the teats, preliminary selection of cows using the CMT test was performed in cowshed conditions. Both cows groups in which the CMT test result in each quarter was negative (control) and animals in which the CMT test result was, at least in one quarter, positive, were qualified for the study.

Milk samples (approx. 10 mL) were taken aseptically from all quarters into sterile test tubes and then transported to the laboratory at approx. + 4 °C. Microbiological tests, hematology, SCC assessment and chemiluminescence measurements were performed no later than 2 h after collection.

2.3. Collection and storage of blood samples

After cows were prequalified for further testing on the basis of routine clinical examinations and the result of the CMT test, blood was collected from the external jugular vein using the Vacuette closed vacuum system (Greiner Bio-One GmbH, Kremsmünster, Austria). Each time the blood was collected in 2 plastic test tubes: 4 mL in a test tube with ethylenediaminetetraacetic acid dipotassium salt (K_2EDTA) and 9 mL in a tube with lithium heparin.

In order to limit the influence of the circadian rhythm on the neurohormonal activity of the body, and thus on the number of blood cells and their functions, blood samples were obtained at a constant hour (9:00 ± 30 min). Blood samples were transported to the laboratory at a temperature of about + 4 °C. Hematological and chemiluminescent determinations were performed not later than 2 h after collection.

2.4. Hematological tests of venous blood

Hematological determinations were one of the elements of health assessment, and thus the classification of animals for further study. In venous blood collected in K_2EDTA tubes, the following hematology indices were determined: red blood cell count (RBC), hemoglobin concentration (HGB), hematocrit (HCT), white blood cell count (WBC) and the percentage of granulocytes (GRA), lymphocytes (LYM) and monocytes (MON).

RBC, HGB, HCT and WBC determinations were performed using an ABC Vet veterinary hematology analyzer (Horiba ABX, Montpellier, France). The percentage of granulocytes, lymphocytes and monocytes in the white blood cell image was determined on the basis of microscopic analysis of blood smears stained by the May-Grünwald-Giemsa method.

2.5. Microbiological tests of quarter milk

The microbiological examination of the milk was performed according to routine laboratory procedures [22]. Milk samples (10 µL) were plated on Columbia and McConkey media and incubated at 37 °C. The growth of microorganisms was assessed after 18–24 and 48 h. Bacteria were identified using conventional methods, i.e. colony morphology, Gram staining, lactose degradation, catalase test, coagulase test, hemolysis and other biochemical properties.

Staphylococci were differentiated from streptococci by catalase assay. Catalase-positive strains were plated on mannitol and 7.5% NaCl medium, and then tested for coagulase. *Staphylococcus aureus* was identified on the basis of morphology, type of hemolysis and coagulase test. Catalase-negative strains were plated on esculin medium and the CAMP test was performed. Colonies growing on McConkey's medium that turned pink (lactose degradation) were identified using the API-20E assay (bioMerieux).

Gram-positive, catalase-positive colonies were identified as *Bacillus sp*. or *Corynebacterium sp*. based on the appearance of the colony.

The basis for the identification of *Trueperella pyogenes* were the morphological features of the colony, Gram staining and biochemical tests (API-Coryne, bioMerieux).

Identification of *Candida sp.* and *Prototheca sp*. was performed based on the cultivation on Sabouraud medium and microscopic evaluation of the preparations.

Samples were classified positive when at least 5 identical *Streptococcus sp*. colonies and 10 CNS (coagulase-negative staphylococci) morphologically similar colonies were grown with 10 µL of milk. The contamination was considered to be the growth of 3 or more different colonies with no major *mastitis* pathogens among them.

2.6. Determination of somatic cell count (SCC) in quarter milk

Precise measurement of SCC was performed in the laboratory by flow cytometry method using a somatic cell counter equipped with an argon laser (Somacount 150, Bentley Instruments, Chaska, MN, USA). About 5 mL of the milk was brought to a temperature of 40.0 ± 1 °C in a water bath, mixed thoroughly and the number of somatic cells was determined.

