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Effect of Lipopolysaccharide and Muramyl Dipeptide on the Inflammatory Cytokine Production Throughout Inflammatory Response of Bovine Mammary Gland

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Abstract: The objective of this study was to get a better knowledge of inflammatory cytokine production (TNF-α, IL-4, IL-10 and TGFβ1) throughout inflammatory response of bovine mammary gland caused by bacterial toxin Lipopolysacharide (LPS) and Muramyl Dipeptide as a structural unit of Peptidoglycan (MDP). In the experiments, there were used LPS and MDP at the specified concentrations (5 and 500 μg, respectively). The inflammatory response was analyzed at selected time points (24, 48, 72 and 168 h) following stimulation of the mammary glands. As a control, the same volumes of physiological solution was used. The mammary gland lavages were collected from the 8 clinically healthy virgin Holsteinx Czech Pied heifers and the concentrations of the cytokines were analyzedby an ELISA method.

Key words: Inflammation, mastitis, dairy cattle, cytokines, ELISA, concentrations

INTRODUCTION

Mastitis is one of the most serious production dairy cattle disease which may be triggered for example by *Staphylococcus aureus*, *Streptococcus agalastiae*, *Streptococcus dysgalactiae* and *Streptococcus uberis* bacteria. It is also influenced by the immunity of a dairy cow.The inflammations of mammary gland are responsible for herd economic impact, decline in milk yield about 10-12%, poor milk quality, culling cows earlier and economic losses up to 300 € per cow per year.

Inflammation is characterized bythe accumulation of cytokines (Feghali and Wright, 1997) which are small proteins produced by cells (Zhang and An, 2009). They play key roles in many biological events related with inflammations and immunity.

Pro-inflammatory cytokines such as Tumor Necrosis Factor alpha (TNF-α) are considered to initiate the inflammatory reactions in mammary tissues and to induce migration of leukocytes into the udder (Persson *et al*., 2003). They are released mainly by activated macrophages (Zhang and An, 2009). TNF-α is one of the most potent and pleiotropic pro-inflammatory cytokine. It is associated with inflammatory response (Tracey and Cerami, 1993) and it is known that the TNF-α is also involved in the process of pathological pain (Zhang and An, 2009).

Anti-inflammatory cytokines regulate the pro inflammatory cytokine response (Opal and Depalo, 2000). One of the best-studied anti-inflammatory cytokine is Interleukin 4 (IL-4) (Brown, 2008). It controls multiple biological functions such as proliferation, differentiation and apoptosis in several cell types (Zamorano *et al*., 2003). Interleukin 10 (IL-10) and Transforming Growth Factor beta 1 (TGF-α1) act as the most important anti-inflammatory cytokines. Interleukin 10 regulates functions of many immune cells (Sabat *et al*., 2010). TGF-α1 manages proliferation and differentiation of cells and other biological responses (Barnard *et al*., 1990).

MATERIALS AND METHODS

Animal selection and trial design: The study procedures were focused on the analysis of the pro-inflammatory cytokine TNF-α and anti-inflammatory cytokines IL-4, IL-10 and TGF-α 1. The cytokine detections were determined using ELISA method. Eight clinically healthy crossbred heifers (Holstein×Czech Pied) were selected for this study. The heifers were group housed in a tie-stall barn and fed a total mixed diet.

The isolated leukocytes from the mammary gland were incubated with LPS (Lipopolysaccharide from *Escherichia coli*; 50 μg/mL) or with MDP (Muramyldipeptide, 500 μg/mL) for 24, 48, 72 and 168 h *in vitro* (at 37°C in 5% CO_2). The cytokines were determined by sandwich ELISA.LPS was used as a Gram-negative bacteria toxin. MDP was used as a Gram-positive bacteria toxin.

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Bacteriological examination: All lavages obtained were bacteriologically examined by culture on 5% washed ram erythrocytes blood agar plates with aerobic incubation at 37°C for 24 h before each experimental procedure.

Sample collection procedures: Samples of cell populations were obtain by lavage of the mammary gland 24 h following the mammary gland stimulation by sterile Buffered Saline Solution (PBS). In total, 20 mL of PBS was used. Fresh mammary gland leukocytes were adjusted (5×10^6/mL) in RPMI. The cell concentration was counted in a Burker chamber in 20 large squares. The cells were smeared on glass slides and stained (Pappenheim). At least 200 leukocytes on each glass slide were counted to determine the differential cell count.

ELISA: The following kits were used to determine the cytokines, Bovine TNF-alpha DuoSet ELISA (R and D systems), IL-4 ELISA Reagent Kit, Bovine (Thermo Fisher Scientific), Bovine interleukin 10 ELISA Kit (MyBioSource) and human/mouse TGF beta 1 ELISA ready-SET-Go! (eBioscience).

