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Production of Interferon Gamma and Transforming Growth Factor Beta 1 from Bovine Mammary Gland Leukocytes

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Abstract: The objective of this trial was to study the Inflammatory cytokinesinterferon gamma (IFN-γ) and Transforming Growth Factor Beta1 (TGF-β1) which are produced by bovine mammary gland leukocytes. The leukocytes were obtained by mammary gland lavage and incubated for 1, 2 and 18 h with lipopolysaccharide (50 μg/mL) cor with muramy l dipeptide (500 μg/mL) *in vitro*. Lipopolyccharide was used as a toxin of Gram-negative bacteria and muramy l dipeptide as a structural unit of a peptidogly can of Gram-positive bacteria. The concentration levels of these cytokines were analyzed by ELISA. Eight clinically healthy crossbred heifers (Holstein x Czech Pied) were selected for this study. The heifers were group housed in a tie-stall barn and fed a total mixed diet. Higher IFN-γ concentration was recorded for lipopolysacharide incubation. The production of IFN-γ started 18 h following the cell incubation with lipopolysaccharide or with muramy l dipeptide. The production of TGF-β1 was higher for lipopolysaccharide incubation than for muramy l dipeptide incubation throughout the all set time points. The TGF-β1 production started 2 h following the cell in cubation with muramy l dipeptide.

Key words: Inflammation, *Escherichia coli*, mastitis, dairy cattle, cytokines, incubation, muramy l

INTRODUCTION

Mastitis is an inflammation of mammary gland. This infectious disease may be caused by many external environment factors. Therefore, it is called multifactorial disease. Mostly, mastitis symptoms are triggered by these bacteria: *Staphylococcus aureus, Streptococcus agalastiae, Streptococcus dysgalactiae* and *Streptococcus uberis* (Rysanek, 2007). The mammary gland inflammation is usually connected with high somatic cell count in milk yield. Approximately 99% of the somatic cells are leukocytes (Kubekova, 2007). 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.

Cytokines are soluble proteins which play key roles in many biological events related with inflammations and immunity (Dinarello, 2007). They are being investigated for use in cows with mammary gland inflammation. Generally, cytokines are used in immunotherapy, diagnosis a prognosis (Alluwaimi, 2004). Interferon gamma (IFN-γ) has multiple functions in innate and adaptive immune responses (Bao *et al*., 2014) and plays an important role in the regulation of early gene expression of Th2 (Torres *et al*., 2004). It is also known to be important in activation of immune responses against viruses (Presti *et al*., 1998). Transforming Growth Factor Beta 1 (TGF-β 1) controls differentiation, proliferation, activation of many cells (Peralta-Zaragoza *et al*., 2001) and gene expression (Ohba *et al*., 1994). Generally, TGF-β1 manages immune response throughout the infectious diseases and cancer. It is produced by lymphocytes, macrophages and dendritic cells (Peralta-Zaragoza *et al*., 2001).

MATERIALS AND METHODS

Animal selection and trial design: The study procedures were focused on the analysis of the inflammatory cytokines IFN-γ and TGF-β1. The cytokine detections were determined using ELISA method. Eight clinically healthy crossbred heifers (Holstein x 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 (Muramy l

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dipeptide; 500 μg/mL) for 1, 2 and 18 h *in vitro* (at 37°C in 5% CO_2). The cytokines were determined by sandwich ELISA.

Apro-Inflammatory (IFN-γ) and anti-inflammatory (TGF-β1) were chosen for this study. LPS was used as a Gram-negative bacteria toxin. MDP was used as a Gram-positive bacteria cell wall component.

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 IFN-γ screening set (Genetica, s. r. o.) and human/mouse TGF beta 1 ELISA Ready-SET-Go! kit (Exbio Praha, a.s.).

Statistical analysis: The absolute count of leukocytes, the differential cell count of leukocytes and 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 (Stat Soft 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 count of the leukocytes 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 IFN-γ: The mammary gland leukocytes concentration level of IFN-γ was determined following 1, 2 and 18 h *in vitro* incubation with LPS or MDP (Table 2).

