Original article

The effect of supportive *E. coli* mastitis treatment on PMN chemiluminescence and subpopulations of T lymphocytes

H. Markiewicz[1], W. Krumrych[1], M. Gehrke[2]

[1] Department of Pathophysiology of Reproduction and Mammary Gland,
National Veterinary Research Institute, Al. Powstańców Wlkp. 10, 85-090 Bydgoszcz, Poland
[2] Department of Animal Reproduction and Animal Health Protection,
University of Technology and Life Sciences, 85-084 Bydgoszcz, Poland

Abstract

The aim of this field study was to assess the impact of a single *i.m.* injection of lysozyme dimer and flunixin meglumine in combination with intramammary and systemic antibiotic on chemiluminescence of PMN (polymorphonuclear leucocytes) and subpopulations of lymphocyte T in blood of cows with *E. coli mastitis*.

Examinations were performed on 30 dairy cows affected with naturally occurring acute form of *E. coli* mastitis. Cows were randomly divided into three groups according to the method of treatment. The first group was treated with approved intramammary antibiotic product, the same antibiotic in *i.m.* injection and one injection of flunixin meglumine on the first day of therapy. Next group was treated with the same antibiotic and additionally one injection of lysozyme dimer on the first day of therapy. The third one was treated only with an antibiotic and served as a control group. Blood samples were taken before treatment and on days 3 and 7. In samples haematology indices were determined, spontaneous and opsonised zymosan stimulated CL and PMA measurements were performed and the subpopulations of T lymphocyte (CD2^{+}, CD4^{+}, CD8^{+}) were assayed in whole blood.

There was no effect of the applied supportive treatment on the value of morphological blood indices. A significant influence of the time of sample collection on the level of CL and dynamics of lymphocytes T subpopulation was demonstrated. A single injection of flunixin meglumine or lysozyme dimer on the day of the beginning of treatment of *E. coli* mastitis, does not affect the level of neutrophil chemiluminescence and the percentage of T lymphocytes in the blood of mastitic cows in the analysed period of time.

Key words: *E.* coli mastitis, treatment, PMN chemiluminescence, lymphocytes T

Correspondence to: H. Markiewicz, e-mail: hannamar@op.pl

Introduction

The treatment of udder inflammation caused by *Escherichia coli* raises a lot of controversy. It was demonstrated repeatedly that the course of *E. coli mastitis* is associated more with the resistance of the animal, than the virulence of a strain causing inflammation. This bacterium does not invade the glandular tissue, but remains in the lumen of a teat duct and lactiferous sinus and the clinical symptoms are associated with the amount of released lipopolysaccharide (LPS) during growth and lysis of the bacterial cells (Burvenich et al. 2003). Although LPS is not a chemoattractant for neutrophils, it induces the production of cytokines mediating the inflammatory response (Carroll et al. 1982). These are mainly interleukins 1, 6, 8 and tumor necrosis factor-α (Alluwaimi 2004). The functional status of neutrophils in the peripheral blood and their flow into the mammary gland also determine the severity of symptoms of *E.coli* mastitis. It is believed that neutrophils play an important role in the detoxification process of LPS with the participation of acyloxyacyl hydrolase (McDermott et al. 1991). The released mediators of inflammation affect neutrophil oxidative metabolism (expressed as the level of chemiluminescence), which is the exponent of their bactericidal properties. They also stimulate T cells. However, the role of T cells in the inflammatory response in *E. coli* mastitis is unclear (Mehrzad et al. 2001, 2008).

During experimental *E. coli* mastitis it was shown that indicators for the disintegration of the blood-milk barrier returned to normal values within 72 h postinfusion (Vangroenweghe et al. 2004). A typical *E. coli* mastitis is characterized by a sudden onset, loss of secretion in the quarter affected by mastitis, swollen and painful infected quarter, an increase in rectal temperature. A severe course was most often observed in cows during the perinatal period, more frequently in multiparous than in primiparous. The treatment is primarily aimed at the reduction of the inflammatory response induced by LPS, and the use of antibiotics is "virtually useless" (Pyöralä and Pyöralä 1998, Hogan and Smith 2003). It has been demonstrated that the intramammary application of antibiotics effective *in vitro*, does not improve the cure rate and does not affect the elimination of bacteria in the mammary gland in case of spontaneous and induced *coliform mastitis* (Pyöralä 2009).

