Dose dependent changes in inflammatory parameters in the milk of dairy cows after intramammary infusion of lipopolysaccharide

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ABSTRACT: The goal of this study was to evaluate the dose dependent changes in common milk and blood parameters for udder health after an intramammary (IM) infusion of five different doses of lipopolysaccharide (LPS, 100, 50, 25, 12.5 and 6.25 µg, respectively). Ten Holstein Friesian cows randomly divided into five groups of 2 cows each were IM infused into one quarter with one LPS dose dissolved in 10 ml of saline. The contralateral quarter was infused with 10 ml saline (9 g/l). Milk samples were taken immediately before and 12, 24, 36, 48 and 60 hours after the treatment. All milk samples were analysed for somatic cell counts (SCC), lactose, sodium (Na), chloride (Cl) and electrical conductivity (EC). Two blood samples were taken immediately after milking for analysing leukocytes (WBC), polymorphonuclear neutrophils (PMN), Na and Cl. The SCC increased maximal at 12 hours after the LPS challenge and differed among LPS doses, as well as the area under curve from 0 to 36 hours (AUC 0–36 h). There were no significant differences among LPS doses in lactose levels for the regression at 12 hours and AUC 0–36 h. Lactose levels in milk from quarters receiving the lowest dose of LPS were lowest after 24 hours, whereas in all other groups lactose levels decreased maximal within the first 12 hours. The regression at 12 hours as well as the AUC 0–36 h showed significant changes for Cl levels but not for Na and EC, respectively. Amongst all groups EC increased maximal within 12 hours and peak EC showed dose dependent differences with highest values at the highest LPS dose. There were no dose differences in WBC. Blood electrolytes showed only tendentially dose dependent differences for blood Na in AUC 0–36 h. The results were possibly due to a great individual variance amongst all cows. In conclusion there are dose dependent differences in the response to LPS especially in milk parameters, which are likely caused by a greater tight junction damage by higher LPS doses. 100 µg LPS seems to be a threshold between low and high doses of LPS. All doses used in this study induced signs of mastitis but there might be a low dose of LPS with only an enhancing effect on mammary gland immune status without inducing mastitis symptoms. This needs to be investigated for developing new ways of mastitis prophylaxis.

Keywords: LPS; dose dependent changes; mammary gland

Lipopolysaccharide (LPS), the endotoxin of Gram negative bacteria, e.g. *Escherichia coli* is responsible for many of the clinical signs, i.e. fever, pain, increasing somatic cell counts (SCC) and loss of milk character in coliform mastitis (Jain et al., 1978; Hill, 1981; Guidry et al., 1983). Therefore, it is used in concentrations up to 500 µg to induce mastitis experimentally to study the inflammatory response in the bovine udder after the release of endotoxin from the infecting pathogens (Hoeben et al., 2000; Mehrzad et al., 2001; Van Oostveldt et al., 2002; Schmitz et al., 2004). Only one study reported on the LPS dose dependent changes in mammary gland health parameters like SCC, lactose, electrical conductivity (EC) and the milk electrolytes sodium (Na) and chloride (Cl) as well as its

influence on blood parameters (Vangroenweghe et al., 2004). Numerous investigators (Kitchen, 1981; Kehrli and Shuster, 1994; Pfaffl et al., 2003; Sarikaya et al., 2006) described SCC as one of the most used parameters in udder health management followed by EC and lactose levels in experimental studies (Stelwagen et al., 1995; Hamann et al., 1995; O'Brien et al., 1999). The composition of SCC changes from mainly macrophages in healthy quarters to a population based on PMN during intramammary inflammation (IMI) (Burvenich et al., 1994; Paape et al., 2002; Sarikaya et al., 2005). The EC reflects the concentration changes of anions and cations (Nielen et al., 1992), in milk particularly Na and Cl levels. The EC in mastitic milk is higher than in milk of healthy udder quarters (Wheelock et al., 1966; Kitchen et al., 1980) and, therefore, it is used as a parameter for mammary gland health status. The interdiffusion between blood and milk constituents is regulated by mammary gland tight junctions (TJ) (Stelwagen et al., 1995, 1997; Nguyen and Neville, 1998). TJ beside the gap junctions and the zonula adherens are forming the junctional complex and are the most apical located structures that surround every adjacent epithelial cell. In healthy lactating mammary glands there is no interdiffusion of milk and blood components through the TJ. In contrast, during involution, milk stasis, or mastitis TJ become leaky which causes the influx of cells and ions into the udder on the one hand and the efflux of milk constituents into the interstitial fluid on the other hand (Stelwagen et al., 1994, 1997; Nguyen and Neville, 1998).