2.7. Measurement of chemiluminescence in venous blood and milk

Tests in whole blood (collected in lithium heparin tubes) and in quarter milk were carried out using luminol-enhanced chemiluminescence (CL) (5-amino-2,3-dihydrophthalazine-1,4-dione; Fluka Chemie GmbH, Buchs, Switzerland) dissolved in 0.4% sodium hydroxide (NaOH) solution to a concentration of 28 mM (basic solution). The final concentration of the CL enhancers in the mixture was 0.3 mM. The assessment was performed using a microplate luminometer (Luminoskan Ascent, Type 392, ThermoLabsystems, Helsinki, Finland) on 96-well microplates (White Cliniplates, Thermo Fischer Scientific Oy, Vantaa, Finland). The kinetic method was used in the assay measuring CL for 60 min at 38.0 °C. CL registration time in each well was 1000 ms at 2-min intervals. Before each measurement, the plate was shaken for 10 s (orbital shaking 300 rpm, diameter of the orbital movement 7 mm). The results were presented as a value of CL integration, which is the area under the curve of light emission as a function of time measured for 60 min in relative light units (RLU). These values were calculated by rectangle method using the kinetic processor function of Ascent Software Version 2.6 (ThermoLabsystems Oy, Helsinki, Finland).

In blood and milk samples, spontaneous luminol-dependent chemiluminescence (without stimulation - CL-WS) and stimulated by the activation of the receptor for the Fc fragment – antibody and complement (CL-Z and CL-OZ), the chemotactic receptor (CL-fMLP), as well as the extra-receptor way (CL-PMA).

The following CL stimulators were used in the study:

· Zymosan (Z): 300 mg zymosan (Sigma Aldrich, Cat. No. Z4520) was suspended in 30 mL phosphate-buffered saline (PBS) to give a 10 mg/mL suspension.

· Opsonized zymosan (OZ): 100 mg of zymosan (Sigma Aldrich, Cat. No. Z4520) was suspended in a mixture of PBS solution (10 mL) and bovine pool plasma

(10 mL), and then incubated for 30 min at 38.0 ± 0.1 °C. After centrifugation (10 min with relative centrifugal force 700 rcf) and washing, the supernatant was decanted and the pellet was resuspended in 10 mL PBS. Centrifugation and washing were repeated 3 times, then the pellet was resuspended in PBS and frozen at –82.0 ± 0.1 °C in tubes. The required volume was thawed for the tests.

· N-formyl-methionyl-leucyl-phenylalanine (fMLP): 5 mg of fMLP (Sigma Aldrich, Cat. No. F3506) dissolved in 5 mL dimethyl sulfoxide (DMSO; Sigma Aldrich, Cat. No. D2650) and diluted in PBS solution to a concentration of 10 µg/mL. The suspension was frozen at –82.0 ± 0.1 °C in tubes. For the tests, the required volume was thawed and, depending on the needs, additionally diluted in PBS.

· Phorbol myristate acetate (PMA): 5 mg of PMA (Sigma Aldrich Cat. No. P8139) was dissolved in 5 mL of ethanol 96% and then diluted in PBS to a concentration of 10 µg/mL). The suspension was frozen at –82.0 ± 0.1 °C in tubes. For the tests, the required volume was thawed and, depending on the needs, additionally diluted in PBS.

The volume of whole blood or milk sample added was 150 µL, while the total volume of the reaction mixture was 450 µL. PBS with pH = 7.4 ± 0.2 was used as buffer.

The tests started immediately after adding blood/milk to the previously prepared reagents (PBS or PBS + stimulator) and transferring 300 µL of the prepared reaction mixture to the microplate well. Each sample was evaluated twice during one test, and the obtained results were used to calculate the arithmetic mean.

2.8. Statistical analysis

The obtained results were presented in the form of an arithmetic mean with standard deviation (SD). Statistical analysis was performed using the nonparametric ANOVA rank Kruskal–Wallis test after checking the assumptions of the parametric test. These assumptions were checked using the Shapiro–Wilk test (normality of distribution) and Levene's test (homogeneity of variance). As the assumptions of the parametric tests were not met in all the studied groups, the nonparametric Kruskal–Wallis test was used. Due to the fact that the CL values of the studied variables observed in group III were significantly different from the CL values in groups I and II, the Mann–Whitney U test (with corrections for continuity) was performed to emphasize the differences within groups I and II. In all analyzes, the significance level was set at $p < 0.05$. The relationship between variables was assessed using Pearson correlation coefficient (r). For the analysis of statistical parameters, Statistica 12PL was used (StatSoft Inc., 2014).

