Early Inflammatory Events of Mastitis - A Pilot Study With the Isolated Perfused Bovine Udder

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Research Article

Keywords: isolated perfused bovine udder, mastitis, lipopolysaccharide, inflammation

DOI: https://doi.org/10.21203/rs.3.rs-701430/v1

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Abstract

Background: Bovine mastitis is an important health and cost factor in the milk industry. To elucidate whether isolated perfused bovine udders can be used to study early inflammatory events of mastitis, 1 mg of lipopolysaccharide (LPS) was instilled into quarters of 10 isolated perfused bovine udders. Three hours and 6 hours after LPS instillation, tissue samples were taken from the gland cistern and base of the udder, subsequently stored in RNAlater and processed for the determination of inflammation-dependent gene regulation by real-time RT-qPCR. Gene expression analysis was performed using delta-delta Ct method. To translate mRNA results to protein, IL-1β and IL-6 were determined in tissue homogenate by ELISA.

Results: The instillation of 1 mg LPS lead to an increased expression of pro-inflammatory cytokines and chemokines like TNF-α, CCL20, CXCL8 as well as of IL-1β, IL-6 and IL-10, lingual antimicrobial peptide (LAP) and S100A9. However, the degree of elevation differed slightly between gland cistern and udder base and markedly between 3 hours and 6 hours after instillation, with a distinct increase in mediator expression after 6 hours. IL-1β protein increased in a time-dependent manner, whereas IL-6 was unchanged within 6 hours of LPS instillation.

Conclusion: Compared to in vivo studies with instillation of LPS into udders of living cows, a similar inflammation-dependent gene regulation profile can be mimicked in the isolated perfused bovine udder, indicating a supplementation of animal experiments.

Background

Mastitis, defined as an inflammatory process of udder tissue mainly due to bacterial infection, is a major health and cost burden in dairy cattle worldwide [1, 2]. Reduced milk production due to mastitis and its associated treatment is a substantial financial burden in the dairy industry. Infections with Escherichia coli, Streptococcus uberis and Staphylococcus aureus in particular differ in their immunological response and can lead to severe and or long lasting damage of the tissue, which is associated with high production losses [1, 3]. Clinical signs of mastitis include swelling due to painful edema, acute inflammation and, following chronic development, fibrosis of udder tissue [2].

Although recent gene expression profiles of tissue from mastitis cows have been performed, early events of infection and inflammation in particular are still not fully understood [4, 5].

Thus, we wanted to perform a proof of principle study to find out whether the isolated perfused bovine udder may be a suitable tool to study early inflammatory events in mammary tissue.

As a first stimulus, lipopolysaccharide (LPS) as part of negative cell walls from e.g. E. coli was used. The signal transduction of LPS is well characterized and includes an interaction with lipopolysaccharide binding protein, CD14, toll like receptor (TLR) 4 and Myeloid Differentiation factor 2 (MD-2) [6]. This in turn leads to the regulation of many genes involved in acute inflammation and infection. Data for LPS
and/or *E. coli* response exist *in vitro* in primary isolates of bovine mammary epithelial cells as well as *in vivo* in LPS instillation experiments in cows [7, 8]. Thus, we intended to assess the value of isolated perfused bovine udders as an *ex-vivo* model ranging in complexity between monolayer cell cultures and a living organism. The selection of possible regulated genes (cytokines TNFα, IL-1β, IL-6, IL-10, chemokines CCL20, CXCL8 and antimicrobial effector molecules LAP and S100A9) were adapted from a recent *in-vivo* study that also focussed on early inflammatory events in mastitis [4].

**Results**

**Regulation of inflammatory genes 6 hours after the instillation of 100 µg LPS**

In pilot experiments, different concentrations of LPS were tested. As 100 µg LPS were used in *in vivo* experiments [5], we started with 100 µg/quarter in 10 ml NaCl 0.9 %. Although there was a slight increase noticeable 6 hours after the instillation (Fig. 1, results for udder base are shown here), the overall response was only moderate. It was thus decided to increase the concentration of LPS to 1000 µg/quarter for the main experiment.

**Regulation of inflammatory genes 3 hours after the instillation of 1000 µg LPS**

There was generally an increase of transcription of pro-inflammatory mediators (particularly CCL20, LAP and S100A9). The overall response was marginally stronger on the udder basis (Fig. 2B) where two mediators (LAP and S100A9) were significantly increased, whereas in the gland cistern, (Fig. 2A), only S100A9 was significantly increased 3 h after the LPS instillation.