The aim of this investigation was to evaluate LPS dose dependent changes in common milk and blood parameters used for the detection of mammary gland health.

MATERIAL AND METHODS

Animals and husbandry

The experiment was approved by the responsible Animal Care and Use Committee (Regierung von Oberbayern).

Ten clinically normal Holstein Friesian cows were used for the experiment. All cows were in late stages (252–510 day) of their first to fifth lactation. Cows were kept in a tethered barn. They were fed hay and concentrate once daily, and water was available *ad libitum*. Milking was performed routinely twice daily at 600 and 1 800 with a periodical air-inlet BIOMILKER (WestfaliaSurge, Oelde, Germany) at a vacuum level of 42 kPa and a pulsation rate of 60 cycles/min at a ratio of 60:40.

Experimental procedure

Treatment

Cows were randomly divided into five groups of 2 cows each. Each group of cows was intramammarily (IM) infused into one randomly selected quarter with one LPS dose (Serotype O26:B6, Nr. L 8274, Sigma Chem. Co., St. Louis, USA; 100, 50, 25, 12.5 and 6.25 μg, respectively) dissolved in 10 ml of saline (9 g/l) immediately after milking (control milking). The contralateral quarter (control quarters) was infused with 10 ml saline. Before every experimental milking all cows received a physical examination including measurement of body temperature, heart rate and reticulo-rumen motility.

Sample collection

To obtain a separate sample of strict foremilk (cisternal milk only) of each investigated quarter before the occurrence of alveolar milk ejection, milking was performed without any udder preparation (Bruckmaier and Blum, 1996). The cisternal milk sample was the milk obtained by hand milking for 30 s because no alveolar milk ejection was to be expected before 40 s of tactile stimulation (Bruckmaier and Hilger, 2001). The remaining quarter milk was collected in one quarter milker (GEMA – Bruno Gelle GmbH, Wangen, Germany) for each investigated quarter.

Moreover, two blood samples (10 ml) were taken from every cow after milking. One sample was stabilized with EDTA for analysing the red and WBC, the other was collected without anticoagulation for serum analysis of ions like Na and Cl.

Laboratory procedures

Blood samples. Total and differential WBC as well as the concentrations of the blood Na and Cl were analysed in the laboratory of the Research Institute of Animal Production, Nitra, Slovak Republic. The number of WBC was investigated

by means of a Burker counting chamber after blood was diluted 1:10 with Turk's solution. The differentiation of WBC was performed under a light microscope with × 1 000 magnification using a Pappenheim panoptic staining. The electrolytes were measured with a MODULAR E170 (Roche Diagnostics GmbH, Mannheim, Germany).

Milk samples. Lactose concentrations and SCC in every quarter milk sample were analysed in the laboratory of the Research Institute of Animal Production, Nitra, Slovakia, by using a Fossomatic 90 Analyser (FOSS Electric**,** Hillerod, Denmark).

Aliquots of each cisternal milk sample were frozen at –20°C immediately after sampling for the determination of electrical conductivity (EC), sodium (Na) and chloride (Cl) concentrations.

Na and Cl ions in milk were measured by potentiometer determination with ion selective electrodes model 9811 and model 9617BN (pH/Ise Meter, Model 720 Aplus, Orion Research, Beverly, MA, USA).

