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Original article

Anti-inflammatory effects of glycyrrhizin on lipoteichoic acid and lipopolysaccharide-induced bovine mastitis

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Abstract

Bovine mastitis is primarily treated with antimicrobial agents. Anti-inflammatory agents are also used to alleviate clinical symptoms or reduce antimicrobial use. Glycyrrhizin is an anti-inflammatory agent used in the treatment of bovine mastitis, but its effects are not fully understood. We therefore examined the anti-inflammatory effects of glycyrrhizin both in vivo and in vitro. We first tested whether glycyrrhizin exerts anti-inflammatory effects using MAC-T cells, an immortalized bovine mammary epithelial cell line. Glycyrrhizin decreased the expression of interleukin (IL)-1β mRNA in a concentration-dependent manner in MAC-T cells stimulated with lipoteichoic acid (LTA). We then investigated the effects of glycyrrhizin in bovine mammary epithelial cells (bMECs), which seem to retain more of the characteristics of actual mammary epithelial cells. Stimulation with LTA or lipopolysaccharide significantly increased cytokine mRNA expression in bMECs. Glycyrrhizin exhibited a slight inhibitory effect, but no significant difference was observed. The effect of glycyrrhizin on LTA-induced mastitis was examined in lactating cows. Quarters were divided into test and control areas (test quarter: n=8, control quarter: n=7). All quarters were stimulated with LTA at the start of the trial (0 h). In the test quarter group, glycyrrhizin was administered via intramammary infusion. The somatic cell count and relative gene expression of IL-1β and tumor necrosis factor–α were significantly lower in test quarters than control quarters. Both the in vitro and in vivo studies showed that glycyrrhizin reduces the expression of proinflammatory cytokine genes in response to LTA-induced inflammation and partially revealed the mechanism of the anti-inflammatory effect of glycyrrhizin on mastitis. Further investigations involving field cases of mastitis with bacterial infections are needed to demonstrate the anti-inflammatory effect of glycyrrhizin on bovine mastitis.

Keywords: anti-inflammatory effect, bovine mammary epithelial cells, bovine mastitis, glycyrrhizin, lipopolysaccharide, lipoteichoic acid

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Introduction

Bovine mastitis causes significant economic harm to dairy farmers (Halasa et al. 2007). As infection of the mammary gland with pathogenic microorganisms is the primary cause of bovine mastitis, the disease is commonly treated with antimicrobial agents (Nobrega et al. 2017). Anti-inflammatory drugs such as steroids or non-steroidal anti-inflammatory agents may also be used in combination with antimicrobial agents. The combined use of anti-inflamma tory and antimicrobial agents reportedly has several benefits, such as early resolution of inflammation, pain reduction, and reduced use of antimicrobial agents due to earlier recovery (Petersson Wolfe et al. 2018, Kurumisawa et al. 2022).

Glycyrrhizin, a triterpenoid compound extracted from licorice, is an anti-inflammatory agent used to treat bovine mastitis. It is commercially available in Japan and used as a therapeutic drug for combined use with antibiotics. However, few reports are available describing the effects of glycyrrhizin on bovine mastitis. Intramammary infusion of glycyrrhizin soon reduced the clinical signs in mild coagulase-negative staphylococcal mastitis (Kai et al. 2003). Administration of glycyrrhizin at the time of initial diagnosis in mild cases of clinical mastitis was shown to reduce the use of antimicrobial agents and promote more-rapid alleviation of local symptoms (Kurumisawa et al. 2022). Glycyrrhizin reportedly exhibits a variety of effects, such as anti-inflammatory, antimicrobial, immunoregulatory, anticancer, anti-ulcer, and hepatoprotective properties (Hosseinzadeh and Nassiri Asl 2015, Yang et al. 2015, Batiha et al. 2020, Hasan et al. 2021). However, the details regarding its anti-inflammatory mechanism remain unclear. Glycyrrhizin reportedly decreases the percentage of neutrophils and reduces the secretion of histamine into milk in lactating cows with mastitis caused by coagulase-negative staphylococci (Kai et al. 2003). In a mouse mastitis model and cells derived from mice, glycyrrhizin inhibited the secretion of inflammatory cytokines and prostaglandins (Yoshida et al. 2007, Kato 2008, Fu et al. 2014).

