INTERPRETIVE SUMMARY

**Effects of local or systemic administration of meloxicam on the mammary gland inflammatory responses to LPS-induced mastitis in dairy cows. by Caldeira et al.**

Meloxicam (MEL), a nonsteroidal anti-inflammatory drug is commonly used for treatment of mastitis in dairy cows to reduce pain and hence support animal welfare. We investigated if MEL administered intramammarily and systemically affects the immune response of the mammary gland. Meloxicam itself did not promote inflammation in the mammary gland. During LPS-induced mastitis, MEL did not diminish the concentrations of markers for blood-milk barrier integrity and immune components of milk, nor the mRNA abundance of pro-inflammatory factors in the mammary tissue. Instead, intramammary MEL expedited the somatic cell count increase during the challenge with low LPS dose.

MELOXICAM AND MASTITIS IN DAIRY COWS

**Effects of local or systemic administration of meloxicam on the mammary gland inflammatory responses to LPS-induced mastitis in dairy cows**

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**ABSTRACT**

Nonsteroidal anti-inflammatory drugs (**NSAID**) are commonly used in combination with antimicrobial mastitis treatments to reduce pain. Little is known if meloxicam (**MEL**), an NSAID designed for a preferential inhibition of cyclooxygenase (**COX**)-2 over COX-1, affects the mammary immune response. The objective was to analyze the mammary immune response to intramammary (local) or intravenous (systemic) administration of MEL with or without immune activation by lipopolysaccharide (**LPS**). We challenged 108 quarters of 30 cows with or without a lower or a higher dosage of LPS from *E. coli* (0.1 or 0.2 µg/ quarter) concomitantly with or without an intramammary administration (50 mg/ quarter) or intravenous (**iv**) injection (0.5 mg per kg of body weight; ~300mg/ cow) of MEL. The intramammary administration of MEL alone did not trigger an acute inflammatory response, verified by unchanged somatic cell count (SCC), lactate dehydrogenase (LDH), serum albumin (BSA) and immunoglobulin (Ig)G concentration in milk, which are normally augmented during mastitis due to an opening of the blood-milk barrier. Similarly, intramammary MEL did not change the mRNA abundance of inflammatory factors in the mammary gland tissue. As expected, quarters challenged with either dose of LPS had increased leukocyte infiltration (SCC), LDH, BSA, IgG, Na, and Cl and diminished K concentrations in milk. Different than hypothesized, the addition of intramammary or iv MEL did not reduce these markers of mastitis in milk. Instead, intramammary MEL appeared to accelerate the SCC response to LPS, but only at the lower LPS dose. Moreover, the mRNA expression of various inflammatory factors in mammary tissue was not modified by the additional intramammary application of MEL compared to contralateral quarters challenged with LPS only. We demonstrated for the first time that intramammary MEL, at a dose of 50 mg/ quarter, did not trigger an immune response in the mammary gland of dairy cows. At the used doses, intramammarily or systemically administered MEL did not lower the inflammatory responses. The intramammary administration of MEL seemed to rather stimulate the leukocyte recruitment into milk in quarters challenged with a low dose of LPS. The blood-milk barrier integrity was not protected by MEL in LPS stimulated quarters. The study provides first indications that MEL does not limit the inflammatory response in the mammary gland, but it also does not impair the mammary immune system.

**INTRODUCTION**

Mastitis in dairy cows is frequently treated with nonsteroidal anti-inflammatory drugs (**NSAID**), in addition to antimicrobials, to decrease the inflammatory response and pain, improving animal welfare (Kayitsinga et al., 2017). In cows diagnosed with mild to moderate clinical mastitis, the addition of the NSAID meloxicam (**MEL**) to parenteral antibiotic treatment resulted in lower somatic cell count (**SCC**), an increased probability of bacteriological cure, and diminished the likelihood of culling compared to cows treated only with antibiotics (McDougall et al., 2009, 2016). Furthermore, cows that received MEL in addition to antibiotics as treatment for mastitis in early lactation showed an improvement in conception rate resulting in economic benefits to the farmer (van Soest et al., 2018).

Inflammation of the mammary gland is mainly caused by various bacterial pathogens entering the teat canal, such as *E. coli* (Bannerman et al., 2004; Bruckmaier and Wellnitz, 2017). The pathogen-associated molecular pattern lipopolysaccharide (**LPS**) from *E. coli* can be used to trigger an immune response comparable to a bacterial intramammary infection (Schmitz et al., 2004). After infusion of LPS into the mammary gland, the common effects of mastitis such as the disruption of the blood-milk barrier are observed. As a consequence, there is a passage of blood components to milk (e.g. SCC, serum albumin [**BSA**], lactate dehydrogenase [**LDH**], and also immunoglobulins [**Ig**]), changing its composition (Wellnitz et al., 2011; Lehmann et al., 2013; Wall et al., 2016). Once the LPS is recognized by pattern recognition receptors, the innate immune system responds by promoting inflammation. This includes an upregulation of pro-inflammatory cytokines (e.g. tumor necrosis factor [**TNF]**),and chemokines (e.g. interleukins [**IL**]) production (Lawrence, 2009; Holdsworth and Gan, 2015). Additionally, the secretion of acute-phase proteins, such as serum amyloid A (SAA) increases up to 1000-fold during inflammation (Uhlar and Whitehead, 1999; Cheng et al., 2018), further promoting cytokines production that increases the influx of leukocytes to the mammary gland facilitating phagocytosis and clearance of bacterial pathogens (Sordillo, 2018). Mastitis severity, duration, and resolution, as well as restoration of mammary gland function, are dependent upon the efficiency of the innate immune response (Aitken et al., 2011).

Prostaglandins (**PG**) also play a key role during intramammary infections, acting as pro-inflammatory mediators. Two cyclooxygenase (**COX**) isoenzymes, COX-1 and COX-2 participate in the conversion of arachidonic acid to PGH2 (precursor for PGE2 synthesis). While COX-1 is constitutively present in most cells, COX-2 is the inflammation-induced form (Gilroy et al., 1999). Nonsteroidal anti-inflammatory drugs are COX inhibitors that block prostaglandin synthesis, alleviating the inflammatory symptoms and pain (Tegeder et al., 2001; Ricciotti and FitzGerald, 2011). Intramammary administration of ketoprofen, a non-selective NSAID, in cows challenged with LPS showed positive results in lessening detrimental effects of mastitis on the blood-milk barrier (Dan et al., 2018). Because non-selective NSAID suppress the constitutive COX-1, side effects such as the disturbance of normal gastrointestinal mucosal protective mechanisms are expected, and alternative NSAIDs have been developed.