The EC was measured at 25°C using the LDM electrode from WTW (LDM 130, Wissenschaftlich-Technische Werkstatten GmbH, Weilheim, Germany).

Statistical analyses

Data are presented as means ± SE. The SCC were converted to logarithm ($\log_{10}$). Regression equations were calculated to relate changes of different tested parameters in milk and blood (depend variables) to LPS doses (independent variable) at 12 hours and during first 36 hours (AUC 0–36 h) after LPS infusion. SAS REG procedure was used to calculate regression coefficients. Results are indicated as statistically significant at $P \leq 0.05$.

RESULTS

All results were normalised to 0 hours sampling time and show the changes after the LPS-/saline (9 g/l)-injection. Because there were no significant changes within all investigated control parameters in each group, controls were all taken together as one group.

There were no significant differences in body temperature, heart rate and reticulo-rumen motility, respectively, 6 hours after the LPS infusion.

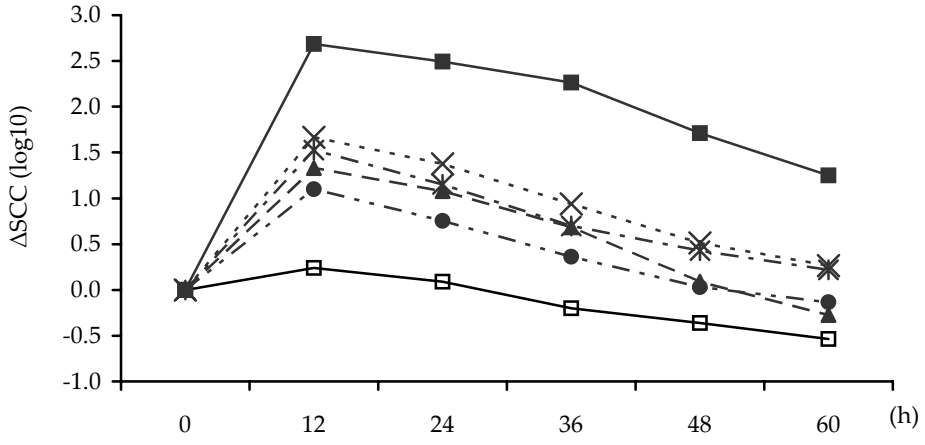

Figure 1. Changes in somatic cell counts ± SEM[1] after an intramammary LPS infusion immediately after the 0 hour sample within the 5 groups

[1]SEM for all control quarters ± 0.07; SEM for all LPS treated quarters ± 0.14

Explantion for Figures 1–3

—□— Controls: values of all quarters infused with 10 ml saline (9 g/l)
—■— LPS 100: values of quarters infused with 100 µg LPS in 10 ml saline (9 g/l)
—▲— LPS 50: values of quarters infused with 50 µg LPS in 10 ml saline (9 g/l)
---×--- LPS 25: values of quarters infused with 25 µg LPS in 10 ml saline (9 g/l)
--∗-- LPS 12.5: values of quarters infused with 12.5 µg LPS in 10 ml saline (9 g/l)
---●--- LPS 6.25: values of quarters infused with 6.25 µg LPS in 10 ml saline (9 g/l)

All values remained within physical limits (results not shown).

Changes in milk components

Somatic cell counts and lactose. The ΔSCC levels in all groups increased to maximal values within the first 12 hours after LPS IM injection (Figure 1). Thereafter they decreased slowly to pre-injection levels within the 60 hours of investigation. As shown in Table 1 the regression was significant ($P \leq 0.05$) at 12 hours as well as the area under curve (AUC) from 0 to 36 hours.

As shown in Table 1, the decrease of Δlactose was maximal at 12 hours after the injection of 12.5 to 100 μg LPS, whereas the lowest dose (6.25 μg) induced maximal Δlactose levels 24 hours after the injection. Levels in all groups slowly returned to normal values within the investigated time period (Figure 2). However, there was no significant dose effect on changes of Δlactose levels at 12 hours as well as during AUC 0–36 h.