However, parenteral antimicrobial treatment seems reasonable in the postpartum period, associated with immunosuppression that occurs physiologically, especially in cows with systemic symptoms. It has been shown that both moderate/mild and acute *colimastitis* may be accompanied by bacteremia (Cebra et al. 1996, Wenz et al. 2001). In the treatment of *E. coli* mastitis supportive medicines such as NSAIDs (nonsteroidal anti-inflammatory drugs) are recommended, because of their anti-inflamatory, anti-pyretic and analgesic effects.

Taking into account the fact that on one hand the course of *E. coli* mastitis is subjected to the cellular resistance of the host cell, and on the other hand the amount of released endotoxins, in the supportive treatment NSAID and an immunomodulator were used. Flunixin meglumine belongs to nonselective COX inhibitors, which changed the clinical picture of coliform mastitis and caused greater DMI (dry matter intake) and milk production after single injection (Rantala et al. 2002, Yeiser et al. 2012). Also combination of parenteral antibiotic and single injection of nonsteroidal anti-inflammatory meloxicam resulted in a lower somatic cell counts and reduced risk of culling cows with clinical mastitis (McDougall 2009). Lysozyme dimer increased indicators of phagocytosis in cows affected by clinical mastitis. A single injection of the lysozyme dimer increased the efficacy of treatment of mastitis caused by *E. coli* (Malinowski et al. 2001, 2006). Disappearance of symptoms and an increase of the effectiveness of treatment after a single administration of the above mentioned drugs may suggest that their effect is long-lasting.

The aim of this field study was to assess the impact of a single *i.m.* injection of lysozyme dimer and flunixin meglumine in combination with intramammary and systemic antibiotic on chemiluminescence (CL) of PMN (polymorphonuclear leucocytes) and subpopulations of lymphocyte T in blood of cows with *E. coli* mastitis. Combined therapy with intramammary and systemic amoxicillin/clavulanic acid for treatment of *E. coli mastitis* is contentious, but on this farm it was used routinely with a good effect.

Materials and Methods

Examinations were conducted on 30 Polish HF cows with naturally-occurring cases of acute form of *E. coli* mastitis that belonged to one farm (average milk yield 9,800 kg per 305 days, free stalls, TMR). All cows were between days 10 and 45 after parturition.

Milk samples were aseptically taken from all cows symptomatic of *coliform* mastitis. The treatment was started within 2 – 4 hours on the notification of disease by the animals' handler. The cows were randomly divided into 3 groups according to the method of treatment. After bacteriological examinations (IDF 1987) only cows with *E. coli* mastitis were included in the study.

Table 1. Morphological indices of blood of mastitic cows treated with the participation of lysozyme dimer and flunixin meglumine ($\bar{x} \pm$SD).

Group	Days of sampling	WBC (G/l)	RBC (T/l)	HGB (mmol/l)	N%
Lysozyme dimer + antibiotic	0	3.49 ± 2.38^a	7.21 ± 1.65	8.37 ± 0.74	44.01 ± 10.11^a
	3	7.72 ± 1.43^b	6.41 ± 1.26	7.87 ± 0.94	69.16 ± 6.08^b
	7	6.87 ± 2.86^b	6.28 ± 1.13	7.52 ± 0.84	50.49 ± 16.66^a
Flunixin meglumine + antibiotic	0	3.17 ± 2.4^a	6.32 ± 0.89	8.12 ± 1.51	39.59 ± 17.89^a
	3	6.18 ± 2.49^b	6.41 ± 1.39	7.84 ± 1.46	58.84 ± 21.7^b
	7	9.99 ± 3.51^c	6.36 ± 1.32	7.44 ± 0.92	65.34 ± 6.97^b
Antibiotic	0	3.41 ± 2.26^a	6.92 ± 1.12	8.27 ± 0.92	41.78 ± 15.02^a
	3	7.09 ± 2.11^b	6.25 ± 1.18	8.05 ± 1.63	65.27 ± 12.23^b
	7	7.82 ± 2.53^c	6.46 ± 1.28	7.64 ± 0.85	58.83 ± 9.08^b
Interaction		G p=0.488 S p=0.0001 GxS p=0.006	G p=0.569 S p=0.166 GxS p=0.498	G p=0.621 S p=0.04 GxS p=0.933	G p=0.991 S p=0.0001 GxS p=0.014

[a, b, c] – significant differences within a group for $p \leq 0.05$; G – group; S – sampling

At the time of first clinical examination mastitic cows had rectal temperature > 39.8ºC, abnormal milk accompanied by swelling in the affected mammary quarter and milk production drop. The first group (n=10) was treated with approved intramammary antibiotic product in label doses (3 x every 12 hours), the same antibiotic intramuscular once a day for 3 consecutive days (also in label dose 8.75 mg/kg) and one injection of flunixin meglumine in a dose of 2.2 mg/kg on the first day of therapy. Next group (n=10) was treated with the same antibiotic and one injection of lysosyme dimer in a dose of 0.02 mg/kg also on the first day of therapy. The third one (n=10) was treated only with an antibiotic and served as a control group.