Meloxicam is an NSAID designed to be a COX-2 selective inhibitor to avoid the adverse effects of COX-1 binding (Engelhardt et al., 1996a; b). *In vitro*, MEL suppressed the increase of inflammatory factors at the mRNA level, and the PGE2 synthesis of bovine mammary epithelial cells challenged with LPS (Caldeira et al., 2019). In the same study, the authors also reported a decrease in mammary epithelial cells viability with the addition of MEL (Caldeira et al., 2019), therefore the possibility of tissue irritation *in vivo* should be considered. Shock et al. (2018) conducted a field trial with 2,653 cows from 20 different herds and showed that animals treated orally with 1.0 mg/kg of body weight of MEL were less likely to have subclinical mastitis at calving. Moreover, cows treated prepartum with MEL orally had greater milk yield compared to non-treated cows (Swartz et al., 2018).

Intramammary administration of therapeutics is routinely used in dairy farms as a method to deliver treatments into the mammary gland. Besides the practicality of the administration through the teat canal, local administration of treatment usually requires lower medication. Although the outcome of systemic administration of MEL together with antibiotics on cows are well compiled, the effect of intramammary administration of MEL on the mammary gland immune response during mastitis is unknown.

Considering the worldwide emphasis to decrease the use of antibiotics in animal production, the aim of this study was to evaluate the immune responses of the mammary gland to local (intramammary) or systemic (intravenous) administration of MEL with or without different levels of immune activation by LPS. We hypothesized that administration of MEL (intramammarily or intravenously) reduces the inflammatory processes of LPS-induced mastitis, while the competence and integrity of the blood-milk barrier is maintained.

**MATERIALS AND METHODS**

***Animals***

The animal trials followed the Swiss Law of animal protection and the Committee of Animal Experiments (Fribourg, Switzerland) approved all procedures involving animals (#27909). Thirty Holstein dairy cows in mid lactation (118 ± 32 DIM) and with milk production > 15 kg/d were enrolled in this study. Parities ranged from 2 to 6. Cow’s body weight averaged 653 ± 33 kg. The cows showed no signs of clinical mastitis and quarters of each cow had a SCC < 150,000 cells/mL before starting the experiment. Animals were housed at the Agroscope Research Station (Posieux, Switzerland) in tie stalls, fed hay *ad libitum* + 1 kg of energy concentrate daily, and had free access to water. Cows were milked twice daily at 0530 and 1600 h.

***Experimental design***

Thirty cows were randomly assigned to one of four experiments (Figure 1A). Six cows each were allocated into experiment 1 – 3. Twelve cows were included into experiment 4, because this experiment included three treatments, requiring six pairs of quarters. The front quarters were used for milk sampling, while the rear quarters were used for mammary gland tissue biopsies.

The treatments administered intramammarily consisted of **CON** (10 mL of 0.9% sterile saline), **MEL-imm** (50 mg of meloxicam sodium salt hydrate [Sigma-Aldrich] diluted in 10 mL of double distilled water as described in Caldeira et al. (2019)), **LPS-L** (0.1 μg/ quarter of LPS from *E. coli* [serotype O26:B6, Sigma-Aldrich, Buchs, Switzerland] diluted in 10 mL of 0.9% sterile saline), and **LPS-H** (0.2 μg/ quarter of LPS from *E. coli* [serotype O26:B6, Sigma-Aldrich, Buchs, Switzerland] diluted in 10 mL of 0.9% sterile saline). The treatment for intravenous injection was **MEL-iv** (0.5 mg of meloxicam [Contacera, Zoetis, Delémont, Switzerland] per kg of body weight) into the jugular vein. All treatments were prepared in different syringes with the final volume of 10 mL each. The treatments were then distributed into the four experiments as following (figure 1A):

***Experiment 1 (n = 6).*** In this experiment, the objective was to analyze the local effects of MEL on the mammary gland without immune activation. In each cow, two quarters received CON and the other two quarters received MEL-imm, all administered intramammarily. Two biopsies (before and after treatment) were performed in both rear quarters.

***Experiment 2 (n = 6).*** With similar aims, the objectives of experiment 2 and 3 were to understand if and how MEL affects the mammary gland that was immune stimulated with different doses of LPS. All four quarters received intramammary injections of LPS-L, followed by CON (two quarters) or MEL-imm treatments (two quarters). No biopsies were taken in this experiment because comparable results as in experiment 3 were expected.

***Experiment 3 (n = 6).*** In this experiment, all four quarters received LPS-H intramammary injections, followed by CON (two quarters) or MEL-imm (two quarters). Two biopsies (before and after treatment) were performed in both rear quarters.

***Experiment 4 (n = 12).*** The objective of this experiment was to report the NSAID effects on the mammary gland when MEL is delivered systemically. Because this experiment included three treatments (refer to figure 1A), which required six pairs of quarters, cows in experiment 4 were divided into two groups: experiment 4TRT (n = 6) and experiment 4CON (n = 6). In experiment 4TRT, two quarters on the one side received CON and two quarters of the other side were infused with LPS-H intramammarily, followed by a systemic MEL-iv injection (0.5 mg of meloxicam [Contacera, Zoetis, Delémont, Switzerland] per kg of body weight iv). From the rear quarters two biopsies (before and after treatment) were taken. In experiment 4CON, two quarters received LPS-H intramammarily without systemic administration of MEL-iv.

All intramammary infusions were performed shortly after the morning milking (0 h). Each teat end was scrubbed with 70% ethanol until clean and intramammary infusions were administered using a sterile teat cannula. All infusions were followed by 20 s massage in cisternal direction. In experiment 2 and 3, the quarters were first infused with the LPS (low or high doses), immediately followed by CON or MEL-imm injections. In experiment 4, the quarters were challenged with CON or LPS-H, concomitantly with an intravenous injection of MEL in one jugular vein.

