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EFFECT OF DIFFERENT LYMPHOCYTE-BACTERIA RATIOS AND BACTERIAL TOXINS ON APOPTOSIS OF BOVINE MAMMARY GLAND LYMPHOCYTES

WPLYW STOSUNKU STEŻEŃ LIMFOCYTÓW DO BAKTERII ORAZ TOKSYN BAKTERYJNYCH NA ŚMIERĆ LIMFOCYTÓW SUTKÓW JAŁÓWEK

Abstract: *Staphylococcus aureus* and *Streptococcus uberis* delay apoptosis of bovine mammary gland lymphocytes following intramammary infusion and in *in vitro* studies with lymphocyte-bacteria ratio 1:1. In this study, we investigated the effect of different lymphocyte-bacteria ratios on apoptosis of bovine mammary gland lymphocytes *in vitro*. We found out that lymphocyte-bacteria (*S. aureus* or *S. uberis*) ratios 1:10, 1:50 and 1:100 have different effect on apoptosis of lymphocytes than ratio 1:1. Lymphocyte apoptosis was induced 6 hours following incubation with *S. aureus* or *S. uberis* with mentioned ratios (1:10, 1:50 and 1:100). In our previous preliminary experiments focused on exploration of chemical components of bacteria on apoptosis of lymphocytes, we established the effect of muramyl dipeptide and lipopolysaccharide on lymphocyte apoptosis only *in vitro*. Therefore, in the second part of the present study we focused our experiments on investigation of the effect of Gram-negative bacterial toxin lipopolysaccharide on apoptosis of bovine mammary gland lymphocytes *in vivo*. The results of these experiments suggest that lipopolysaccharide induces apoptosis of lymphocytes following intramammary application. These data need next exploration to reveal detail effects of bacteria or bacterial toxins on lymphocyte programmed cell death in connection with inflammatory process.

Keywords: bacteria, bacterial toxin, apoptosis, lymphocyte, mammary gland

Bacterial pathogens modulate apoptosis of cells [1]. Pathogens which are involved in the cell death of lymphocytes include Gram-positive or Gram-negative bacteria [1-3] and their components, especially bacterial toxins such as *lipopolysaccharide* (LPS) [4-6]. *Staphylococcus aureus* and *Streptococcus uberis* are the most important pathogens causing clinical and subclinical bovine mastitis [7, 8]. Resident and incoming leukocytes play an important role in the mammary gland's defence system against invading pathogens. Macrophages and lymphocytes are the predominant resident cells in the healthy mammary gland, while intramammary infections induce the recruitment of leukocytes (and especially neutrophils) from blood into the mammary gland [9, 10]. During bouts of acute mastitis caused by staphylococcal or streptococcal infections, an increased number of lymphocytes are detected in the mammary glands [11].

Bacterial toxins and components are common constituents of environment entry to food chain. Thus they can be posing a risk for human population. Bovine strains of *S. aureus* associated with intramammary infection produce staphylococcal enterotoxins [12, 13]. Staphylococcal enterotoxins are known as superantigens [14]. The interactions of superantigens with the T cells lead to their activation [15] and apoptosis [16, 17]. Haslinger et al [18] reported that *S. aureus* α -toxin induced apoptosis in human peripheral blood

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mononuclear cells. Park et al [19] investigated apoptosis of CD4⁺ and CD8⁺ lymphocytes of bovine blood during *in vitro* cultivation with staphylococcal enterotoxin C, after previously demonstrating that staphylococcal infections induce immunosuppressive CD8⁺ T cells *in vivo* [20, 21]. LPS is as a toxin released from the cell wall of Gram-negative bacteria. Infusion of LPS into the bovine mammary gland induces an inflammatory response [22-24].

In our previous study, we have demonstrated the effect of *S. aureus* and *S. uberis* on apoptosis of bovine mammary gland lymphocytes. We found out that apoptosis of lymphocyte was delayed during an experimentally induced infection with *S. aureus* or *S. uberis*. These results were confirmed by *in vitro* experiment with lymphocyte-bacteria ratio 1:1. Some authors described a possible role of different lymphocyte-bacteria ratio on apoptosis of lymphocytes such as Perticarari et al [25]. They focused their experiments on exploration of the effect of *Borrelia burgdorferi* on apoptosis of human lymphocytes *in vitro* with lymphocyte-bacteria ratios 1:10, 1:20, 1:50, 1:100.

The aim of our study was to determine the effect of different lymphocyte-bacteria ratios and *Escherichia coli* LPS on apoptosis of bovine mammary gland lymphocytes.

Materials and methods

The experiments were carried out on 16 mammary glands of 4 clinically healthy Holstein × Bohemian Red Pied crossbred heifers aged 16 to 18 months - *in vivo* experiments followed *in vitro* experiments in the same animals. The heifers were housed in an experimental tie-stall barn and fed a standard ration consisting of hay and concentrates with mineral supplements. The experimental tie-stall used in this study is certified and animal care conformed to good care practice protocols. All heifers were free of intramammary infections, as demonstrated through a bacteriological examination of mammary lavages.