In the present study, we conducted three experiments to evaluate the anti-inflammatory effects of glycyrrhizin on bovine mastitis. In experiment 1, we tested whether glycyrrhizin exerts anti-inflammatory effects in bovine mammary epithelial cells, using an immortalized bovine mammary epithelial cell line (MAC-T cells) (Huynh et al. 1991). Only one inflammatory parameter, interleukin-1β (IL-1β) expression, was evaluated in experiment 1. We then compared the response of cells to inflammation in the presence and absence of glycyrrhizin using bovine mammary epithelial cells (bMECs), which seem to retain more of the characteristics of actual mammary epithelial cells (experiment 2). In experiment 2, the number of parameters examined was expanded to provide a more detailed study. Finally, we examined whether glycyrrhizin exerts anti-inflammatory effects in lipoteichoic acid (LTA)-induced mastitis in lactating cows in vivo (experiment 3).

Materials and Methods

Experiment 1

MAC-T cells were cultured in Dulbecco's Modified Eagle Medium (Sigma-Aldrich Co. LLC., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Biosera, Cholet, France), 5 μg/mL insulin (Sigma-Aldrich), and 1 μg/mL hydrocortisone (Sigma-Aldrich). MAC-T cells were stimulated with 30 μg/mL LTA (*Staphylococcus aureus*, Sigma-Aldrich) and treated with 0, 0.2, 2, 20, and 200 μg/mL glycyrrhizin (Sigma-Aldrich). The cells were cultured at 37°C in a humidified incubator (5% CO_2) for 24 h. Relative gene expression levels of IL-1β were determined. Total RNA (tRNA) was isolated from the cells using ISOGEN (Nippon Gene, Tokyo, Japan) following the manufacturer's instructions. Reverse transcription (RT) was carried out on 0.5 μg of the recovered RNA using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan) to obtain first-strand cDNA. The resulting cDNA was stored at -20°C until analysis. Quantitative PCR (qPCR) was conducted using a LightCycler 96 System (Roche Diagnostics K.K., Tokyo, Japan) with FastStart SYBR Green Master Mix (Roche Diagnostics, Mannheim, Germany). Primer sequences are shown in Table 1. PCR conditions were as follows: 10 min at 95°C, followed by 45 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 15 s. *β*-Actin served as an internal control (Bougarn et al. 2011). Gene expression levels were determined according to the $\Delta\Delta C_T$ method. All tests were conducted in quadruplicate.

To determine the effect of glycyrrhizin on cell viability, MAC-T cells were plated at a density of 10^4 cells/cm^2 in 24-well plates (AS ONE Corp., Osaka, Japan) and stimulated with 200 μg/mL glycyrrhizin for 24 h. Cell viability was measured using a Luna-FL™ system (Logos Biosystems, South Korea).

Experiment 2

The protocol for this experiment adhered to the guidelines of the Rakuno Gakuen University Animal Experimentation Committee and was approved by the committee (no. VH21C2). bMECs were originally isolated from three healthy multiparous lactating Holstein

Table 1. Sequences of oligonucleotide primers used in this study.

Gene product	Primer sequence	Amplicon size (bp)	Accession No.	Experiment No.	References
β-actin	F: AGC AAG CAG GAG TAC GAT GAG	241	NM_173979.3	exp. 2	Robinson et al. 2007
	R: ATC CAA CCG ACT GCT GTC A				
	F: ACC GTG AGA AGA TGA CCC AGA	90	NM_173979.3	exp. 1, 3	Griesbeck-Zilch et al. 2008
	R: AGG CAT ACA GGG ACA GCA CA				
YWHAZ	F: GCA TCC CAC AGA CTA TTT CC	120	GU817014.1	exp. 2	Goossens et al. 2005
	R: GCA AAG ACA ATG ACA GAC CA				
IL-1β	F: AGT GCC TAC GCA CAT GTC TTC	114	M_37211	exp. 1, 2, 3	Griesbeck-Zilch et al. 2008
	R: TGC GTC ACA CAG AAA CTC GTC				
IL-6	F: ATC AGA ACA CTG ATC CAG ATC C	145	NM_173923.2	exp. 2	O'Gorman et al. 2006
	R: CAA GGT TTC TCA GGA TGA GG				
IL-8	F: GAA GAG AGC TGA GAA GCA AGA TCC	142	NM_173925.2	exp. 2	O'Gorman et al. 2006
	R: ACC CAC ACA GAA CAT GAG GC				
	F: ACA CAT TCC ACA CCT TTC CAC	149	AF_232704	exp. 3	Griesbeck-Zilch et al. 2008
	R: ACC TTC TCG ACC CAC TTT TC				
TNF-α	F: TCT TCT CAA GCC TCA AGT AAC AAG C	418	NM_173966.3	exp. 2	Lee et al. 2006
	R: CCA TGA GGG CAT TGG CAT AC				
	F: CCA CGT TGT AGC CGA CAT C	155	NM_173966	exp. 3	Griesbeck-Zilch et al. 2008
	R: CCC TGA AGA GGA CCT GTG AG				
TLR2	F: CAT TCC CTG GCA AGT GGA TTA TC	195	NM_174197.2	exp. 2	Imaizumi et al. 2024
	R: GGA ATG GCC TTC TTG TCA ATG G				
TLR4	F: CTT CCC GGG GGA TGT TTC AA	169	NM_174198.6	exp. 2	Imaizumi et al. 2024
	R: CCT GAG GCG GTT TCT ACT CG				

bp – base pair, F – forward, R – reverse, YWHAZ – tryptophan 5-monooxygenase activation protein zeta polypeptide, IL – interleukin, TNF – tumor necrosis factor, TLR – toll-like receptor.