***Milk Sampling and Analyses***

Quarter milk sampling (~10mL) was performed by hand. At 0 h (before intramammary injections) milk samples were taken and used as baseline samples. After that, milk samples were collected every 30 minutes up to 6 h, and at 9 h post-challenge (Figure 1B). Milk somatic cells count (**SCC**) was measured immediately after each collection using DeLaval cell counter (DCC, DeLaval, Tumba, Sweden) and then samples were stored at -80°C until further analyses. Milk concentrations of BSA and total IgG were measured in duplicates in Synergy Mx plate reader (BioTek Instruments, Winooski, VT) using ELISA kits E10-113-39 and E10-118-34 (Bethyl Laboratories, Montgomery, TX), respectively, according to the manufacturer’s protocol. Inter- and intra-assay coefficient of variation were 3.7% and 7.5% for BSA, respectively, and 6.1% and 6.0% for IgG, respectively.

For the other measurements, the milk was thawed, and serum was obtained by two steps centrifugation: 1,900 × g for 15 min at 4°C and then at 20,800 × g for 30 min at 4°C. The LDH activity and electrolyte concentrations (Na, K, Cl) in milk serum were determined with commercial kits AXON00025 (Axon-Lab AG, Baden, Switzerland) and Mira ISE module DIA50100A1 072008 (Diatools AG, Villmergen, Switzerland), respectively, following the manufacturer's instructions using an automated analyzer (Cobas Mira, Roche Diagnostics, Basel, Switzerland).

***Mammary Gland Tissue Biopsies***

Mammary gland tissue biopsies (approximately 40 mg) of the rear quarters were taken from cows in experiments 1, 3, and 4TRT at –24 h prior treatments and at 6 h post-challenge. The udders were cleaned and clipped. To avoid injuring large blood vessels and cisternal region, the biopsy location was carefully selected by sonography. The biopsy site was cleaned and rubbed with alcohol 70%. Ten minutes before the procedure, rear quarters received local anesthesia using a subcutaneous injection of ~ 3 ml of lidocaine HCl 2% (Streuli Pharma AG, Uznach, Switzerland). A small incision (~ 0.5 cm) was made in the skin for easier penetration of the biopsy needle. Two biopsies (at -24 h and 6 h post-challenge) per rear quarter were performed using the Bard Magnum biopsy instrument (Bard GmbH, Karlsruhe, Germany) and a 12 ga x 13 cm biopsy needle (Bard Magnum Core Tissue Biopsy Needle; Bard GmbH). For the second mammary tissue biopsy, the angle of the needle entrance was slightly changed to obtain tissue from a close but not disturbed region of the udder. Immediately after acquiring the tissue, samples were placed into an RNA stabilization solution (RNAlater; Invitrogen, Carlsbad, CA, USA) and stored at −80°C until RNA extraction.

***RNA Extraction and Quantitative Real-time PCR***

 To obtain the RNA from the biopsy tissue, the samples were placed into a 1.5 mL screw cap microtube (BRAND GmbH, Wertheim, Germany) with glass microbeads and 1 ml of peqGOLD Trifast (PEQLAB Biotechnologie GmbH, Erlangen, Germany). After 5 min in a homogenizer (Mini-BeadBeater, BioSpec Products, Bartlesville, OK, USA), the majority of tissue was homogenized and the RNA extraction was performed using Direct-zol RNA MiniPrep (Zymo Research Corp. Irvine, CA, USA) following manufacturer’s instructions. Samples were eluted with 20 µL of RNA free water and total RNA concentrations and purity were determined using a NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) with an absorbance at 260 nm and 280 nm. The cDNA, quantitative real-time PCR, calculation of cycle threshold (**Ct**) values, and normalization of quantitative real-time PCR were performed as previously described by Caldeira et al. (2019). Primer sequences are shown in Table 1. The ∆Ct of target genes were normalized using the equation: ∆Ct = [Ct (gene of interest) – Ct (mean of housekeeping genes) \* -1] + 20. The ∆∆Ct were calculated by subtracting the ∆Ct of 6 h post-challenge biopsies from the ∆Ct of -24 h prior treatment biopsies of each quarter.

***Statistical Analyses***

All data were analyzed with SAS (version 9.4; SAS Institute Inc., Cary, NC, USA). Differences were considered significant at *P* < 0.05, and data are reported as means ± SEM. The SCC and LDH measurements were transformed and are presented in a log10 scale. For the milk analyses, a repeated measurement mixed model analysis using the MIXED procedure was performed to examine the effects of MEL on the SCC, BSA, and IgG concentrations, and LDH activity. Each analysis was performed within the respective experiment. The model included treatment, time, and their interaction as fixed effects, and cow as the experimental unit. The repeated statement had cow as the specific term and the compound symmetry structure was used in the model. Differences between LSMEANS were determined by Tukey-Kramer test.

To investigate the effects of MEL on the mammary tissue mRNA expression of different pro- and anti-inflammatory factors, the TTEST procedure was used. Factors were evaluated separately, and treatments within experiments were compared as paired observations.

**RESULTS**

***Milk Composition***

Results of the statistical evaluation of blood-milk barrier integrity and inflammatory markers in milk are presented in Table 2.

In all challenged quarters, LPS consistently induced local inflammation and opening of the blood-milk barrier as indicated by increased SCC and concentrations of blood components (BSA, LDH, and IgG) in milk.

***Experiment 1.*** Treatment (CON vs MEL-imm), time and their interaction (treatment × time) had no significant effect on SCC, LDH, and BSA (Figures 2A, 3A, and 4A, respectively). The IgG concentrations were greater (Figure 5A) in milk from quarters intramammarily injected with CON (0.35 ± 0.03 mg/mL) compared to the MEL-imm (0.31 ± 0.02 mg/mL) treatment. Electrolytes (Na, K, and Cl) were not affected by treatment or treatment × time. Time, however, significantly affected milk Na, K, and Cl concentrations (Table 2 and Figure 6). The Na concentrations increased (Figure 6A) and K concentrations decreased (Figure 6D) at 0.5 h (Na: 34.33 ± 3.36 mmol/L and K: 31.12 ± 1.50 mmol/L) compared to 0 h (Na: 22.42 ± 3.36 mmol/L and K: 37.41 ± 1.50 mmol/L; *P <* 0.02).