3. Results

The basic criterion for the classification of the tested animals into particular groups (I, II and III) was the somatic cell count (SCC) in the quarter milk. The mean values of this index were as follows: I - 32,000 ± 17,000; II - 185,000 ± 42,000 and III - 3,159,000 ± 1,159,000 cells/mL of quarter milk. The performed statistical analysis showed the significance of differences between all the examined groups of cows.

The results of hematological indices (WBC, RBC, HGB, HCT, GRA, MON, LYM) of the tested animals are presented in Table 1. Their analysis showed a significantly higher mean number of white blood cells (WBC) in the cows from group II compared to animals from group III. However, no statistically significant differences were found between the studied groups of animals in terms of other blood parameters.

Table 2 presents the results of microbiological tests of quarter milk of the tested cows, taking into account the type of microorganisms present. In group I (control), no microorganisms were found, which corresponds to

Table 1. Mean values of the determined hematological indices in the studied groups of cows (mean ± SD).

Group of cows	WBC (G/l)	RBC (T/l)	HGB (mmol/l)	HCT (l/l)	GRA (%)	MON (%)	LYM (%)
I (n = 35)	[ab] 7.6 ± 1.1	6.2 ± 0.8	6.8 ± 0.5	0.32 ± 0.1	45.4 ± 7.2	5.0 ± 0.9	49.6 ± 7.3
II (n = 30)	[a] 8.1 ± 1.1	6.1 ± 0.8	6.7 ± 0.4	0.33 ± 0.1	50.1 ± 9.6	4.9 ± 0.7	45.0 ± 9.7
III (n = 43)	[b] 6.0 ± 2.5	6.2 ± 0.5	6.8 ± 0.6	0.31 ± 0.1	44.9 ± 15.7	5.1 ± 1.4	50.0 ± 16.9

Explanations: n - number of animals; I, II, III - test groups of animals; WBC - white blood cell count; RBC - red blood cell count; HGB - hemoglobin; HCT - hematocrit; GRA - granulocytes; MON - monocytes; LYM - lymphocytes; [a, b,] - means marked with different letters differ significantly at $p < 0.05$.

Table 2. Number of individual microorganisms in the quarter milk of the tested cows.

Pathogens	Group of cows		
	I (n = 35)	II (n = 30)	III (n = 43)
Staphylococcus aureus	0	10	8
Coagulase-negative staphylococci (CNS)	0	12	6
Streptococcus uberis	0	3	7
Streptococcus agalactiae	0	2	2
Streptococcus dysgalactiae	0	3	3
Escherichia coli	0	0	15
Klebsiella pneumoniae	0	0	3
Pseudomonas aeruginosa	0	0	2
Enterobacter sp.	0	0	1
Trueperella pyogenes	0	0	1
Candida sp.	0	0	1
Prothoteca sp.	0	0	1

Explanations: n, I, II, III - as in Table 1.
In group III, the greater number of microorganisms found than the number of animals results from a greater number of infected quarters in a single animal.

the clinical condition of these animals. The presence of pathogens, typical in the course of *mastitis*, was found in the quarter milk of diseased cows (groups II and III). Five different microorganisms were found in group II individuals, with the dominant coagulase-negative staphylococci (CNS) and *Staphylococcus aureus*. In turn, in cows with clinical signs of *mastitis* (group III), the presence of 12 different microorganisms was recorded, with the highest share of *Escherichia coli*.

The mean values of CL measurements in whole blood of the studied groups of animals are presented in Table 3. The highest mean values of all examined parameters (CL-WS, CL-Z, CL-OZ, CL-fMLP, CL-PMA) were found in cows with clinical symptoms of the disease (group III). In comparison with the results recorded in healthy animals (group I), these differences were statistically significant. However, apart from CL-PMA, no significant differences in the examined indicators were recorded between cows from groups I and II. This result indicates the greatest usefulness of CL-PMA determinations in blood as an indicator of ongoing inflammation in animals with subclinical *mastitis* (group II).

