

## EFFECT OF PEPTIDOGLYCAN OF *STAPHYLOCOCCUS AUREUS* ON APOPTOSIS OF BOVINE MAMMARY GLAND LYMPHOCYTES

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### ABSTRACT

The goal of this study was to analyze whether apoptosis of lymphocytes is modulated by experimental infection caused by peptidoglycan of *Staphylococcus aureus*. Lymphocytes were obtained by lavage of the mammary glands at four intervals (24, 48, 72 and 168 hours) following experimental stimulation of mammary glands. The portion of apoptotic lymphocytes peaked at 48 hours following infection with peptidoglycan of *Staphylococcus aureus*. The results show that during the mentioned infection the apoptosis of lymphocytes is induced.

**Keywords:** peptidoglycan, lymphocyte, apoptosis, *Staphylococcus aureus*, mammary gland

### INTRODUCTION

*Staphylococcus aureus* is one of the most important pathogens which is able to cause bovine mastitis (Pettersson-Wolfe et al. 2010). These bacteria contain peptidoglycan in their cell wall. Peptidoglycan is also found in the cell wall of Gram-negative bacteria but in different structure than in Gram-positive bacteria (Vollmer et al., 2008). This natural molecule of bacteria is able to induce inflammatory response of bovine mammary gland (Furukava et al., 2018). Sulabh et al. (2019) have confirmed the stimulant effect of peptidoglycan of *S. aureus* on peripheral blood mononuclear cells obtained from blood samples of crossbred cattle, Tharparkar cattle, and Murrah buffaloes. Peptidoglycan stimulates leukocytes and also epithelial cells during mastitis (Im et al., 2014).

*S. aureus* is also able to produce enterotoxins. These enterotoxins (staphylococcal enterotoxin C,  $\alpha$ -toxin) induce activation and apoptosis of T cells (Damle et al., 1993; Webb and Gascoigne, 1994; Boshell et al., 1996) and apoptosis of peripheral blood mononuclear cells (Haslinger et al., 2003).

In context of previous information, changes of programmed cell death can be caused by different inducers in frame work of bacteria – by components of cell wall or product of bacteria mainly toxins. We previously studied the effect of *S. aureus* and *Streptococcus uberis* on lymphocyte apoptosis of bovine mammary gland (Slama et al., 2009a). We have found that apoptosis of lymphocytes was delayed by those bacterial species during experimental infection and in *in vitro* study, too. Therefore, we would like to know whether peptidoglycan as the molecule naturally found in the cell wall of *S. aureus* can also modulate lymphocyte apoptosis in the same manner or in different way.

The aim of this study was to determine whether apoptosis of bovine mammary gland lymphocytes is modulated during an inflammatory response of bovine mammary gland induced by peptidoglycan.

### MATERIAL AND METHODS

For our experiments, we used eight clinically healthy virgin heifers (Holstein x Bohemian Red Pied crossbred) in age 16 to 18 months. All animals were free of infection of the mammary glands. Bacteriological examination of mammary gland lavages was executed by culturing on blood agar with aerobic incubation at 37 °C for 24 hours.

For the experimental infection, there were used urethral catheter (AC5306CH06, Porges SA, France) to insert into the teat canal after disinfection of the teat orifice (Sladek et al., 2005; Slama et al., 2009a). Each mammary gland in each

quarter of the udder was injected with 20 ml phosphate buffered saline (PBS) with 50  $\mu$ g of peptidoglycan of *S. aureus* (Sigma, USA). Before experimental infection, the mammary glands were used for preparation of control samples through treatment by PBS as previously described (Sladek et al., 2005).

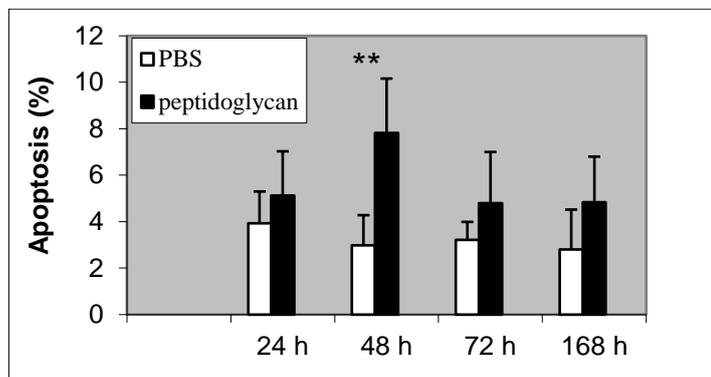
The lymphocytes obtained by lavages of mammary glands were analysed by flow cytometry (FACS Calibur Apparatus, Becton Dickinson, CA, USA) and subsequently by software WinMDI 2.8 (Trotter, 2000) as in previous studies (for example Slama et al., 2006). Proportion of apoptotic lymphocytes was enumerated by staining with Annexin-V (FITC) and propidium iodide (PE) previously described by Vermes et al. (1995).