***Experiment 2.*** Treatment × time was not different (Table 2) in SCC, BSA, IgG (Figure 2B, 4B, and 5B, respectively) or electrolytes (Figure 6B, 6E and 6H). At 9 h post-challenge, LDH concentrations were significantly different (*P <* 0.01; Figure 3B) between treatments (LPS-L + CON: 5.74 ± 0.21 log10U/L vs LPS-L + MEL-imm: 6.12 ± 0.28 log10U/L). There was an overall treatment effect on SCC, where quarters that received LPS-L + MEL-imm as treatment (5.85 ± 0.09 log10/mL) had a greater SCC (*P <* 0.05) than LPS-L + CON quarters (5.71 ± 0.06 log10/mL). Compared to the baseline (0 h), the SCC was significantly greater (*P <* 0.05) at 6 h and 9 h in LPS-L + CON, while in LPS-L + MEL-imm, the SCC was already elevated at 3.5 h and 4.5 h until 9 h (Figure 3B). There was an overall treatment effect (*P =* 0.03; Table 2) on Na concentrations (Figure 6B), showing that LPS-L + MEL-imm (40.38 ± 2.16 mmol/L) quarters had higher Na concentrations compared to LPS-L + CON (34.34 ± 1.51 mmol/L). Time significantly influenced all milk components in response to LPS stimulation (Table 2). The electrolytes Na and Cl were increased at 9 h (61.75 ± 5.25 mmol/L and 57.42 ± 3.05 mmol/L, respectively), while K was decreased at 9h (27.21 ± 2.65 mmol/L) compared to 0h (Na: 36.60 ± 5.40 mmol/L, Cl: 39.82 ± 3.18 mmol/L, and K: 34.40 ± 2.68 mmol/L; *P <* 0.02; Figure 6).

***Experiment 3.*** Treatment (LPS-H + CON vs LPS-H + MEL), and treatment × time had no impact on all milk constituents evaluated (Figures 2 to 6). There was a time effect for all analyzed milk components (Table 2) in response to the immune activation by LPS. In both treatments, the SCC increased from 3.5 h and remained high until 9 h post-challenge (Figure 2C), when compared to the baseline (0 h; *P <* 0.0001). For LDH, this effect was only seen at 9 h post-challenge (Figure 3C), while in IgG, it occurred at specific timepoints (3 h, 4 h and 9 h post-challenge; Figure 5C) when compared to 0 h (*P <* 0.05). For the electrolytes, there was an overall time effect on Na, Cl, and K (*P <* 0.0001; Figure 6C, 6F, and 6I). The Na and Cl concentrations in milk were increased (*P <* 0.05) at 9 h (49.67 ± 3.69 mmol/L and 49.92 ± 3.83 mmol/L, respectively) compared to the baseline, and K concentrations were decreased (*P <* 0.05) at 9 h (29.14 ± 1.65 mmol/L) in comparison to 0 h (Na: 24.75 ± 3.69 mmol/L, Cl: 34.83 ± 3.83 mmol/L, and K: 36.99 ± 1.64 mmol/L, respectively).

***Experiment 4.*** Somatic cell count was similar (*P =* 0.16) in quarters with LPS-H + MEL-iv and LPS-H (without MEL systemically). Both treatments had statistically greater SCC from 3 h (LPS-H + MEL-iv) and 3.5 h (LPS-H) post-challenge until the end of the experiment (9 h) in comparison to CON + MEL-iv (*P <* 0.0001; Figure 2D). Compared to the baseline (0 h), the mammary quarters that received LPS-H started increasing SCC at 3 h and remained elevated until the end of experiment (*P <* 0.05), as expected after LPS challenges. The LDH concentrations were greater in LPS-H + MEL-iv from 4.5 h up to 6 h post-challenge and in LPS-H at 9 h post-challenge when both are compared to CON + MEL-iv (*P <* 0.0001; Figure 3D). A treatment × time difference (LPS-H + MEL-iv vs CON + MEL-iv) was only detected in BSA and IgG concentrations at 4.5 h after challenge (*P <* 0.05; Figures 4D and 5D, respectively). At 4.5 h, BSA (1.19 ± 0.50 mg/mL) and IgG (0.88 ± 0.25 mg/mL) concentrations in LPS-H + MEL-iv were significantly greater (*P <* 0.05) compared to 0 h (0.20 ± 0.04 mg/mL and 0.37 ± 0.07 mg/mL, respectively). Unfortunately, the BSA and IgG results for LPS-H could not be evaluated as samples were lost.

***Mammary Gland Gene Expression***

 Results of the gene expression (∆∆Ct) of pro- and anti-inflammatory factors analyzed in the mammary gland tissue collected from experiment 1 (CON vs MEL-imm), experiment 3 (LPS-H + CON vs LPS-H + MEL-imm) and experiment 4 (CON + MEL-iv vs LPS-H + MEL-iv) are presented in Table 3.

 Besides the increased mRNA abundance of IL-6 (*P =* 0.01; Table 3) and HSP70 (*P =* 0.02; Table 3) in the MEL-treated mammary gland compared to CON, no other significant difference could be found in experiment 1 (*P >* 0.05). In experiment 3, the mRNA abundance of all factors analyzed were similar between LPS-H + CON and LPS-H + MEL-imm treatments. In experiment 4, the TNF (*P =* 0.02), IL-1 (*P =* 0.02), and IL-10 (*P =* 0.01) mRNA expressions were upregulated and COX-1 (*P =* 0.04) mRNA expression was downregulated in LPS-H + MEL-iv when compared to CON + MEL-iv treatments (Table 3).

**DISCUSSION**

Our study was, to our knowledge, the first to examine short term effects of local meloxicam administration on the immune system of the mammary gland and to relate these results to the standard systemic MEL administration. In dairy farms, the intramammary administration of medication is a common practice due to its easiness, safety, and lower labor intensity compared to iv drug administration. Furthermore, a local administration usually requires a lower dose of the drug.

The intramammary administration of meloxicam, at a dose of 50 mg/ quarter without additional LPS challenge, did not activate an immune response itself as the number of somatic cells, IgG and BSA concentrations, and LDH activity in milk, which are typically augmented when the immune system is triggered (Wellnitz et al., 2010, 2011, 2013), remained unchanged. In addition, gene expression of key inflammatory factors, such as TNF, IL-8, SAA3, and INOS were similar in MEL-imm and CON. Solely the mRNA abundance of the cytokine IL-6 and HSP70 were upregulated in treated quarters, which may indicate a slight immunological response of the mammary tissue to the intramammary application of MEL. In the used dose, MEL alone did not cause a local inflammatory reaction of the mammary gland tissue. The selected intramammary dose of 50 mg was approximately a sixth compared to the iv dosage (0.5 mg/kg body weight); however, the MEL distribution throughout the body after the intramammary administration is yet not known. In conclusion, our results confirm those of an earlier *in vitro* experiment, where mammary epithelial cells were cultivated in the presence of different doses of MEL, with or without stimulation by bacterial antigens, and the NSAID alone did not activate an immune response, i.e. increased cytokine expression (Caldeira et al., 2019).