The heifers were used as mammary gland cell donors for *in vitro* studies. Lymphocytes from the mammary glands were harvested following the *phosphate buffered saline* (PBS) intramammary injection, using a procedure previously described by Rysanek et al [26] that employs a model of an induced influx [27]. Fresh mammary gland leukocytes were adjusted (1.0×10^7 cells/cm³) in RPMI 1640 medium (Sigma, MO, USA), both with and without *S. aureus* and *S. uberis*, at a ratio of 1:1, 1:10, 1:50 and 1:100. The two bacterial strains used were *S. aureus* Newbould 305 (CCM 6275) and *S. uberis* (CCM 4617). The samples were incubated at 37°C in a 5% CO₂ atmosphere for 1, 3 and 6 hours, as described by Scaife et al [28]. After incubation, apoptotic lymphocytes were detected through *flow cytometry* (FCM) (FACS Calibur apparatus, Becton Dickinson, CA, USA).

Before experimental infection, the mammary glands were treated with PBS prepared with apyrogenic water. All 4 mammary gland sinuses of each heifer were rinsed stepwise with PBS to obtain a cell suspension using the following procedure. The first cell sample was obtained by lavage of the left forequarter 1 day after administration of PBS. The remaining quarters were rinsed stepwise at two 1-day intervals and one 4-days interval in the following order: left-rear (2 days) → right-front (3 days) → right-rear (at 7 days). These PBS-treated mammary glands were set as a control for the infections, as undertaken in previous studies [29, 30]. LPS of *E. coli*, serotype 0128:B12 (Sigma, USA) in a concentration of 5 µg in 20 cm³ PBS was used for inducing inflammatory response. Modified urethral catheters (AC5306CH06, Porges S.A., France) were inserted into the teat

canal following thorough disinfection of the teat orifice with 70% ethanol. Through the catheter, each mammary quarter was injected with 20 cm³ of PBS and 2 cm³ of lavage solution was immediately collected back through the catheter directly to the syringe and subsequently used for bacteriological examination.

Apoptotic lymphocytes were analysed by FCM following simultaneous staining with Annexin-V labelled with *fluorescein isothiocyanate* (FITC) and propidium iodide, as described by Vermes et al [31]. The commercial Annexin-V-FLUOS staining kit (Boehringer Mannheim, GmbH, Mannheim, Germany) was used according to the manufacturer's instructions. Briefly, 500 mm³ of the incubation buffer (10 mM Hepes/NaOH, pH 7.4; 140 mM NaCl; 2.5 mM CaCl₂) was mixed with 10 mm³ of PI and 10 mm³ of FITC-Annexin-V solution. After 15 min of incubation at room temperature with fresh buffer containing PI and FITC-Annexin-V, the cell suspension was analysed by FCM with differentiation of at least 20,000 cells. After labelling with Annexin-V-FITC and PI, lymphocyte cells were distributed over three different quadrants of a dot plot analysis (with FL1 and FL3 axes), representing viable (Annexin V⁻/PI⁻), apoptotic (Annexin V⁺/PI⁻), and necrotic cells (Annexin V⁺/PI⁺). Dot plots were evaluated qualitatively and quantitatively using WinMDI™ software.

Arithmetic means and standard deviations were used to describe the apoptotic lymphocytes. Statistically significant differences in the proportions of apoptotic lymphocytes were determined using the paired *t*-test. The data were processed using Statistica 8.0 software (StatSoft CR Ltd, Prague, Czech Republic).

Results and discussion

The objective of this study was to determine the effect of different lymphocyte-bacteria (*S. aureus* and *S. uberis*) ratios on apoptosis of bovine mammary gland lymphocytes *in vitro*. In our previous study, we described that a significantly lower percentage of apoptotic lymphocytes were observed following cultivation of cells with *S. aureus* and *S. uberis* than following cultivation without bacteria (initial ration of lymphocyte to bacteria 1:1) [3]. In this study, we investigated an effect of different lymphocyte-bacteria ratios on apoptosis of lymphocytes. We found out that apoptosis of lymphocytes is significantly increased following 6 hours of cultivation with lymphocyte-bacteria ratios 1:10, 1:50 and 1:100, contrary to the ratio 1:1 (Figs 1 and 2). These results correspond with other experimental work focused on lymphocyte apoptosis co-cultured with *B. burgdorferi* [25]. The authors of that article showed that increasing lymphocyte apoptosis corresponds with increasing ratio (1:10 → 1:100).

The aim of this study was also to determine whether apoptosis of bovine mammary gland lymphocytes is modulated during an inflammatory response of bovine mammary gland induced by LPS of *E. coli*. Lymphocytes were obtained by lavage of the mammary gland at 4 intervals (1, 2, 3 and 7 days) following stimulation with LPS. Stimulation of mammary gland with LPS resulted in a significant increase in proportion of apoptotic lymphocytes in comparison with the control. The portion of apoptotic lymphocytes peaked at 2 days following stimulation. In previous studies, there was demonstrated that apoptosis of bovine mammary gland lymphocytes is delayed during an experimentally induced infection of bovine mammary gland with *S. aureus* and *S. uberis* and during an *in vitro*

cultivation with LPS and MDP [3, 6, 32]. However, results of this part of the study suggest that LPS intramammary application induces apoptosis of lymphocytes.