The intramammary product (amoxycillinum trihydrate 200 mg/clavulanic acid 50 mg/prednisolone 10 mg) was selected on the basis of sensitivity of bacteria to antibiotics earlier isolated in this herd. The amoxicillin with clavulanic acid is authorized for lactating cattle. Blood samples were taken before treatment (day 0), and on days 3 and 7 from the coccygeal vein using vacutainer tubes. Sampling scheme was dictated by monitoring the course of treatment and regression of clinical symptoms. Haematological assay was performed in blood samples collected on potassium versenate using haematological analyser Horiba ABC. Measurements of luminol-dependent chemiluminescence (CL) using the area under the curve for a certain period of time (integrate) expressing the total amount of light emitted by the cell at this time were performed with a BioOrbit 1251 luminometer (Pharmacia-LKB). The assay was performed by the kinetic method at the temperature of 38ºC, recording luminometer indications for 40 min.

at 5-minute intervals. Spontaneous and opsonised zymosan stimulated CL and PMA (Phorbol 12-myristate 13-acetate) measurements were performed. Each measurement was carried out simultaneously in triplicate. The detailed methodology was described in an earlier work (Markiewicz et al 2006).

The subpopulations of T lymphocytes (CD2+, CD4+, CD8+) were determined in whole blood by means of flow cytometry (Facs Calibur, Becton Dickinson) using mice monoclonal antibodies produced by Serotec Ltd. (MCA833F, clone CC42, MCA1653F, clone CC8; MCA836P, clone CC63).

The data within groups were evaluated statistically by one-way analysis of variance. Two-factorial ANOVA was performed to evaluate the effect of supportive treatment and sampling time from Statistica v.6.0. by StatSoft® Poland. All parameters are presented as means ($\bar{x} \pm$SD).

Results

Mean values of haematological indices in the study cycle are shown in Table 1. On the day of the beginning of treatment the groups were similar. In all groups on the day 3 and 7 a significant increase of WBC (white blood cells) was observed as compared to the first assay. This was accompanied by an increase in the percentage of neutrophils (% GR) in white blood cells count. The number of red blood cells and haemoglobin concentration were similar in all groups and did not change in subsequent days of the study. There was no effect of the applied supportive treatment on the value of morphological blood indices. The dy-

Table 2. Level of PMN chemiluminescencje (CL) of blood of mastitic cows treated with the participation of lysozyme dimer and flunixin meglumine ($\bar{x} \pm$SD).

Group	Days of sampling	BS (mV/min)	ZO (mV/min)	PMA (mV/min)
Lysozyme dimer + antibiotic	0	2095.1 ± 1448.8[a]	2674.5 ± 1495.1	2465.3 ± 1560.7
	3	896.2 ± 518.2[b]	1646.3 ± 791.5	1719.1 ± 664.5
	7	879.8 ± 335.7[b]	1667 ± 882.8	1607.9 ± 684.6
Flunixin meglumine + antibiotic	0	1910.6 ± 684[a]	2300.4 ± 1113[a]	2108.5 ± 1120[a]
	3	1211.2 ± 532[ab]	1547.7 ± 480[ab]	1543.6 ± 794[ab]
	7	665 ± 559[b]	903.9 ± 463[b]	1085.2 ± 518[b]
Antibiotic	0	1985.9 ± 971[a]	2439.5 ± 1207[a]	2207.1 ± 1285[a]
	3	1005.4 ± 589[b]	1538.2 ± 622[ab]	1672.5 ± 697[ab]
	7	744.6 ± 421[b]	1244 ± 539[ab]	1371.5 ± 602[ab]
Interaction		G p=0.876 S p=0.0001 GxS p=0.501	G p=0.089 S p=0.0001 GxS p=0.443	G p=0.136 S p=0.003 GxS p=0.839

[a, b, c] – significant differences within a group for $p \leq 0.05$; G – group; S – sampling

Table 3. Subpopulations of T lymphocytes of blood of mastitic cows treated with the participation of lysozyme dimer and flunixin meglumine ($\bar{x} \pm$SD).