The challenge with LPS is a model of mastitis comparable to an infection with a maximal stimulus at the time of LPS infusion. Therefore, meloxicam and LPS were administered simultaneously. Cows did not develop fever (data not shown) after the LPS challenge indicating that the immune activation was restricted to the mammary gland. As expected, all quarters that were challenged with LPS responded with an increase of SCC in milk, which agrees with previous works (Wellnitz et al., 2011). Contrary to our hypothesis, intramammary injection of MEL did not diminish leukocyte infiltration (measured as SCC) into quarters that received LPS-H or LPS-L. Surprisingly, MEL-imm increased the overall SCC response, but only at the lower LPS dose, even though MEL alone (without additional LPS challenge) did not induce a greater SCC. This could indicate that intramammary MEL may stimulate the leukocyte recruitment into the mammary gland improving its immune competence, but only if the immune system is already activated. We speculate that after the challenge with the higher LPS dose, the leukocyte recruitment was already at a maximum, and, therefore, an accelerated increase of SCC could not be detected. However, additional research is needed to confirm this hypothesis.

As demonstrated before, the integrity of blood-milk barrier is affected during acute bacterial (Bannerman et al., 2004) or LPS-induced (Wall et al., 2016) mastitis, and as consequence, a transfer of blood constituents into milk is observed (Lehmann et al., 2013). This includes immunoglobulins, which are increased in milk during mastitis, acting as opsonins to improve recognition and phagocytosis of bacteria by macrophages and neutrophils (Aitken et al., 2011). Our results showed that in quarters that were immune activated by LPS the concentrations of IgG increased over time, but when MEL was intramammarily injected into the same quarters, milk IgG concentrations were not lowered. Moreover, milk concentrations of LDH and BSA increased over time showing the disruption of the blood-milk barrier in quarters challenged with LPS with high variations between quarters at the end of the experiment. However, when MEL-imm was added to the LPS challenge, it did not prevent the opening of the barrier. These findings differ considerably from the results of Dan et al. (2018) that had a similar experimental design to ours, but infused a non-selective NSAID (50 mg of ketoprofen) into quarters that were immune activated by LPS. In their study, ketoprofen was able to reduce the increase in SCC and to maintain the blood-milk barrier integrity. The main differences between the two mentioned NSAID are the COX-binding preference (MEL is selective towards COX-2, and ketoprofen binds to COX-1 and COX-2), the bioavailability (orally; MEL, 89% and ketoprofen, 100%), and MEL has a longer half-life (15-20 h) compared to the 2 h of ketoprofen (Calatayud and Esplunges, 2016). Although nothing is known about the bioavailability of these two drugs after intramammary administration, it is possible that these particularities of ketoprofen and MEL may be the reason for discrepancies in the results of the two studies.

Further markers for the blood-milk barrier integrity are electrolytes in milk. The relationship between mastitis and milk electrolytes, such as Na, K and Cl, was well reported by Fernando et al. (1985). In healthy quarters, the ion concentrations of Na and Cl in milk is much lower than in blood. During mammary inflammation, due to disruption of the blood-milk barrier, blood components travel freely to milk, increasing concentrations of Na and Cl, and decreasing K concentrations in milk (Nguyen and Neville, 1998; Bruckmaier et al., 2004). The initial differences (0 h to 0.5 h post-challenge) of the milk electrolyte concentrations in our experiments likely occurred due to the intramammary injections of MEL and LPS that were dissolved in 10 mL of 0.9% of saline solution or double distilled water. After this period, our results showed that MEL alone did not change Na, Cl, and K concentrations indicating that, at the dose of 50 mg/ quarter, the NSAID does not alter the integrity of the blood-milk barrier.

In veterinary practice, NSAIDs are usually administered iv, and for this reason, we compared our results from intramammary administration of MEL to the iv administration. Using the dose recommended for cattle by the manufacturer (0.5 mg MEL/ kg of body weight), no difference was observed between the SCC and LDH of cows that were challenged with LPS and received MEL-iv or not.

McDougall et al. (2009) analyzed the effects of MEL at 7-, 14- and 21-days after the NSAID treatment and detected lower SCC when cows received MEL subcutaneously together with intramammary antibiotics. In general, we did not detect lower SCC concentrations after MEL administration. One possible explanation is that our cows challenged with LPS had an SCC above one million/mL and maybe at this threshold, MEL could not counterbalance these harmful effects. Besides that, our study was designed to evaluate if MEL plays a role in the short term changes (one day) of milk components during LPS-induced mastitis. In a more recent study, McDougall et al. (2016) treated cows diagnosed with mild to moderate mastitis with MEL subcutaneously (together with antibiotics) at the same dose as in our experiment 4 (MEL-iv). Similar to our results, the authors found no significant change in SCC of cows treated with the NSAID compared to the non-treated cows. When these studies are considered together, intravenous administration of MEL does not markedly affect SCC in the short-term, acute inflammatory response.

The present study shows for the first time the effects of intramammary MEL administration to dairy cows on mammary gene expression. The mRNA abundance of cytokines (TNF, IL-1, -6, -10, and TGFB1) and chemokines (IL-8 and CXCL5) were similar in quarters that received LPS-H with or without MEL-imm. Contrary to what was found in this study, the addition of MEL *in vitro* to mammary epithelial cells downregulated the activation of pro-inflammatory cytokines upon LPS challenge in a dose dependent manner (Caldeira et al., 2019). Cytokines guide and direct the host’s immune response during phagocytosis and bacterial clearance (Aitken et al., 2011). The increase of pro-inflammatory cytokines in LPS-challenged cells and tissues from the mammary gland of dairy cows was widely demonstrated *in vitro* (Wellnitz and Kerr, 2004; Pareek et al., 2005; Dan et al., 2018; Caldeira et al., 2019) and *in vivo* (Wellnitz et al., 2011; Zarrin et al., 2014). Moreover, the mRNA abundance of analyzed enzymes (COX-1, COX-2, PTGES, ALOX5, INOS, and CASP3), acute phase protein (SAA3) and heat shock protein (HSP70), which are important players in the inflammatory response, was not affected by LPS-H with or without MEL-imm. Although MEL inhibits COX-2 function, it did not affect the COX-2 mRNA expression in mammary cells indicating that the binding to the protein and inhibition of its function does not influence the level of transcription. This result was also shown previously *in vitro* (Caldeira et al., 2019). Accordingly, the lack of effects on pro-inflammatory gene expression is consistent with the physiological responses found in cows infused with LPS-H. Because biopsies were not performed in mammary glands that received LPS-L, we cannot infer that MEL-imm affects the mammary gene expression.