Statistical analysis: The concentration level of the cytokines were expressed as arithmetic mean (x)±Standard Deviation (SD). Data were analyzed using Statistical Software program Statistica 8.0 (StatSoft CR, s.r.o.). The paired t-test was used.

RESULTS AND DISCUSSION

Leukocytes count: The leukocytes count was counted in a Burker chamber. The absolute leukocyte count obtained by the mammary gland lavage following 24 h mammary gland stimulation by PBS was 2.5×10^9/l (±0.35).

Differential cell count of leukocytes: The differential cell count was counted using light microscopy. At least 200 leukocytes on each glass slide were counted (Table 1).

Concentration level of TNF-α: The mammary gland leukocytes concentration level of TNF-α was determined following 24, 48, 72 and 168 h *in vitro* incubation with LPS or MDP (Fig. 1).

Concentration level of IL-4: The mammary gland leukocytes concentration level of IL-4 was determined following 24, 48, 72 and 168 h *in vitro* incubation with LPS or MDP (Fig. 2).

Concentration level of IL-10: The mammary gland leukocytes concentration level of IL-10 was determined following 24, 48, 72 and 168 h *in vitro* incubation with LPS or MDP (Fig. 3).

Table 1: Differential cell count of mammary gland leukocytes (obtained by the mammary gland lavage following 24 h mammary gland stimulation by PBS)

Type of leukocytes	Arithmetic mean (%)	Standard Deviation (SD)
Neutrophils	62.5	7.2
Macrophages	31.7	9.8
Lymphocytes	5.8	3.2

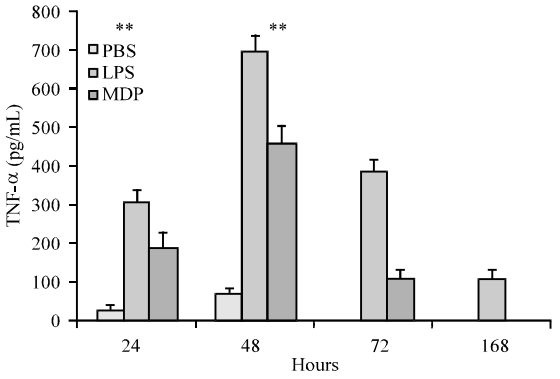

Fig. 1: Mammary gland leukocytes concentration Level of TNF-α following incubation with LPS or MDP (24, 48, 72 and 168 h of incubation)

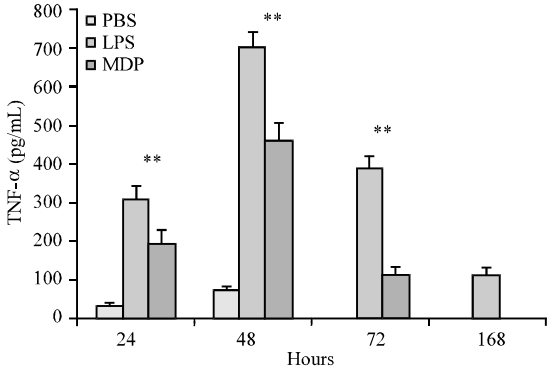

Fig. 2: Mammary gland leukocytes concentration level of IL-4 following incubation with LPS or MDP (24, 48, 72 and 168 h of incubation)

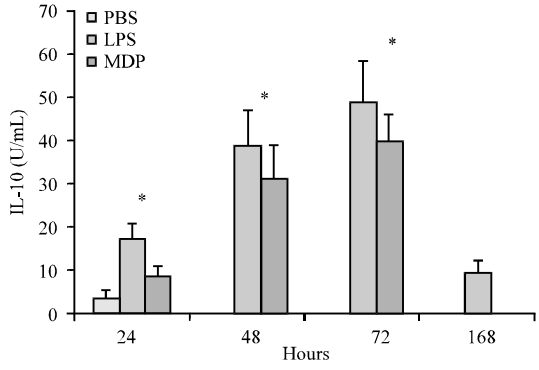

Fig. 3: Mammary gland leukocytes concentration level of IL-10 following incubation with LPS or MDP (24, 48, 72 and 168 h of incubation)

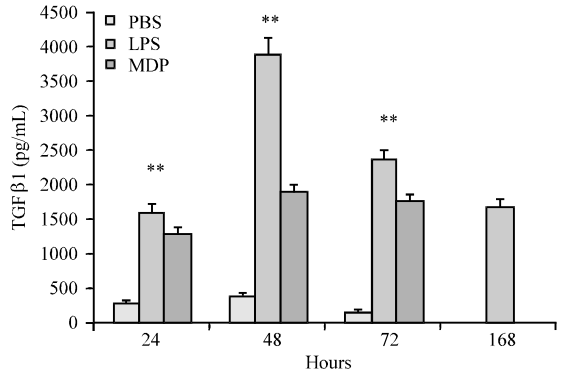

Fig. 4: Mammary gland leukocytes concentration level of TGF-α1 following incubation with LPS or MDP (24, 48, 72 and 168 h of incubation)

Concentration level of TGF-α 1: The mammary gland leukocytes concentration level of TGF-α 1 was determined following 24, 48, 72 and 168 h *in vitro* incubation with LPS or MDP (Fig. 4).