In turn, Table 4 presents the results of CL measurements in the quarter milk of the tested cows. They indicate, as in the case of whole blood tests, the highest values of all the tested indicators in animals of group III. The statistical analysis showed that they differed significantly from the results recorded in animals from groups I and II. The lowest values of CL indices were found in milk of healthy cows (group I), and they differed significantly from the results of diseased animals (group II) in terms of CL-Z, CL-OZ and CL-PMA.

The results of the analysis of the correlation between the somatic cell count (SCC) in milk and CL results of blood and milk of the examined cows are presented in Table 5. They show much higher values of correlation coefficients (r) in the case of measurements made in quarter milk. The positive correlation between SCC and all CL results was statistically confirmed at the level of $p < 0.0001$. In turn, in the case of CL results recorded in the blood, this relationship was statistically confirmed only in the case of CL-Z, CL-OZ and CL-PMA.

4. Discussion

In the presented studies, the mean values of hematological indices of healthy and diseased cows (with subclinical and clinical *mastitis*) of both groups were within the ranges of physiological values for cattle [23]. In general, they were characterized by relatively little differentiation. As other authors' studies show, significant changes in the value of cow blood morphology indices depend mainly on the type of pathogen that causes *mastitis* and on the intensity of inflammation [24]. Infections of the udder with gram-positive bacteria increase the total number of white blood cells (WBC) and the percentage of PMN cells. In turn, inflammation caused by gram-negative bacteria (often acute) causes a decrease in WBC in the peripheral blood [24–26]. This seems to be confirmed by the results of own research, showing the highest mean values of WBC and

Table 3. Influence of the intensity of local inflammation on the chemiluminescence (CL) values in the whole blood of the tested cows (mean ± SD).

Group of cows	CL-WS	CL-Z	CL-OZ	CL-fMLP	CL-PMA
I (n = 35)	[a] 4.46 ± 2.20	[a] 68.88 ± 29.76	[a] 71.73 ± 29.68	[a] 5.84 ± 2.59	[a] 41.19 ± 18.23
II (n = 30)	[ab] 6.41 ± 4.56	[a] 66.25 ± 33.79	[a] 67.77 ± 35.61	[ab] 6.59 ± 3.73	[b] 94.59 ± 61.84
III (n = 43)	[b] 7.97 ± 6.50	[b] 129.06 ± 74.09	[b] 135.53 ± 81.22	[b] 12.59 ± 13.54	[b] 99.62 ± 63.60

Explanations: CL-WS - spontaneous CL; CL-Z - CL stimulated with zymosan; CL-OZ - CL stimulated with opsonized zymosan; CL-fMLP - CL stimulated with N-formyl-methionyl-leucyl-phenylalanine; CL-PMA - CL stimulated with phorbol myristate acetate; n, I, II, III, [a, b,] - as in Table 1.

Table 4. Influence of the intensity of local inflammation on the chemiluminescence (CL) results in the quarter milk of the studied cows (mean ± SD).

Group of cows	CL-WS	CL-Z	CL-OZ	CL-fMLP	CL-PMA
I (n = 35)	[a] 15.12 ± 3.84	[a] 22.20 ± 13.65	[a] 22.47 ± 14.44	[a] 16.99 ± 4.08	[a] 16.73 ± 3.68
II (n = 30)	[a] 19.92 ± 6.53	[b] 67.27 ± 22.82	[b] 68.97 ± 23.40	[a] 21.88 ± 8.98	[b] 22.84 ± 5.87
III (n = 43)	[b] 1120.70 ± 1514.53	[c] 4395.20 ± 43160.5	[c] 3729.06 ± 4054.18	[b] 1111.08 ± 1451.52	[c] 1399.98 ± 1770.38

Explanations: as in Table 3.
The number of tested samples of quarter milk in the following groups was: I - 140, II - 30, III - 50.

GRA in animals from group II (subclinical *mastitis*), where gram-positive bacteria turned out to be a 100% pathogenic factor, and the lowest values of these parameters in cows from group III (clinical *mastitis*), where both gram-positive and gram-negative bacteria were found to be the main pathogen. Although the differences in the values of the discussed indices between the animals of groups II and III were not very large, it seems that it could be caused by the presence of various pathogens (gram-positive and gram-negative bacteria, single cases of *Prothoteca* algae and yeast-like *Candida* fungi) in cows with clinical symptoms of the disease (group III). It should be added that all microorganisms isolated from the milk of diseased cows are typical pathogens of *mastitis* [27,28].