There is also a possibility that the used dose of 50 mg/quarter is not sufficient to affect more proximal regions of the parenchyma. Even though the NSAIDs are easily distributed throughout the tissues (Calatayud and Esplunges, 2016), its dilution with the residual milk that remains in the udder after milking, and the unknown bioavailability after intramammary administration, do not allow for a precise calculation of MEL concentrations in the mammary parenchyma.

**CONCLUSION**

 In our study intramammary administration of 50 mg/ quarter of MEL did not trigger an immune response in the mammary gland. The simultaneous intramammary administration of MEL with an immune challenge by LPS did not inhibit an SCC increase in milk and did not contribute in maintaining the blood-milk barrier integrity. Meloxicam seemed to promote an earlier increase in SCC response when the mammary gland was stimulated with a lower dose of LPS; however, it needs further investigation to verify this finding. The intravenous administration of MEL, at the recommended dose by the manufacturer, did not diminish SCC in LPS-activated mammary glands. Furthermore, MEL did not vastly alter the mRNA abundance of inflammatory factors in the mammary gland tissue. Therefore, it seems that MEL does not limit the inflammatory response, but it also does not impair the mammary immune system.

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Table 1. Primer sequences for PCR (F = forward, R = reverse), GenBank accession number, annealing temperature, and product size.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Gene1 | Primer sequence2 (5’- 3’) | GenBank accession number | Annealingtemperature (°C) | Product size(bp) |
| GAPDH | F | GTC TTC ACT ACC ATG GAG AAG Ga | NM001034034.2 | 60 | 197 |
| R | TCA TGG ATG ACC TTG GCC AGa |
| Ubiquitin | F | AGA TCC AGG ATA AGG AAG GCA Ta | NM174133.2 | 62 | 198 |
| R | GCT CCA CCT CCA GGG TGA Ta |
| COX-1 | F | AGA TGC GGA GTT TCT GAG TCGd | AF004943.1 | 60 | 313 |
| R | GGG TAG TGC ATC AGC ACG Gd |
| COX-2 | F | TCC TGA AAC CCA CTC CCA ACAd | AF031698.1 | 62 | 242 |
| R | TGG GCA GTC ATC AGG CAC AGd |
| PTGES | F | AAA TGT ACG TGG TGG CCG TC | NM174443.2 | 60 | 124 |
| R | CGT TCC ACA TCT GGG TCGT T |
| ALOX5 | F | GCC CTT CTA CAA CGA CTT CGA | AJ306424 | 62 | 332 |
| R | CAG GGT TCC ACT CCA TCC A |
| TNF | F | CCA CGT TGT AGC CGA CAT Ca | NM173966.3 | 60 | 155 |
| R | CCC TGA AGA GGA CCT GTG AGa |
| IL-1 | F | AGT GCC TAC GCA CAT GTC TTC | M37211 | 60 | 114 |
| R | TGC GTC ACA CAG AAA CTC GTC |
| IL-6 | F | CTT CAC AAG CGC CTT CAC TC | NM173923.2 | 62 | 132 |
| R | GTC AGA AGT AGT CTG CCT GG |
| IL-8 | F | ATG ACT TCC AAG CTG GCT GTT Ga | AF232704.1 | 60 | 149 |
| R | TTG ATA AAT TTG GGG TGG AAA Ga |
| IL-10 | F | CCT GGA AGA GGT GAT GCC AC | NM174088.1 | 60 | 133 |
| R | GTT TTC GCA GGG CAG AAA GCG |
| SAA3 | F | GGG CAT CAT TTT CTG CTT CCTc | NM181016.3 | 60 | 106 |
| R | TTG GTA AGC TCT CCA CAT GTC TTT AGc |
| INOS | F | ACC TAC CAG CTGA CGG GAG AT | U14640.1 | 62 | 316 |
| R | TGG CAG GGT CCC CTCT GAT G |
| CXCL5 | F | ACC ACA CCG GGA ATT CAT CC | NM174300.2 | 60 | 368 |
| R | CAG AAA GCT GCA AGG GCA AG |
| TGFB1 | F | TGG AGC TGT ACC AGA AAT ATA GCA A | NM001166068.1 | 60 | 120 |
| R | GCC ACT GCC GCA CAA CTC |
| CASP3 | F | GAC CAT AGC AAA AGG AGC A | NM001077840.1 | 55 | 211 |
| R | CAC TGT CTG TCT CAA TAC CAC |
| HSP70 | F | ACA TGA AGA GCG CCG TGG AGG | NM203322.3 | 60 | 170 |
| R | GTT ACA CAC CTG CTC C |

a (Griesbeck-Zilch et al., 2008), b (Goossens et al., 2005), c (Mukesh et al., 2010), d (Pfaffl et al., 2003).

1GAPDH = glyceraldehyde 3-phosphate dehydrogenase; COX = cyclooxygenase; PTGES = prostaglandin E synthase; ALOX5 = arachidonate 5-lipoxygenase; TNF = tumor necrosis factor alpha; IL = interleukin; SAA3 = serum amyloid A 3; INOS = inducible nitric oxide synthase; CXCL5 = C-C motif chemokine ligand 5; TGFB1 = transforming growth factor beta 1; CASP3 = caspase 3; HSP70 = heat shock protein 70

Table 2. Summary of statistical analysis for milk markers depending on treatment (Trt), time, and their interaction (Trt × Time).