cows using a procedure described previously and used at the 6th-7th passage (Hu et al. 2009). The characteristics of the three cows varied; the parity was in the range 3-4, and the number of days in milk (DIM) ranged from 6-39. A total of six experiments were performed using cells from these three cows (n=6). Cells were pre-incubated with or without 200 μg/mL glycyrrhizin for 16 h and then stimulated with 50 μg/mL lipopolysaccharide (LPS) (*Escherichia coli* O111:B4, Sigma-Aldrich) or 20 μg/mL LTA (*S. aureus*, Sigma-Aldrich) for 24 h. Gene expression levels of cytokine (IL-1β, IL-6, IL-8, and tumor necrosis factor-α [TNF-α]) and toll-like receptor (TLR)2 and TLR4 were determined under each condition. tRNA was isolated from bMECs, and cDNA was synthesized using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH, USA), TURBO DNA-free DNAse (Ambion), ReverTra Ace reverse transcriptase (Toyobo, Japan), and oligo dT primers (Toyobo). Thunderbird SYBR qPCR mix (Toyobo) and a CFX ConnectMyiQ-iCycler (Bio-Rad Laboratories, USA) were used for qPCR analyses. Primer sequences are shown in Table 1. The qPCR cycling conditions were as follows: denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. Melting curve analysis consisted of heating the PCR product from 55°C to 95°C and monitoring the change in fluorescence at every increase of 0.5°C. Transcripts encoding β-actin and tyrosine 3-monooxygenase / tryptophan 5-monooxygenase activation protein – zeta polypeptide (YWHAZ) were analyzed as reference genes (Bougarn et al. 2011). Gene expression levels were determined according to the $\Delta\Delta C_T$ method.

Experiment 3

The study protocol for the animal trial involving a lactating dairy cow adhered to the guidelines of the Azabu University Animal Experimentation Committee and was approved by the committee (no. 230120-1). One Holstein dairy cow in late-lactation (200 DIM) and producing 20 kg/day of milk was evaluated for this trial. The cow was milked twice each day, at 8 AM and 4 PM, and showed no signs of clinical mastitis. The four quarters were divided into test and control quarters, and the allocation was replaced for a total of four trials. Each trial was conducted at 2-week intervals, and a total of 15 quarters were tested, except for one quarter that did not recover from inflammation (test quarters: n=8, control quarters: n=7). All quarters were stimulated with 50 μg/quarter LTA (*S. aureus*, Sigma-Aldrich) diluted in 10 mL of 0.9% sterile saline via the intramammary route at the start of the trial (0 h). Treatment was administered at 0 h and 8 h with an intramammary infusion of 10 mL of Mastrytine® (Kyoritsu Seiyaku Corp., Tokyo, Japan) containing 600 mg of glycyrrhizin for the test quarters and 10 mL of 0.9% sterile saline for the control quarters. Using sterile techniques, a milk sample was collected from each quarter into a sterile culture tube at 0, 4, 8, 24, and 72 h. The milk somatic cell count (SCC) was determined using a DeLaval Cell Counter (DeLaval International AB, Tumba, Sweden). The activity of β-N-acetylglucosaminidase (NAGase) was determined using a β-N-acetylglucosaminidase assay kit (Sigma-Aldrich). Whey obtained by centrifugation at 3,500 rpm for 10 min was used for NAGase activity measurements. To control for the