Group	Days of sampling	CD2%	CD4%	CD8%	CD4/8
Lysozyme dimer + antibiotic	0	46.46 ± 13.01[a]	26.05 ± 9.44[a]	16.95 ± 6.19	1.71 ± 0.812[a]
	3	61.76 ± 10.09[b]	39.77 ± 8.64[b]	17.46 ± 4.25	2.35 ± 0.5[b]
	7	53.44 ± 10.91[ab]	31.66 ± 8.79[ab]	18.48 ± 4.26	1.73 ± 0.47[ab]
Flunixin meglumine + antibiotic	0	48.7 ± 9.66[a]	28.68 ± 8.46[a]	15.35 ± 5.91[a]	1.85 ± 0.923[ab]
	3	59.73 ± 5.91[b]	37.41 ± 7.58[b]	16.32 ± 3.23[a]	2.43 ± 0.86[b]
	7	60.01 ± 8.6[b]	33.27 ± 8.66[ab]	23.87 ± 8.73[b]	1.56 ± 0.651[a]
Antibiotic	0	47.05 ± 11.4[a]	27.98 ± 8.12[a]	16.07 ± 6.41[a]	1.78 ± 0.782[a]
	3	61.98 ± 8.75[b]	37.07 ± 7.24[b]	16.87 ± 5.01[a]	2.37 ± 0.641[b]
	7	58.25 ± 8.07[b]	32.57 ± 7.94[ab]	20.53 ± 7.19[b]	1.62 ± 0.534[a]
Interaction		G p=0.39 S p=0.0001 GxS p=0.43	G p=0.074 S p=0.0001 GxS p=0.63	G p=0.564 S p=0.023 GxS p=0.132	G p=0.521 S p=0.045 GxS p=0.443

[a, b, c] – significant differences within a group for $p \leq 0.05$; G – group; S – sampling

namics of changes depended only on the time of sample collection. Regarding WBC and %GR, an interaction between the time of sample collection and group was demonstrated.

On the day of the beginning of treatment oxidative metabolism of PMN cells, expressed as the level of CL, was similar in all groups. Spontaneous chemiluminescence was significantly decreased in the group treated with lysozyme dimer on days 3 and 7 of treatment compared to the first assay. A similar tendency was observed for opsonised zymosan-stimulated CL and PMA. However, these differences were not

significant. In the group receiving flunixin meglumine, on day 3 after medicine administration an insignificant decrease of CL was noticed. A significant CL decrease was noted on the day 7 of the study. A similar dynamics of CL changes was observed in the control group. A significant influence of the time of sample collection on the level of spontaneous and stimulated CL was demonstrated, but not the type treatment (Table 2). This also concerned the dynamics of lymphocytes T subpopulation, which was also not influenced by the supportive treatment. In all groups a significant increase of lymphocytes CD2^{+} and CD4^{+}

was observed on the day 3 of the study in comparison with the first assay. The percentage of CD8$^+$ subpopulation on day 3 was similar to the first day of treatment, however, an increase was observed on day 7 of the study. In the group receiving flunixin meglumine and in the control group the difference was significant. Also CD4/CD8 ratio increased significantly on day 3, then it was decreased on day 7 to the level as at the beginning of the treatment (Table 3).

Discussion

Binding of LPS – LBP (lipopolisacharide binding protein) complex with receptor CD14$^+$ (located on neutrophils, macrophages, monocytes) induces the release of e. g. TNFα and lipid mediators in arachidonic acid cascade (Paape et al. 2002). The lysozyme dimer modulates TNFα concentration both *in vitro* and *in vivo* (Kiczka 1994). The flunixin meglumine inhibits activity of cyclooxygenase and shows anti-prostaglandin activity (Anderson et al. 1986). During endotoxin-induced mastitis the concentrations of TNFα returned to level before LPS infusion after 24 h, but higher values of serum amyloid A were evident after 72 h.

The course of *E. coli mastitis* in an early phase (0 – 18 hours following infection) is associated with neutropenia. The population of T lymphocytes also decreased significantly at this time (Mehrzad et al. 2008). In experimental endotoxin mastitis it was shown that the number of lymphocytes was the lowest between 8 and 12 hours post challenge and returned to baseline level after 48 h (Springer et al. 2006). Similar behaviour of WBC was observed in our study. Neutropenia observed at the beginning of the treatment disappeared and on day 3 of the study white blood cell counts returned to physiological levels. Other authors also demonstrated that in the experimentally induced *colimastitis* white blood cell count, rectal temperature, somatic cell count and all the symptoms signifying the disintegration of the blood/milk barrier returned to normal within 24 – 72 hours after inoculation with E. coli P4:O32 (Vangroenweghe et al. 2004). After 72 hours from the beginning of the treatment an increase of CD2$^+$ and CD4$^+$ lymphocytes was observed in the peripheral blood, which was accompanied by a higher CD4$^+$/CD8$^+$ ratio. Helper cells are the dominant population in blood, in contrast to the mammary gland, where CD8$^+$ subpopulation plays major role (Shafer-Weaver and Sordillo 1997).