**Regulation of inflammatory genes 6 hours after the instillation of LPS**

The increase of transcription was more pronounced 6 hours after LPS the instillation. This became significant for CXCL8, IL-10, and antimicrobial effector molecules LAP and S100A9 in the gland cistern (Fig. 3A) and for CCL20, IL-10, LAP and S100A9 in the udder base (Fig. 3B).

**IL-1β and IL-6 in tissue homogenate 3 and 6 hours after the LPS instillation**

The concentration of bovine IL-1β increased in a time dependent manner in both the gland cistern and the udder base (Fig. 4A/B) and became significantly increased 6 hours after the LPS instillation. Although a measurable concentration of bovine IL-6 was observed in control samples, there was no further increase of IL-6 after 3 or 6 hours of incubation with LPS (Fig. 4C/D).

**Altered gene regulation in “mastitis” quarters**
During the study, five udder quarters were identified with a highly elevated cell count. These were not used for LPS experiments, but tissue samples were taken directly at the beginning of the perfusion, in order to evaluate whether these “mastitis” quarters show an altered expression of the selected cytokines.

Interestingly, the pattern of alterations were similar to that seen after the LPS instillation. However, the alterations in the udder base were much more pronounced compared to the altered expression at the gland cistern (Fig. 5A/B).

**Discussion**

The aim of the present study was to gain first proof of principle data in isolated perfused bovine udders as a potentially feasible model for early inflammatory events during mastitis. The hypothesis of the current study was that inflammatory responses in isolated perfused bovine udders range between simple two-dimensional cell cultures and complex *in vivo* experiments [4, 7].

It was stressed in former studies that the response of mammalian epithelial cells (MECs) to LPS or inactivated bacteria do not mimic all responses observed in the infected udder *in vivo*. One striking difference is the lack of IL-10 mRNA upregulation in MECs, whereas a strong induction was described in udders [11]. Even in the SV40 immortalized and often used bovine mammary alveolar cell line MAC-T, IL-10 was not detectable [12]. Thus, the increase of IL-10 observed in the isolated bovine udder indicates that the response is closer to the *in vivo* situation compared to simple 2D cells cultures [4]. In addition, the up-regulation of S100A9 is also very moderate in MEC compared to *in vivo* in udders. A first hint that S100A9 is significantly secreted by the cells of the udder was given by the results of Lind et al. (2015) [13]. After LPS stimulation, an up-regulation of S100A9 was observed using teat explants. Taken together, the response in the isolated udder might be closer to the *in vivo* situation. On the other hand, LAP, CCL20 and CXCL8 did belong to the top 20 upregulated genes in the pBMECs [11], as well. Although recent publications indicate that TNF-α is associated with an LPS-induced inflammatory response, Günther et al., (2009) [11] could not identify TNF-α as one of the top 20 up-regulated genes in their pbMEC studies. However, studies are contradictory: TNF-α detection was possible in experiments with the LPS-stimulated cell line MAC-T [14–16]. Lind et al (2015) [13] showed a TNF-α baseline and, in addition, a LPS-dependent TNF-α expression in primary cells using a teat explant model. In our results, only a slight increase in TNF-α was observed, however, results obtained by Rabot et al. (2007) [17] show similar findings. Based on literature, these results seem surprising and may be attributed to variable individual immune responses.

When first experiments in udders were accomplished, study results of *ex vivo* infection experiments in isolated perfused udders have been published that support the feasibility to use udders as a meaningful model [18, 19]. Nevertheless, we believe that the current data are still valuable, as the LPS stimulation allows a direct comparison with former *in vitro* and *in vivo* studies and helps to further evaluate the values of isolated perfused bovine udders in mastitis research.

In contrast to *in vivo* infection with *E. coli*, where an early response (1 and 3 hours after infection) was observed in the glandular cistern in particular, in the present LPS stimulation, there are only marginal
differences between responses in udder base and gland cistern after 3 hours. This might be explainable by the different cause of stimulation, instilled E. coli still grow during infection (5x 10^6 CFU at the beginning of infection to roughly 5x 10^8 CFU three hours after infection) [4]. This initial proliferation might mainly occur in the cistern, whereas the instillation of LPS in NaCl might lead to a faster more even distribution up to the udder base.