For statistical analysis, there were used statistical software STATISTICA 8.0 (StatSoft, Czech Republic). Arithmetic means and standard deviations were used to describe apoptosis of lymphocytes. Statistically significant differences in the proportion of apoptotic lymphocytes were determined by paired *t*-test.

### RESULTS AND DISCUSSION

The aim of this study was to evaluate the effect of peptidoglycan on apoptosis of bovine mammary gland lymphocyte during experimentally induced inflammation.

In this study, the apoptosis of lymphocytes was changed during the inflammatory response. Experimental infusion of peptidoglycan into the mammary gland led to increase of lymphocyte apoptosis with the maximum in 48 hours following stimulation of the mammary glands compare to the control (Figure 1).



**Figure 1** Apoptosis of mammary gland lymphocytes (%) following stimulation by peptidoglycan in four timepoints (24, 48, 72, 168 hours). PBS – phosphate buffered saline; \*\*  $P < 0.01$

These results suggest that peptidoglycan can induce apoptosis of lymphocytes. Contrary to that, our previous results shown that *S. aureus* or *S. uberis* can delay apoptosis of lymphocytes (Slama et al., 2009a). Slight increase in apoptosis of T lymphocytes was detected by Park et al. (2006). Those authors cultivated lymphocytes with staphylococcal enterotoxin C *in vitro*. Those mentioned information suggest that components of bacterial cell wall could be more effective in induction of lymphocyte apoptosis in the initial stage of inflammation. Later, bacteria produce toxins which can be effective apoptotic inducer but in the late stage of inflammation. Apoptosis of cells is very important to protect mammary gland tissue damage during mastitis. Apoptotic cells have stable cell membrane and the content of the cell is not spread into surrounding tissue. On the other hand, necrosis of cells is dangerous to tissue because the cell membrane of these cells is damaged. If the necrotic cells are neutrophils, they can harm the mammary tissue by releasing reactive oxygen intermediates and proteolytic enzymes (Zhao and Lacasse, 2008).

In our study, we used peptidoglycan of *S. aureus* as Gram-positive bacteria. Question remains, if Gram-negative bacteria or the components of their cell wall are able to affect the lymphocyte apoptosis in the same way. Our preliminary results (unpublished data) indicate that lipopolysaccharide of *Escherichia coli* has similar effect on apoptosis of lymphocyte *in vivo*. Yokochi et al. (1996) also shown that lipopolysaccharide induced apoptosis of B lymphocytes. In contrary to that, we reported that lipopolysaccharide can delay apoptosis of lymphocytes in *in vitro* conditions (Slama et al., 2009b). We also used muramyl dipeptide to stimulate lymphocytes *in vitro*. Muramyl dipeptide is the minimal structural unit of peptidoglycan. We found out that apoptosis of lymphocytes was also delayed using muramyl dipeptide (Slama et al., 2009c). Contrast in results of *in vitro* and *in vivo* studies can be possible. *In vitro* study only says what cells are able to do but in *in vivo* conditions there are more variables which can play important role in final results of experiments. Moreover, there are produced different cytokine during mastitis by immune cells. These cytokines are able to modulate apoptosis of cells. In the initial stage of mastitis, there is produced tumor necrosis factor alpha by neutrophils (Sohn et al., 2007). That cytokine is able to induce apoptosis of cells (Rath and Aggarwal, 1999) and therefore the highest percentage of lymphocyte apoptosis is found out in 48 hours following the start of inflammation.

## CONCLUSION

The results suggest that the cell wall components as peptidoglycan are able to modulate lymphocyte apoptosis during the process of inflammation of the mammary gland. We studied whole population of lymphocytes and therefore it is necessary to continue to investigate different subpopulation of lymphocytes. Different subpopulation of lymphocytes could have different resistance to bacterial compounds and toxins.

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