In this study, there was no effect of a single injection of both lysozyme dimer and flunixin meglumine, on the day of the beginning of antibiotic treatment in cows with *colimastitis*, on oxidative metabolism of PMN cells and subpopulations of T lymphocytes. No response after a single injection of flunixin meglumine was also observed by other authors. It was shown that a single injection of flunixin meglumine does not affect haptoglobin concentration in cows with *mastitis*. There was also no effect of NSAID on the number of WBC in cows with experimentally induced inflammation of the mammary gland (Young et al. 2000, Rantala et al. 2002, Vangroenweghe et al. 2004).

In vitro studies demonstrated an increase of chemiluminescence of neutrophils in blood and mammary inflammatory secretion of cows under the influence of lysozyme dimer (Malinowski 2001). In our study, in the analysed period of time, no such relationship was observed. A decrease of spontaneous and induced CL on day 3 and 7 of the study is connected with the decrease of neutrophil oxygen metabolism. It is assumed that the level of CL is a measure of inflammation intensity. The response of PMN cells to the stimuli used (opsonised zymosan and PMA) suggests that the way of neutrophils stimulation, both the receptor-mediated and non-receptor-mediated, is working in cows with *colimastitis*. *In vitro* study showed suppressive influence of antibiotics (except enrofloxacin) on PMN functionality (Hoeben 1998). In our study the application of antibiotics, both intrammamary and intramuscular, could be one of the reasons for the lack of changes of the observed indicators.

The results obtained in our studies indicate the activation of T lymphocytes in cows with acute *E. coli* mastitis. This is confirmed in the literature (Kornalijnslijper et al. 2003). The increase in the percentage of CD4$^+$ lymphocytes is associated with a change in the immunological profile towards Th1 cells. They determine an increase of the cellular immune response by activating macrophages and an increase of adhesion and diapedesis by PMN cells (Dosogne 2002). A significant increase in the percentage of CD4$^+$ lymphocytes is the result of ongoing inflammation. This is accompanied by a significant increase in the ratio of CD4$^+$/CD8$^+$. This state is confirmed by the increase in the percentage of granulocytes. CD4$^+$/CD8$^+$ ratio increase is associated with an increased proportion of helper lymphocytes. The observed changes in the distribution of T lymphocytes in the peripheral circulation indicate that in the initial phase of *E. coli mastitis* T helper lymphocytes play a dominant role. The opposite happens in the mammary gland, where the dominant cells are CD8$^+$ lymphocytes. In the experimentally induced inflammation of the mammary gland it can be noticed that the time after inoculation with *E. coli* is significant in the inflammatory response (Mehrzad et al. 2008). In the field studies, in the

samples collected on the first day of treatment, there was already an effect of ongoing inflammation for at least several hours in the form of leukopenia, increased oxidative metabolism of neutrophils and reduced T-cell subpopulations. The decreased percentage of T lymphocytes and reduction in the proportion of CD4^{+} T-cells could be related to systemic immunosuppresion. CL of neutrophils is associated with their bactericidal ability. The high level of CL of neutrophils in peripheral blood on the first day of treatment indicates the intensification of metabolism in these cells connected with intracellular killing of microorganisms. In the initial phase of *E. coli mastitis* it is interesting to observe a multiple increase of oxidative metabolism of neutrophils with at the same time observed neutropenia.

Attention has been drawn to a large variability of CD8^{+} lymphocyte subpopulation in 3 weeks postpartum. *Mastitis* occurring during this period also has an influence on the variability of this subpopulation (Van Kampen and Mallard 1997). Our study, however, did not confirm this observation. A significant decrease in neutrophil respiratory burst between 1 and 3 weeks after parturition has been indicated in comparison with those observed before parturition. From 1 to 5 weeks after parturition, a gradual increase in CL of neutrophils can be observed, which, however, does not reach the values observed before parturition (Dosogne 1999). The reduction of production of reactive oxygen species by PMN cells after parturition can be one of the factors affecting the increase of the incidence of *mastitis* in the period.

A single injection of flunixin meglumine or lysozyme dimer on the day of the beginning of treatment of *E. coli* mastitis, does not affect the level of neutrophil chemiluminescence and the percentage of T lymphocytes in the blood of mastic cows in the analysed period of time. The supportive treatment in the form of a single injection of the above-mentioned drugs did not fulfil the assumptions. Perhaps, it should be elongated, but this thesis needs to be supported by further studies.

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