Limitations of the study

The great variability of response in isolated udders has to be seen as a limitation. Interestingly, there were no “low” and “high” responding udders in general (i.e. in one udder the CCL20 response was high, but the CXCL8 response low) but rather a variability within and between the udders. Thus, a sampling size of around 10 udders seems reasonable to gain robust responses.

Protein data show that mRNA and protein correlate, at least as shown here for IL-1β. Interestingly, we saw at least the tendency of an IL-6 increase, particularly after 6 hours (however, this did not become significant). This underscores that mRNA and protein data do not always have to correlate. Nevertheless, we investigated the presence of IL-6 and IL-1β by ELISA in one “mastitis” quarter. IL-6 was increased 7-fold in the gland cistern and a 3-fold increase was seen in the udder base compared to a healthy udder quarter. In contrast, IL-1β could not be detected (data not shown).

It is intended to use isolated udders for possible prevention experiments (e.g. testing of new anti-infective or anti-inflammatory strategies like biofilm preventer or bacteriophages, as well. Although there is a definite time limitation of maximal 8 hours of perfusion, these inflammatory response results support the usefulness of isolated perfused udders to study and modify early inflammatory processes of mastitis. For long-term examinations, the model of precision-cut bovine udder slices (PCBUS) can be used [20]. In this model, cells remain to their original location-specific composition and also resemble the in vivo situation. Additionally, more trails can be performed with tissue of one udder, since the generation of approximately 200 PCBUS from one udder quarter is possible. Filor et al (2021) [20] demonstrated a stimulability of PCBUS by LPS through the differential release of immune mediators by ELISA. The combined use of both models can achieve complementary results in the field of bovine mastitis for future studies.

**Conclusion**

These first proof of principle data indicate that early inflammatory responses of mastitis can be monitored in isolated perfused bovine udders and that this model can be used to supplement in vivo mastitis experiments.

**Material And Methods**

**Isolated perfused bovine udder**
The perfusion of bovine udders was performed as described for former studies [9, 10]. In brief, immediately after healthy cows had been slaughtered, the organs were collected. Only udders that had no visible skin lesions or pathological changes of the milk and glandular tissue (macroscopic and palpatoric examination) were utilized. To avoid clot formation, a first perfusion was performed at the slaughterhouse with roughly 2 liters of heparinized tyrode solution after the cannulation of the right and left external pudendal arteries. After transport to the institute, the mamma complexes were perfused via the external pudendal arteries with carbogen saturated tyrode solution possible by means of a peristaltic pump (Masterflex 7518 10; Cole Parmer Instr., Chicago, U.S.A.) with flux rate 100 to 120 ml per minute. The perfusion fluid was tempered at 38.5°C. The perfusion started within 45 to 60 minutes after slaughtering. For the determination of glucose consumption, a venous drainage via the *vena epigastrica cranialis superficialis* was performed. The viability of the perfused udder skin was demonstrated by a nearly unchanged glucose consumption.

**Challenge with lipopolysaccharide**

To exclude subclinical mastitis, cell count in milk was measured in each quarter with a Neubauer chamber. Only udder quarters < 100,000 somatic cells/ml were taken for instillation experiments.

LPS (O55:B5, Sigma-Aldrich, Steinheim, Germany), diluted in 10 ml sterile NaCl 0.9 % was instilled in two quarters of the udder. Different quarters were used for 3 hours experiments and 6 hours experiments. To exclude any influence of LPS on the untreated quarters, it was decided to take control samples from glandular cistern and udder base from one quarter directly before the instillation of LPS. In pilot experiments it was verified that the expression of the studied genes did not alter during 6 hours of perfusion (data not shown).

Udder tissue samples were taken from the glandular cistern and udder base 3 hours and 6 hours after LPS instillation. To minimize leakage after the incision of udders, the incisions for control samples and 3 hours after the instillation were closed by a simple suture technique.

**Samples from quarters with high cell count**

A few udder quarters had high or very high somatic cell counts (between $2 \times 10^5$ and $5 \times 10^6$ cells/ml) and thus were not used for LPS instillation trials. However, tissue samples were taken directly at the beginning of the perfusion from these quarters to evaluate whether these “mastitis” quarters show an altered expression of the selected inflammation associated genes.