Concentration level of TGF-β 1: The mammary gland leukocytes concentration level of TGF-β 1was determined following 1, 2 and 18 h *in vitro* incubation with LPS or MDP (Table 3). The objective of thistrial was to study the objective of this trial was to study the pro-Inflammatory (IFN-γ) and anti-inflammatory (TGF-β1) cytokines *in vitro* which are produced by bovine mammary gland leukocytes.

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 (x) (%)	SD
Lymphocytes	62.45	5.77
Neutrophils	31.23	3.15
Monocytes	4.120	0.45
Eosinophils	2.200	0.28

Table 2: Mammary gland leukocytes concentration level of IFN-γ following incubationwith LPS or MDP (1, 2 and 18 h of incubation)

Bacterial toxin/Incubation (h)	Arithmetic mean (x) (pg/mL)	SD
LPS		
1	-	-
0.00	-	-
2	0.00	-
18	345.67	31.87
MDP		
1	0.00	-
2	0.00	-
18	305.21	38.89

Table 3: Mammary gland leukocytes concentration level of TGF-β1 following incubation with LPS or MDP (1, 2 and 18 h of incubation)

Bacterial toxin/Incubation (h)	Arithmetic mean (x) (pg/mL)	SD
LPS		
1	184.82	32.11
2	1337.06	158.21
18	4223.58	342.77
MDP		
1	0.00	-
2	899.00	87.82
18	2167.78	101.65

Leukocytes count: The absolute count of leukocytes 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*., 2001). Hereby, the correct mammary gland experimental interventionhas been confirmed.

Concentration level of IFN-γ: The production of IFN-γ started 18 h following the cell incubation with LPS or MDP. The difference between the production level of IFN-γ following the LPS or the MDP stimulation was not statistically significant. However, the production was slightly higher following the LPS stimulation. Regards no significant difference, it may be labeled as the equal cell stimulation by LPS and MPD. Conversely these results, Alluwaimi *et al.* (2003) confirmed significant difference in IFN-γ production throughout mastitis caused by Gram-positive and negative bacteria. Surbatovic *et al.* (2015) published 13-fold higher IFN-γ level in the Gram-negative bacteria group compared

with the Gram-positive bacteria group. According to Hisaeda *et al*. (2001), the IFN-γ level is elevated during all duration of coliform mammary gland mastitis.

Concentration level of TGF-β1: The production of TGF-β1was analyzed 1, 2 and 18 h following the cell incubation with LPS and MDP. The difference between the production of TGF-β 1 following the in cubation with LPS or with MDP was statistically significant ($p<0.05$) in all set time points (1, 2 and 18 h). It was higher following the incubation with LPS than with MDP. The production of TGF-β 1 started till 2 h following the cell incubation with MDP. Also, Slama *et al*. (2012) studied the leukocyte incubation with LPS. The TGF-β1 production was elevated following 4 h of incubation. Those findings are similar with our results we got following 1 and 2 h of incubation.

Based on the results, it is very likely that Gram-negative bacteria stimulate mammary gland leukocytes to produce TGF-β1 more intensively than Gram-positive bacteria. These study results are in agreement with findings by Surbatovic *et al*. (2015).

CONCLUSION

In this trial, we studied the pro-Inflammatory (IFN-γ) and anti-inflammatory (TGF-β1) cytokines *in vitro* which are produced by bovine mammary gland leukocytes.The production of IFN-γ started 18 h following the cell incubation with LPS or MDP. The difference between the production level of IFN-γ following the LPS or the MDP stimulation was not statistically significant. The difference between the production of TGF-β1 following the incubation with LPS or with MDP was statistically significant ($p<0.05$) in all set time points (1, 2 and 18 h). It was higher following the incubation with LPS than with MDP.

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

SUGGESTION

Results of this study may guide future research on early mastitis detection using cytokines. Unfortunately, a fast and cheap method in not known yet.

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