It is assumed that the relatively high number of leukocytes (WBC) in cows with subclinical disease states (group II) was mainly due to the increase in the number of PMN cells in the peripheral blood, which in turn could be the result of an increased release of these cells from the bone marrow into the bloodstream as a result of a generalized inflammatory response. It should be emphasized that the regulation of circulating PMN cells is important to ensure the optimal immune status of the cow's organism. An increase in the number of these cells is also found at the site of inflammation, i.e. the mammary gland. It is worth adding that the appearance of inflammation in the udder leads to the release from the bone marrow of largely immature cells with limited phagocytic abilities [29].

On the other hand, the decrease in WBC in the blood (caused mainly by a lower proportion of PMN cells) in the case of clinical *mastitis* caused by gram-negative bacteria is largely related to the properties of endotoxins, which are one of the components of the outer cell membranes of these microorganisms [30]. Endotoxins induce the release of strong proinflammatory mediators (cytokines), which in turn results in a marked increase of SCC in the mammary gland, mainly caused by the influx of PMN cells with a simultaneous decrease in their number in the peripheral blood [25,26].

The presented studies showed relatively low values of

Table 5. Values of correlation coefficients (r) between the somatic cell count in milk (SCC) and CL results of peripheral blood and quarter milk of the tested cows.

CL	Whole blood (n, N = 108)	Quarter milk (n = 108, N = 220)
CL-WS	0.0363	0.7931****
CL-Z	0.5075****	0.8523****
CL-OZ	0.4727****	0.7913****
CL-fMLP	0.1598	0.8004****
CL-PMA	0.2402*	0.8010****

Explanations: n - number of animals, N - number of samples, CL-WS, CL-Z, CL-OZ, CL-fMLP, CL-PMA - as in Table 3;
*, **, ***, **** – significance level (respectively $p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$).

spontaneous chemiluminescence (CL-WS) in peripheral blood in control cows. This result, proving the resting metabolic activity of PMN cells, in combination with the effective stimulation of these cells by the receptor and nonreceptor method, indicates the good health of animals from group I. It is believed that in diseased individuals, phagocytic cells may be in a preactivated state, activated or exhausted, which means that despite stimulating them with various stimulators, they do not respond with a significant increase in ROS production [31].

The clinical form of *mastitis* was manifested in comparison to the group of healthy animals by significantly higher CL values of peripheral blood for all the examined stimulators. The highest blood CL values were observed after stimulation with Z and OZ. Interesting is the relatively small, albeit significant, increase in CL after stimulation with fMLP. This result seems to confirm the opinion of some authors, according to which the receptors for fMLP on bovine neutrophils may be latent and the mechanisms of their expression are still unknown [32]. Other authors even believe that bovine PMN cells lack fMLP receptors [33,34].

On the other hand, in animals with subclinical *mastitis*, it was shown that only stimulation by the nonreceptor route caused a significant (by about 150%) increase in blood CL in comparison to control cows. This result suggests the greatest usefulness of studies using PMA as a CL stimulator of PMN cells in the blood in the context of the diagnosis of ongoing local inflammation in cows. The obtained results are consistent with the results of previous studies carried out in the peripheral blood of diseased lactating cows between 15 and 70 days after calving [35].

The results of own research indicate that the spontaneous quarter milk CL in cows, both healthy and diseased, was characterized by significantly higher values compared to blood CL. This result corresponds with the research results Paape et al. [36], who registered a higher CL of unstimulated PMN cells isolated from milk than cells isolated from blood. They attribute this phenomenon to the presence of components in the secretion of the mammary gland (fats, casein) that stimulate PMN cells to produce ROS. However, it is believed that this reaction does not translate into greater phagocytic efficiency of milk PMN cells, as their lysosomes connect with the phagosomes containing the aforementioned milk components, instead of the phagosomes containing the absorbed pathogens. Thus, casein and milk fat reduce the effectiveness of phagocytosis. In addition, milk PMN cells contain lower (by approx. 38%) glycogen reserves than blood PMN cells, which also translates into their lower phagocytic efficiency [36]. However, it is worth bearing in mind that the higher CL value of milk PMN cells before infection and the rapid increase in SCC in the infected quarter (mainly PMN cells) and the increase in milk CL at the beginning of the pathogen invasion are key in the rapid elimination of the source of infection. According to Mehrzad et al. [19], the low CL value of milk PMN cells prior to infection is a risk factor for *Escherichia coli mastitis*.