|  |  |  |
| --- | --- | --- |
|  |  | Milk Markers |
| Experiment1 | Factor | SCC | LDH | BSA | IgG | Na | K | Cl |
| 1 |  |  |  |  |  |  |  |  |
| Trt | ns2 | ns | ns | 0.0068 | ns | ns | ns |
| Time | ns | ns | ns | ns | <0.0001 | <0.0001 | <0.0001 |
| Trt × Time | ns | ns | ns | ns | ns | ns | ns |
| 2 |  |  |  |  |  |  |  |  |
| Trt | 0.0481 | ns | ns | ns | 0.0331 | ns | ns |
| Time | <0.0001 | <0.0001 | 0.001 | 0.0125 | <0.0001 | 0.0006 | <0.0001 |
| Trt × Time | ns | 0.028 | ns | ns | ns | ns | ns |
| 3 |  |  |  |  |  |  |  |  |
| Trt | ns | ns | ns | ns | ns | ns | ns |
| Time | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 |
| Trt × Time | ns | ns | ns | ns | ns | ns | ns |
| 4 |  |  |  |  |  |  |  |  |
| Trt | <0.0001 | 0.0033 | 0.0022 | 0.0045 | - | - | - |
| Time | <0.0001 | <0.0001 | 0.0299 | ns | - | - | - |
| Trt × Time | <0.0001 | <0.0001 | 0.0191 | 0.0393 | - | - | - |

1Treatments – Experiment 1: CON (saline solution, intramammarily) vs MEL-imm (50 mg/ quarter of meloxicam diluted in double distilled water, intramammarily); Experiment 2: LPS-L (0.1 μg/ quarter of LPS from *E. coli*, intramammarily) + CON vs LPS-L + MEL-imm; Experiment 3: LPS-H (0.2 μg/ quarter of LPS from *E. coli*, intramammarily) + CON vs LPS-H + MEL-imm; Experiment 4: CON + MEL-iv (0.5 mg of meloxicam per kg of body weight, administered iv) vs LPS-H + MEL-iv vs LPS-H without MEL-iv (included in SCC and LDH only).

2ns: not significant (P > 0.05)

Table 3. Relative abundance of mRNA (ΔΔ threshold cycle; mean ± SEM) in tissue from mammary gland biopsies.

|  |  |
| --- | --- |
|  | Treatments1 |
|  | Experiment 1 | Experiment 3 | Experiment 4 |
| Factor2 | CON | MEL-imm | LPS-H + CON | LPS-H + MEL-imm | CON + MEL-iv | LPS-H + MEL-iv |
| COX-1 | 0.00 ± 0.32 | -0.34 ± 0.58 | -1.79 ± 0.44 | -0.99 ± 0.44 | 0.28 ± 0.52 | -0.31 ± 0.44\* |
| COX-2 | 0.54 ± 0.36 | 0.18 ± 0.10 | 1.20 ± 0.52 | 1.37 ± 0.80 | 0.02 ± 0.44 | 0.57 ± 0.27 |
| PTGES | 1.08 ± 0.41 | 1.18 ± 0.38 | 0.11 ± 0.39 | 0.68 ± 0.28 | 0.01 ± 0.28 | 0.50 ± 0.66 |
| ALOX5  | 0.49 ± 0.37 | -0.22 ± 0.33 | -1.88 ± 0.46 | -1.70 ± 0.24 | -0.06 ± 0.58 | -0.42 ± 0.58 |
| TNF | 1.97 ± 0.36 | 1.54 ± 0.78 | 3.43 ± 0.58 | 3.85 ± 0.93 | -0.16 ± 0.40 | 2.11 ± 0.82\* |
| IL-1 | 2.28 ± 1.01 | 1.58 ± 0.99 | 4.94 ± 0.93 | 5.13 ± 1.01 | 0.63 ± 0.33 | 3.36 ± 0.82\* |
| IL-6 | -1.26 ± 0.75 | 1.58 ± 0.98\* | 4.16 ± 1.07 | 4.26 ± 1.24 | -1.84 ± 0.87 | 1.75 ± 0.71 |
| IL-8 | 1.23 ± 0.41 | 0.84 ± 0.99 | 4.34 ± 0.35 | 4.12 ± 0.67 | 0.28 ± 0.35 | 2.64 ± 0.69 |
| IL-10 | 4.79 ± 1.30 | 2.59 ± 0.18 | 6.20 ± 0.97 | 6.80 ± 1.19 | 0.34 ± 1.17 | 5.46 ± 0.70\* |
| SAA3 | 4.38 ± 1.39 | 3.52 ± 1.31 | 6.29 ± 1.24 | 7.18 ± 0.94 | 1.31 ± 1.24 | 4.85 ± 0.84 |
| INOS | 1.05 ± 1.22 | -0.24 ± 0.85 | 2.25 ± 0.84 | 2.01 ± 0.37 | -0.33 ± 0.31 | 1.27 ± 1.11 |
| CXCL5 | -0.24 ± 0.46 | 0.58 ± 0.74 | 0.67 ± 0.42 | 0.77 ± 0.25 | -1.13 ± 0.29 | -0.10 ± 0.48 |
| TGFB1 | 0.45 ± 0.46 | -0.05 ± 0.30 | -0.29 ± 0.27 | 0.38 ± 0.15 | -0.53 ± 0.30 | 0.07 ± 0.27 |
| CASP3 | -0.26 ± 0.33 | -0.07 ± 0.23 | 0.08 ± 0.39 | -0.37 ± 0.23 | -0.51 ± 0.30 | -0.11 ± 0.43 |
| HSP70 | -0.90 ± 0.50 | 0.42 ± 0.79\* | 2.09 ± 1.14 | 2.22 ± 1.36 | -0.72 ± 0.27 | -0.12 ± 0.25 |

\*Means within experiments are significantly different (*P <* 0.05).

1Treatments – Experiment 1: CON (saline solution, intramammarily) vs MEL-imm (50 mg/ quarter of meloxicam diluted in double distilled water, intramammarily); Experiment 3: LPS-H (0.2 μg/ quarter of LPS from *E. coli*, intramammarily) + CON vs LPS-H + MEL-imm; Experiment 4: CON + MEL-iv (0.5 mg of meloxicam per kg of body weight, administered iv) vs LPS-H + MEL-iv.