Electrolytes and electrical conductivity. Changes of ΔEC for LPS doses of 12.5 to 100 μg increased to maximum at 12 hours after the LPS infusion then values in these groups decreased to beginning

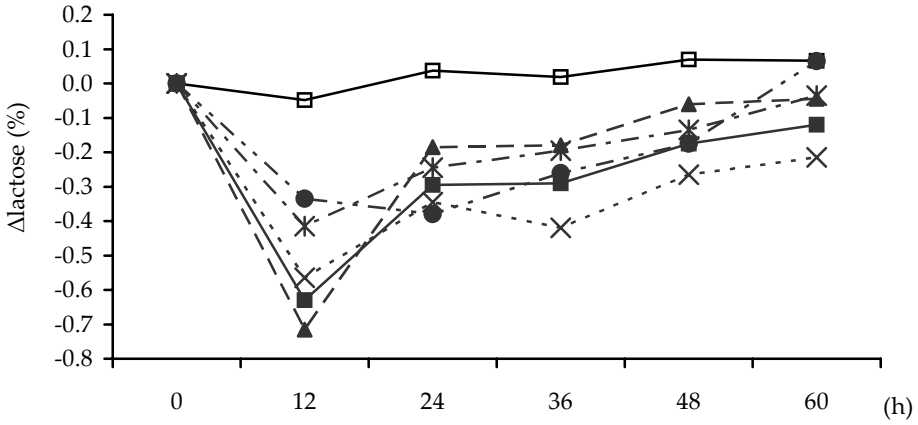

Figure 2. Changes in lactose ± SEM[1] after an intramammary LPS infusion immediately after the 0 hour sample within the 5 groups

[1]SEM for all control quarters ± 0.02; SEM for all LPS treated quarters ± 0.12

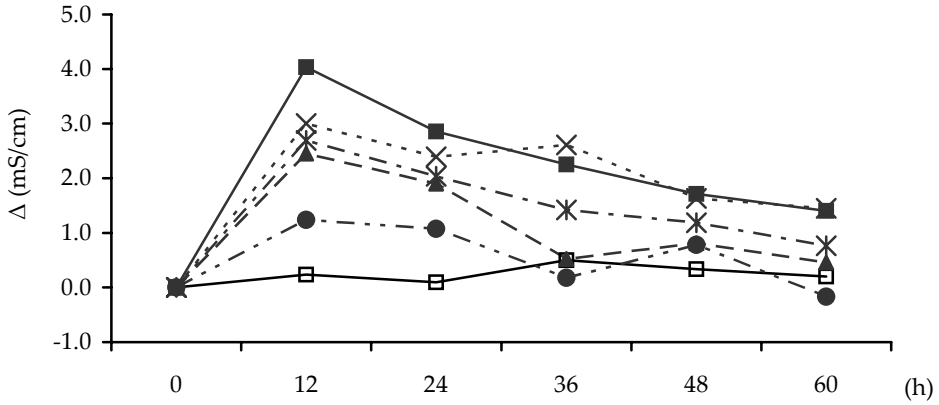

Figure 3. Changes in electrical conductivity ± SEM[1] after an intramammary LPS infusion immediately after the 0 hour sample within the 5 groups

[1]SEM for all control quarters ± 0.61; SEM for all LPS treated quarters ± 0.15

Table 1. Regression coefficients ± SE of the tested parameters in milk and blood at 12 h and during first 36 h (AUC 0–36 h) after intramammary infusion of LPS[1]