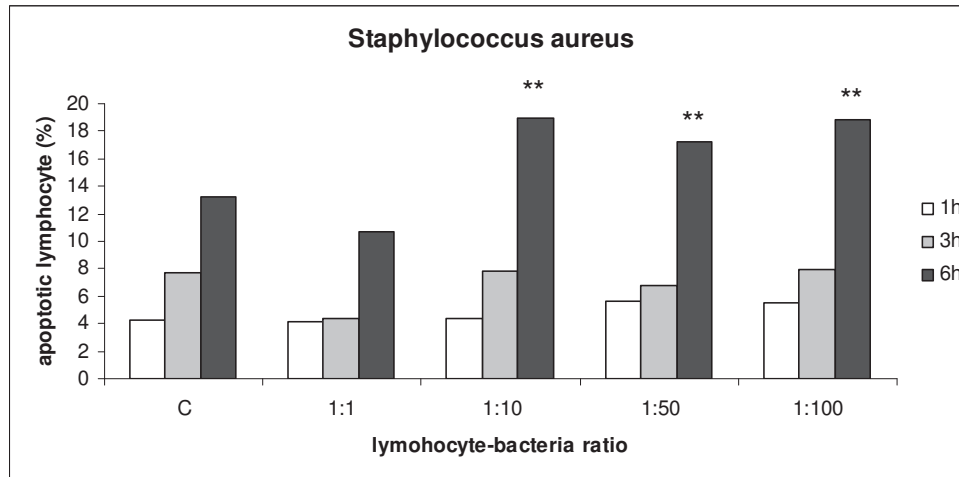


Fig. 1. The proportion of apoptotic lymphocytes in *in vitro* experiment with lymphocyte-bacteria (*S. aureus*) ratios 1:1, 1:10, 1:50, 1:100. C - control (cells only). Significant differences among the lymphocyte-bacteria ratio 1:1 and lymphocyte-bacteria ratios 1:10, 1:50, 1:100 are marked with asterisks (** $p < 0.01$)

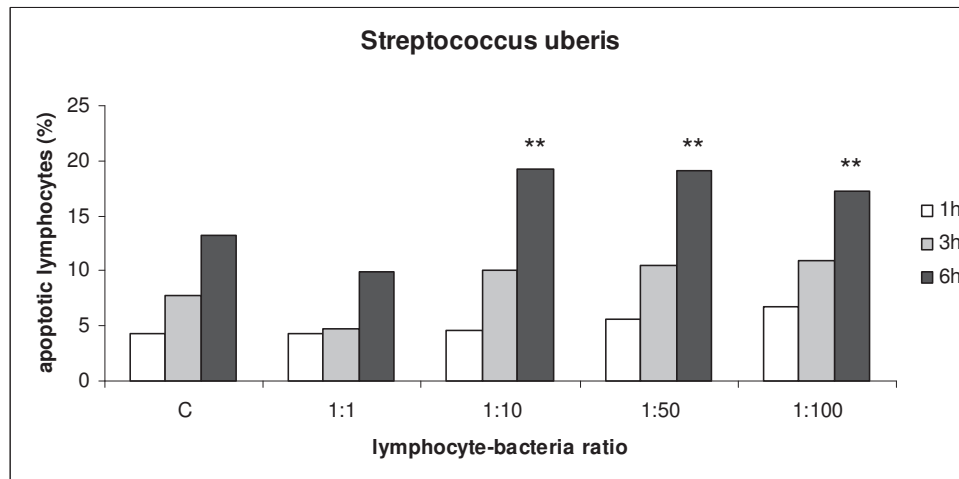


Fig. 2. The proportion of apoptotic lymphocytes in *in vitro* experiment with lymphocyte-bacteria (*S. uberis*) ratios 1:1, 1:10, 1:50, 1:100. C - control (cells only). Significant differences among the lymphocyte-bacteria ratio 1:1 and lymphocyte-bacteria ratios 1:10, 1:50, 1:100 are marked with asterisks (** $p < 0.01$)

Conclusions

The effect of bacterial pathogens on apoptosis of lymphocytes is depended on cell-bacteria ratio. Apoptosis of lymphocytes is induced in lymphocyte-bacteria ratios 1:10, 1:50 and 1:100, contrary to the ratio 1:1 which is connected with delaying of lymphocyte apoptosis.

Stimulation of bovine mammary gland lymphocytes *in vivo* with *E. coli* LPS leads to increase in apoptotic lymphocytes at variance with previous *in vitro* experiment with *E. coli* LPS that showed delaying of lymphocyte apoptosis.

To confirm these data, there is necessary next exploration to reveal detail effects of bacteria or bacterial toxins on lymphocyte programmed cell death in connection with inflammatory process.

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