The objective of this trial was to study the pro inflammatory (TNF-α) and anti-inflammatory (IL-4, IL-10 and TGF-α1) cytokines *in vivo* following the mammary gland stimulation by LPS and MDP.

The absolute leukocyte count and the differential cell count of mammary gland leukocytes obtained by lavage of the mammary gland 24 h following the mammary gland stimulation by sterile buffered saline solution agree well with other published results (Sladek *et al*., 2008). Hereby, the correct mammary gland experimental intervention has been confirmed.

Cytokines act as a mediator of host defense. They manage communication between antigen-presenting cells, lymphocytes and other host cells throughout an immune response (Belardelli and Ferrantini, 2002). TNF-α, an important pro-inflammatory cytokine, plays a key role in driving innate immunity (Aggarwal *et al*., 1999). It is a main pro-inflammatory cytokine which activates innate and adaptive immune response (Weinberg and Buchholz, 2006). In this trial, we observed the significant ($p<0.01$) differences in the TNF-α production induced by LPS and MDP mammary gland stimulation in comparison to the control (PBS). There was also significant ($p<0.01$) difference between LPS and MDP usage when LPS caused higher TNF-α production. The highest production of TNF-α was reached 24 h following the stimulation. Yokomizo *et al*. (1995) reached the highest production 48 h after the stimulation by *Staphylococcus aureus* exoproteins. Then the authors noted gradual recession of the TNF-α level. LPS has been well known as a major inducer of TNF-α secretion in monocytes. It may encourage neutrophils to release TNF-α as well (Dubravec *et al*., 1990) which was proved by Yokomizo *et al*. (1995) and by this study.

IL-4 is ananti-inflammatory cytokine shaping the innate immune responses (Choi and Reinser, 1998). It is produce by activated Th2 lymphocytes (Deo *et al*., 2010). Its function is to maintain homeostasis throughout inflammation and immune response when the organism is attacked by a bacterial infection (Tumbleson and Schook, 1997). IL-10 is also anti-inflammatory cytokine whose production by macrophages may be stimulated by some bacterial pathogens (Sing *et al*., 2002). In our study, the level of IL-4 peaked 48 h following the LPS stimulation. Then the level decreased by the time while the level of IL-10 increased by the time and peaked 72 h following the stimulation. Those results are contra to Luan *et al*. (2012)'s findings which claim LPS reduces the IL-4 production. Delogu *et al*. (2001) demonstrated the elevated IL-10 production is related with lymphocytes apoptosis. Also, Hessle *et al*. (2000) proved Gram-negative bacteria stimulate IL-10 production. In our trial, we may confirm higher IL-10 production following LPS stimulationagainst MDP stimulation as well.

TGF-α1 anti-inflammatory cytokine participates on the immune response during infectious diseases (Peralta-Zaragoza *et al*., 2001). We recorded its elevated level following LPS stimulation. The maximum level was reached 48 h after stimulation. Following MDP stimulation, the maximum level was reached 48 h lateras well. However, the levels reached were lower than following LPS stimulation. Those findings are in agreement with Cross *et al*. (2004) who noted different TGF-α1 levels following Gram-negative or positive bacteria stimulation. Zheng *et al*. (2002) claim elevated TGF-α production is related with lowered IFN-α and IL-10 production.

CONCLUSION

In this trial, we studied the pro-inflammatory (TNF-α) and anti-inflammatory (IL-4, IL-10 and TGF-α1) cytokines *in vivo* following the mammary gland stimulation by LPS and MDP which are produced by bovine mammary gland leukocytes.

We recorded the significant differences in the TNF-α production induced by LPS and MDP mammary gland stimulation in comparison to the control. The concentration level of TNF-α was higher following the incubation with LPS than with MDP. The highest production of TNF-α was reached 24 h following the stimulation.

We confirmed a higher IL-10 production following LPS stimulation against MDP stimulation. The level of IL-4 peaked 48 h following the LPS stimulation. Then, the level decreased by time, while the level of IL-10 increased by time and peaked 72 h following the stimulation.

Further, we recorded an elevated level of TGF-α1 following the LPS stimulation. The maximum level was reached 48 h after the stimulation. Following, the MDP stimulation, the maximum level was reached after 48 h as well. However, the levels reached were lower than following LPS stimulation.

All cytokines showed a higher concentration level following the cells incubation with LPS. Based on this, we assume that Gram-negative bacteria stimulate mammary gland leukocytes more intensively than Gram-positive bacteria.

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