**Isolation of mRNA and RT-qPCR technique**

Excised udder tissue (ca. 50 mg) was stored in RNAlater (Thermo Fisher Scientific GmbH, Dreieich, Germany) at 4°C overnight and then frozen at -80°C until further analysis. For mRNA extraction, samples were homogenised by means of a T 25 Ultra-Turrax (Ika, Staufen, Germany) on ice, followed by a further homogenization with a sterile syringe and cannula. Lysis and mRNA isolation (including DNA digestion) was performed with RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer’s protocol. Transcription to cDNA was carried out with QuantiTect Rev. Transcription Kit (Qiagen) and cDNA was
stored at -20°C. The quality and purity of cDNA was evaluated at 260/280 nm wave-length (~ 1.8) and
260/230 nm wave-length (~ 2.0–2.2).

For RT-qPCR Maxima, SYBR Green/Fluorescein RT-qPCR-Mastermix was used with a cDNA concentration
of 200 ng/sample. Selected primers and sources are presented in Suppl. Table 1. For RT-qPCR 40 cycles
were conducted, after an initial denaturation at 95°C for 10 min as follows: denaturation at 95°C for 15
sec. followed by annealing and extension at 60°C for 60 sec. At completion, melting curves were
monitored for quality assurance and also, gels were run to confirm the correct amplification size.

An analysis of experiments was performed according to the delta/delta CT method. As house-keeping
genes Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18s rRNA were compared. GAPDH led
to very robust results and thus was used for all further analysis as house-keeping gene.

**Protein extraction and determination of IL-1β and IL-6 by ELISA**

About 50 mg of glandular tissue sample were taken from udders before, 3 hours and 6 hours after LPS
instillation and instantly frozen at -80°C. As it was decided to take samples for protein analysis after the
experiments already started, only samples from 5 udders were available for 6 hours LPS instillation.

Samples were thawed in 1 ml T-PER™ Tissue Protein Extraction Reagent + Protease Inhibitor (Thermo
Fisher Scientific) and homogenised with the T 25 Ultra-Turrax (Ika) on ice.

After centrifugation (3000g, 10 min, 4°C) protein content was measured (BCA Protein Assay Kit, Cell
Signaling Technology Europe B.V., Frankfurt am Main, Germany) in supernatant and samples stored at
-20°C. Concentrations of bovine IL-1β (Thermo Fisher Scientific GmbH) and IL-6 (Duo Set, R&D Systems,
Minneapolis, USA) were determined by ELISA according to the manufacturer’s protocol.

**Statistical analysis**

The results were analysed to determine whether there was a significant difference in mRNA and protein
content between untreated and LPS treated udder quarters (gland cistern and udder base) 3 hours and 6
hours after the instillation. Three hours vs. control and 6 hours vs. control were compared with the non-
parametric test (Mann Whitney U-test) for significant difference. P < 0.05 was set as significance level.

**Abbreviations**

GAPDH
glyceraldehyde-3-phosphate dehydrogenase, IL:interleukin, LAP:lingual antimicrobial peptide,
LPS:lipopolysaccharide, MEC:mammalian epithelial cell, NaCl:sodium chloride, PCBUS:precision-cut
bovine udder slices, TLR:toll like receptor

**Declarations**
Competing interests

The authors declare no financial or non-financial competing interests.

Funding

KB was supported by a grant of the Federal Office of Consumer Protection and Food Safety within a pharmacovigilance program (FUB no. 2017000383).

Author contributions

KB performed most of the experiments, was involved in data analysis and interpretation.

VF performed part of the experiments, was involved in data analysis and interpretation, wrote part of the manuscript.

WB: designed the study, supervised KB during her experiments, performed interpretation of data, wrote most of the manuscript. All authors read and approved the manuscript.

Acknowledgements

We thank Wiebke Gentner for proofreading the manuscript.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

Wolfgang Bäumer is a member of the editorial board of BMC Veterinary Research.

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Figures
Figure 1

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Figure 2

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Figure 3

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Figure 4

Concentration of IL1-β in tissue of gland cistern (A) and udder base (B) and of IL-6 in tissue of gland cistern (C) and udder base (D) 3 h and 6 h after instillation of 1000 µg LPS into the udder quarters. N= 5 (6 h) to 10 (control and 3 h), data are given as median and all the samples analysed are represented as dots. *p<0.05, **p< 0.01
Figure 5
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