The viability of PMN cells is also extremely important in the elimination of the source of infection. It was demonstrated that the milk PMN cells viability was significantly higher in the infected quarter compared to the cells in the uninfected quarters [18]. There was also a clear increase in the free radical activity of milk PMN cells after infection of the udder quarter by e.g., *Escherichia coli* already in the first day after infection [18].

The presented research showed that milk CL values of the quarters infected by pathogen were significantly higher in both groups of diseased cows compared to healthy animals. This reaction, which is a response to the invasion of the pathogen, is in line with previous observations on isolated PMN cells [18,19]. It was also found that the milk CL values in cows with clinical *mastitis* were significantly higher than in cows with subclinical *mastitis*. This phenomenon was clearly related to SCC in milk and, as mentioned earlier, higher viability and phagocytic activity of milk PMN cells in response to pathogen invasion.

The presented research showed a strong positive correlation between SCC and all tested CL parameters of milk and some CL indicators of peripheral blood (CL-Z, CL-OZ, CL-PMA). This result confirms the thesis that *mastitis* is not only a local process, limited to a given quarter, but also affects the entire body of the cow [35]. This is manifested, among others, by increased CL activity not only of milk, but also of peripheral blood in response to stimulating factors.

The demonstration of a clearly greater relationship between SCC and CL of milk than SCC and CL of peripheral

blood suggests a potential diagnostic significance of the presented studies, especially in terms of early detection of *mastitis*. Attempts to use CL measurements of milk for this purpose were previously undertaken by Takahashi et al. [37]. Their method was based on the measurement of the luminol-dependent CL of a milk sample stimulated with OZ in a cell culture solution (Hepes Eagle's Minimal Essential Medium). These researchers showed that the increase in the chemiluminescent activity of PMN cells occurs at the beginning of the infection of the mammary gland. Such an early detection of *mastitis* is beyond the range of classic methods, such as SCC measurement and CMT test [37].

5. Conclusion

The conducted studies have shown that the inflammation of the mammary gland of cows results in an increase of the spontaneous and stimulated luminol-dependent chemiluminescence not only of the quarter milk but also of the peripheral blood. It was found that there is a greater correlation between SCC and milk CL results than there is a correlation between SCC and blood CL results. Thus, the CL determination of milk better reflects the intensity of the inflammation of the udder, which suggests its usefulness in the early detection of the disease. On the other hand, blood CL tests, especially with the use of PMA as a PMN cell stimulator, can be a useful tool, especially in the diagnosis of subclinical *mastitis*.

Funding

This study was funded by the Polish Minister of Science and Higher Education, under the program "Regional Initiative of Excellence" in 2019–2022 (Grant No. 008/RID/2018/19).

The funding body did not play a role in the design, analysis and reporting of the study.

Ethics approval and consent to participate

All animal procedures described were conducted under ethical approval from the Local Ethics Committee for Animal Experimentation at the Faculty of Animal Breeding and Biology, UTP University of Science and Technology in Bydgoszcz (Poland) gave consent to the research (license no. 59/2012). Laboratory procedures were carried out in accordance with the standards of good laboratory practice developed in the National Veterinary Research Institute in Pulawy (Poland).

Availability of data and materials

All of the data generated or analyzed during this study are included in this published article, and the supplementary information files will be freely available upon request via e-mail with the corresponding author.

Conflict of interest

The authors declare that they have no conflict of interest.

Contribution of authors

Conceived and designed the experiments: R.S. and W.K. Performed the experiments: R.S., W.K. and H.M. Analyzed the data: R.S., W.K., H.M. and E.S. Wrote the paper: R.S. and W.K. Supervised the study: W.K. Edited the final manuscript: R.S. and W.K. All authors read and approved the final manuscript.

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