2COX = cyclooxygenase; PTGES = prostaglandin E synthase; ALOX5 = arachidonate 5-lipoxygenase; TNF = tumor necrosis factor alpha; IL = interleukin; SAA3 = serum amyloid A 3; INOS = inducible nitric oxide synthase; CXCL5 = C-C motif chemokine ligand 5; TGFB1 = transforming growth factor beta 1; CASP3 = caspase 3; HSP70 = heat shock protein 70

**FIGURE LEGENDS**

**Figure 1.** Experimental design. Thirty cows were randomly assigned to one of four experiments and treatments were performed in all quarters. The front quarters were reserved for milk samples and rear quarters for biopsies. (A) In Exp. 1, individual mammary quarters were infused with either CON (10 mL of 0.9% sterile saline, intramammarily) or MEL-imm (50 mg/ quarter of meloxicam diluted in 10 mL of double distilled water, intramammarily). In Exp. 2, mammary quarters were individually infused with either LPS-L (0.1 μg/ quarter of LPS from *E. coli* diluted in 10 mL of 0.9% sterile saline, intramammarily) followed by CON or MEL-imm. In Exp. 3, individual mammary quarters were infused with either LPS-H (0.2 μg/ quarter of LPS from *E. coli* diluted in 10 mL of 0.9% sterile saline, intramammarily) followed by CON or MEL-imm. In Exp. 4, quarters were individually infused with either CON or LPS-H with MEL-iv (0.5 mg of meloxicam per kg of body weight, iv) or LPS-H without MEL-iv. (B) Timeline of sample collection.

**Figure 2.** Milk somatic cell count (SCC; mean ± SEM) presented in log scale. All treatments were performed at 0 h the milk sample collection. (A) In Exp. 1, quarters were individually infused with either CON (saline solution, intramammarily) or MEL-imm (50 mg/ quarter of meloxicam diluted in double distilled water, intramammarily); (B) In Exp. 2, individual mammary quarters were infused with either LPS-L (0.1 μg / quarter of LPS from *E. coli,* intramammarily) + CON or MEL-imm; (C) In Exp. 3, quarters were individually injected with either LPS-H (0.2 μg/ quarter of LPS from *E. coli,* intramammarily) + CON or MEL-imm, (D) In Exp. 4, individual mammary quarters were infused with either CON or LPS-H followed by the administration of MEL-iv (0.5 mg of meloxicam per kg of body weight, administered iv) or mammary quarters were individually infused with LPS-H only without the MEL-iv treatment. Different letters (a-b) represent significant treatment effect within timepoint. Pound sign (#) indicates significance compared to baseline (0 h). Double pound sign (##) indicates timepoint of significant elevation (compared to 0 h) until the end of the experiment. Significance was declared at *P* < 0.05.

**Figure 3.** Milk lactate dehydrogenase (LDH; mean ± SEM) presented in log scale. All treatments were performed at 0 h the milk sample collection. (A) In Exp. 1, quarters were individually infused with either CON (saline solution, intramammarily) or MEL-imm (50 mg/ quarter of meloxicam diluted in double distilled water, intramammarily); (B) In Exp. 2, individual mammary quarters were infused with either LPS-L (0.1 μg / quarter of LPS from *E. coli,* intramammarily) + CON or MEL-imm; (C) In Exp. 3, quarters were individually injected with either LPS-H (0.2 μg/ quarter of LPS from *E. coli,* intramammarily) + CON or MEL-imm, (D) In Exp. 4, individual mammary quarters were infused with either CON or LPS-H followed by the administration of MEL-iv (0.5 mg of meloxicam per kg of body weight, administered iv) or mammary quarters were individually infused with LPS-H only without the MEL-iv treatment. Different letters (a-b) represent significant treatment effect within timepoint. Pound sign (#) indicates significant difference compared to baseline (0 h). Significance was declared at *P* < 0.05.

**Figure 4.** Bovine serum albumin (BSA; mean ± SEM) concentrations in milk. All treatments were performed at 0 h the milk sample collection. (A) In Exp. 1, quarters were individually infused with either CON (saline solution, intramammarily) or MEL-imm (50 mg/ quarter of meloxicam diluted in double distilled water, intramammarily); (B) In Exp. 2, individual mammary quarters were infused with either LPS-L (0.1 μg / quarter of LPS from *E. coli,* intramammarily) + CON or MEL-imm; (C) In Exp. 3, quarters were individually injected with either LPS-H (0.2 μg/ quarter of LPS from *E. coli,* intramammarily) + CON or MEL-imm, (D) In Exp. 4, individual mammary quarters were infused with either CON or LPS-H followed by the administration of MEL-iv (0.5 mg of meloxicam per kg of body weight, administered iv). Pound sign (#) indicates significant difference compared to baseline (0 h). Asterisks (\*) represent significant treatment effect within timepoint. Significance was declared at *P* < 0.05.

**Figure 5.** Immunoglobulin G (IgG; mean ± SEM) concentrations in milk. All treatments were performed at 0 h the milk sample collection. (A) In Exp. 1, quarters were individually infused with either CON (saline solution, intramammarily) or MEL-imm (50 mg/ quarter of meloxicam diluted in double distilled water, intramammarily); (B) In Exp. 2, individual mammary quarters were infused with either LPS-L (0.1 μg / quarter of LPS from *E. coli,* intramammarily) + CON or MEL-imm; (C) In Exp. 3, quarters were individually injected with either LPS-H (0.2 μg/ quarter of LPS from *E. coli,* intramammarily) + CON or MEL-imm, (D) In Exp. 4, individual mammary quarters were infused with either CON or LPS-H followed by the administration of MEL-iv (0.5 mg of meloxicam per kg of body weight, administered iv). Pound sign (#) indicates significant difference compared to baseline (0 h). Asterisks (\*) represent significant treatment effect within timepoint. Significance was declared at *P* < 0.05.

**Figure 6.** Electrolyte concentrations in milk (mean ± SEM). (A, B, C) Sodium (Na), (D, E, F) potassium (K), and (G, H, I) chloride (Cl). All mammary quarters were challenged at 0 h after the milk sample collection and treatments were: (A, D, G) In Exp. 1, quarters were individually infused with either CON (saline solution, intramammarily) or MEL-imm (50 mg/ quarter of meloxicam diluted in double distilled water, intramammarily); (B, E, H) In Exp. 2, individual mammary quarters were infused with either LPS-L (0.1 μg / quarter of LPS from *E. coli,* intramammarily) + CON or MEL-imm; (C, F, I) In Exp. 3, quarters were individually injected with either LPS-H (0.2 μg/ quarter of LPS from *E. coli,* intramammarily) + CON or MEL-imm. No significant treatment effect within timepoint was found (*P >* 0.05).

Figure 1. Caldeira et al.



Figure 2. Caldeira et al.



Figure 3. Caldeira et al.



Figure 4. Caldeira et al.



Figure 5. Caldeira et al.



Figure 6. Caldeira et al.