Parameter	Unit	At 12 hours				AUC 0–36 hours			
		tested parameter	intercept	*P*-value	R^2	tested parameter	intercept	*P*-value	R^2
Quarter milk									
SCC[2]	Δlog10	0.01 ± 0.003	1.12 ± 0.18	0.004*	0.67	0.013 ± 0.003	0.68 ± 0.15	0.002*	0.72
Lactose	Δg/100ml	−0.003 ± 0.002	−0.42 ± 0.11	0.21	0.17	-7.99×10^{-4} ± 0.002	−0.29 ± 0.08	0.63	0.03
Cisternal milk									
Na^+	Δmmol/l	0.16 ± 0.29	48.70 ± 14.93	0.60	0.04	0.08 ± 0.17	34.62 ± 8.94	0.67	0.02
Cl^-	Δmmol/l	0.58 ± 0.23	45.37 ± 11.87	0.04*	0.44	0.37 ± 0.15	35.33 ± 7.55	0.04*	0.44
EC[3]	ΔmS/cm	2.11 ± 1.18	186.68 ± 60.84	0.11	0.29	1.32 ± 0.84	129.98 ± 43.25	0.15	0.24
Blood									
WBC[4]	ΔG/l	−0.01 ± 0.03	−0.67 ± 1.29	0.59	0.04	−0.02 ± 0.02	−0.17 ± 0.91	0.39	0.09
PMN[5]	Δ%	−0.07 ± 0.03	4.44 ± 1.79	0.08**	0.33	−0.06 ± 0.03	1.93 ± 1.59	0.10	0.26
Na^+	Δmmol/l	0.07 ± 0.04	−1.86 ± 2.22	0.14	0.25	0.04 ± 0.02	−0.27 ± 1.13	0.08**	0.33
Cl^-	Δmmol/l	0.01 ± 0.06	3.15 ± 2.98	0.93	0.001	0.002 ± 0.03	2.25 ± 1.52	0.95	0.0001

*significance for $P \leq 0.05$

**tendency for $P < 0.1$

[1]lipopolysaccharids: five different doses were used (100, 50, 25, 12.5 and 6.25 μg LPS in 10 ml saline 9 g/l)

[2]somatic cell counts

[3]electrical conductivity

[4]leukocytes

[5]polymorphonuclear neutrophils

levels within 60 hours (Figure 3). For the 6.25 μg dose of LPS the ΔEC increased slightly within the first 12 hours then showed a plateau for another 12 hours and decreased to basic levels 36 hours after the LPS injection. The ΔEC only tended to increase with increasing LPS doses (P = 0.11 for 12 hours and P = 0.15 for AUC 0–36 hours).

There were no dose dependent changes ($P \leq 0.05$) in ΔNa levels at 12 hours and within the first 36 hours (Table 1). However, ΔCl values increased ($P \leq 0.05$) with increasing dose of LPS at 12 hours and in AUC 0–36 hours.

Changes in blood components

As shown in Table 1, all investigated blood parameters did not show significant dose depend responses to IM LPS infusion. Among the investigated blood parameters dose depend changes were observed only tendentially ($P = 0.08$) in the ΔPMN levels at 12 hours and in the Δblood Na values in AUC 0–36 h of investigation. Thereby ΔPMN levels at 12 hours tended to decrease with increasing LPS doses and Δblood Na values in AUC 0–36 h tended to increase with increasing LPS doses.

DISCUSSION

To date, high IM injections of LPS at high concentration up to 500 μg/mammary gland quarter were generally used to induce IMI. Vangroenweghe et al. (2004) assayed the different effect of two high IM doses of LPS in primiparous cows. In order to show that also low doses of LPS provoke altered effects in udder parameters, this study evaluated dose dependent changes after IM infusion with five different doses (100 μg, 50 μg, 25 μg, 12.5 μg, and 6.25 μg LPS).

Two cows per LPS dose were infused in one mammary gland rear quarter the contra lateral quarter was infused with 10 ml saline as control. In one cow front quarters had to be used because only one rear quarter was milkable. Because of the small number of animals per group only the regression at 12 hours and over 0 to 36 hours (AUC 0–36 h) were tested for significance.

Our findings of a dose dependent increase in SCC within the first 36 hours of investigation and a significant regression to the 12 hours point agrees with results of Vangroenweghe et al. (2004). The significant regression of dose dependently increasing SCC within the first 36 hours (AUC 0–36 h) implies that there was a dose dependent TJ damage which means that the higher the dose of LPS the greater the influx of somatic cells. But different from the results in SCC of Vangroenweghe et al. (2004) and our study in quarters treated with 100 μg LPS, SCC values in LPS treatments with 6.25, 12.5, 25, and 50 μg had their maximal values after 12 hours of infusion. It is likely that the innate immune response was able to eliminate doses under 100 μg LPS faster than doses beyond.

The disaccharide lactose is only synthesized in the mammary gland and it is typical of all mammalians (Wheelock and Rook, 1966; Kuhn and Linzell, 1970). Our results of decreasing lactose values within the first 12 hours after IM LPS at doses from 12.5 to 100 μg LPS might be due to damaged TJ after IMI due to LPS (Stelwagen et al., 1997; Nguyen and Neville, 1998). In quarters infused with 6.25 μg LPS lactose values reached their maximal decrease 24 hours after the LPS challenge. In this case the TJ damage might be due to the cell influx and not by the infused LPS. Lactose with its small molecular weight is chiefly responsible for the osmotic activity of the milk and consequently it controls the influx of water into the milk (Linzell and Peaker, 1971). Changes of lactose in milk run always in contrast to Na and Cl levels and is responsible for the osmotic balance of milk (Mielke, 1986; Ontsouka et al., 2003).

Although Vangroenweghe et al. (2004) did not investigate EC levels, increasing Na and Cl levels in both groups (group A: 1×10^4 cfu; group B: 1×10^6 cfu) challenged with *E. coli* are similar to our changes in Na and Cl ($P \leq 0.05$) values. Therefore, because 60% of the EC of milk is due to Na, Cl and potassium levels (Schulz and Sydow, 1957) it explains the dose dependent increases we found in EC levels. Albeit our results of EC showed maximal changes for all doses 12 hours after IM injection of LPS. Our findings of increasing EC levels after an IM infusion of LPS also agree with results of others (Kitchen, 1981; Nielen et al., 1992; Blum et al., 2000; Bruckmaier et al., 2004).

It is well known that in infected quarters PMN are the predominant cell population whereas SCC in healthy quarters consist mainly of macrophages (Burvenich et al., 1994; Paape et al., 2002; Sarikaya et al., 2004). This may explain the tendential decrease we found in circulating PMN at 12 hours of investigation. Due to only small changes in the milk electrolytes the influence of different IM LPS doses showed only tendentially dose dependent changes

in blood Na levels within the first 36 hours (AUC 0–36 h) of investigation. We have to assume that the wide range of individual variance amongst all animals led to these unspecific results.

An explanation for our findings that higher IM LPS doses led to higher SCC might be attributable to the production of chemoattractants by cells in the mammary gland by LPS (Hoeben et al., 2000; Wittmann et al., 2002). Therewith, it led to a dose dependent damage of TJ. The damaged TJ itself potentiated the efflux of lactose and diametrical the influx of Na and Cl ions into the udder. This explained the higher EC after IM LPS (Wheelock et al., 1966). Although similar changes were observed after the IM infusion of 6.25μg LPS it seemed that lower LPS doses needed more time to establish endotoxin-like changes in the investigated parameters than higher doses.

CONCLUSION

In conclusion, the influx of cells and ions into the mammary gland after LPS treatment is dose dependent which is possibly caused by increased secretion of chemoattractants and cytokines like TNFα (Vangroenweghe et al., 2004), causing greater damage of TJ in high doses of LPS. Our results suggested that the dose of 100 μg LPS is like a threshold between high and low doses of IM LPS. Every LPS dose used in this study induced factors that contributed to subclinical mastitis such as increased SCC, decreased lactose and increased EC. To use the enhancing effect of LPS on the innate immune response for mastitis prophylaxis it might be necessary to investigate the dose, which has no clinical effects but may have a positive influence on the immune status of the bovine mammary gland.

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