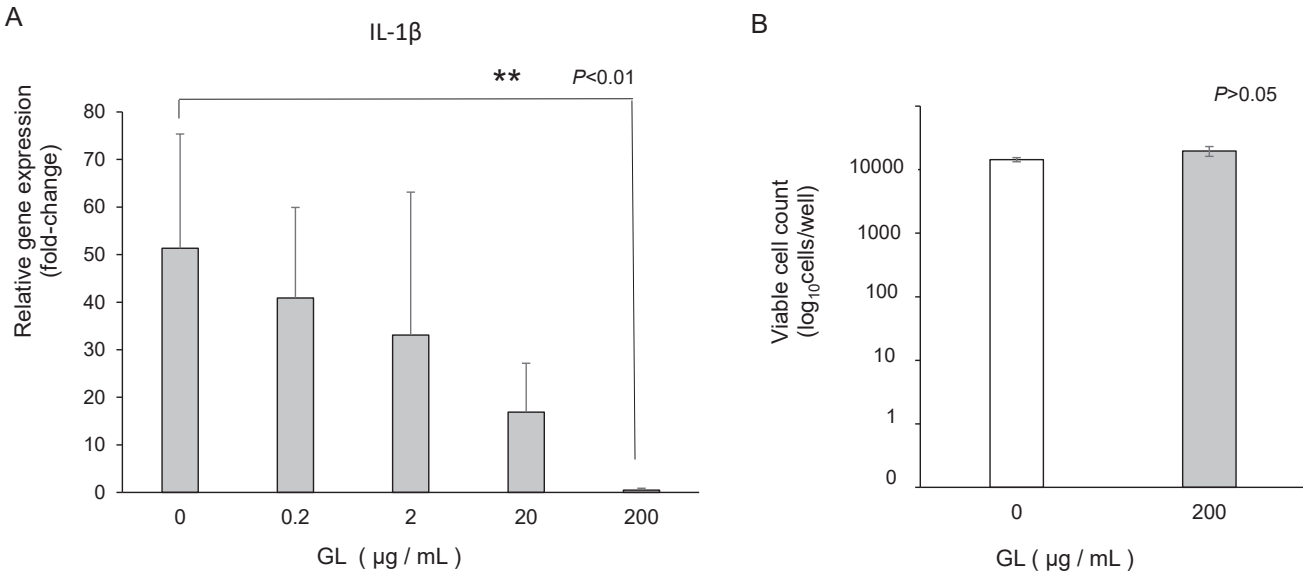

Fig. 1. Effect of glycyrrhizin on lipoteichoic acid (LTA)-induced inflammation in mammary epithelial cells (MAC-T cells).

effect of whey coloration on measurements, the absorbance of an unreacted sample was subtracted from the measured value. Gene expression levels of IL-1β, IL-8, and TNF-α were measured for each milk sample. tRNA was extracted from milk samples based on the report by Wellnitz et al. (2011), with some modifications. Briefly, a 50-mL milk sample was immediately centrifuged (1,500 × g, 30 min). The fat layer and supernatant were removed by aspiration, and the cell pellet was recovered with PBS (pH 7.4) and centrifuged (460 × g, 15 min). After removal of the supernatant, the cells were suspended in ISOGEN and stored at -20°C until total RNA extraction. RT-qPCR analyses were conducted in the same manner described for Experiment 1.

Statistical analyses

Statistical analyses were performed using the Dunnett test for gene expression of IL-1β in MAC-T cells, and the Student *t*-test was used to assess differences in cell viability. Differences in gene expression levels in bMECs were analyzed using the Smirnov-Grubbs test for outliers, followed by the Kruskal-Wallis test. A generalized linear mixed model was used to assess differences in SCC, NAGase activity, and expression of cytokine gene in milk samples of LTA-induced mastitis. P-values of <0.05 were considered statistically significant.

Results

Effect of glycyrrhizin on LTA-induced inflammation in MAC-T cells

The gene expression of IL-1β in MAC-T cells stimulated with LTA decreased in a concentration-dependent manner following the addition of glycyrrhizin. A significant difference in gene expression was observed between 0 and 200 μg/mL glycyrrhizin ($p<0.01$). MAC-T cell viability was not affected by glycyrrhizin (Fig. 1).

Effect of glycyrrhizin on LTA- or LPS-induced inflammation in bMECs

Stimulation with LPS or LTA significantly increased cytokine gene expression, but the suppressive effect of glycyrrhizin was slight, with no significant differences observed (Fig. 2). TLR gene expression levels did not change significantly following stimulation with LPS or LTA.

In vivo effect of glycyrrhizin on LTA-induced mastitis in a lactating cow

SCC and NAGase activity in milk after intramammary injection of LTA are shown in Fig. 3(A) and (B). In the test quarters, SCC levels were significantly lower than in the control quarters, indicating an interaction ($p<0.01$). There were no significant differences in NAGase activity between groups or over time. No visible abnormalities in milk properties were observed in either the test or control quarters. Mild swelling was observed between 4 h and 8 h in some of the control and test quarters. The relative gene expression of IL-1β, IL-8, and TNF-α is shown in Fig. 3(C). IL-1β and TNF-α gene expression in the control quarters increased rapidly and peaked during the first 4-8 h. In contrast, the increase in the test quarters was slight, and the difference between the two groups was significant. Maximum IL-8 expression was observed after 24 h, but the difference was not significant.

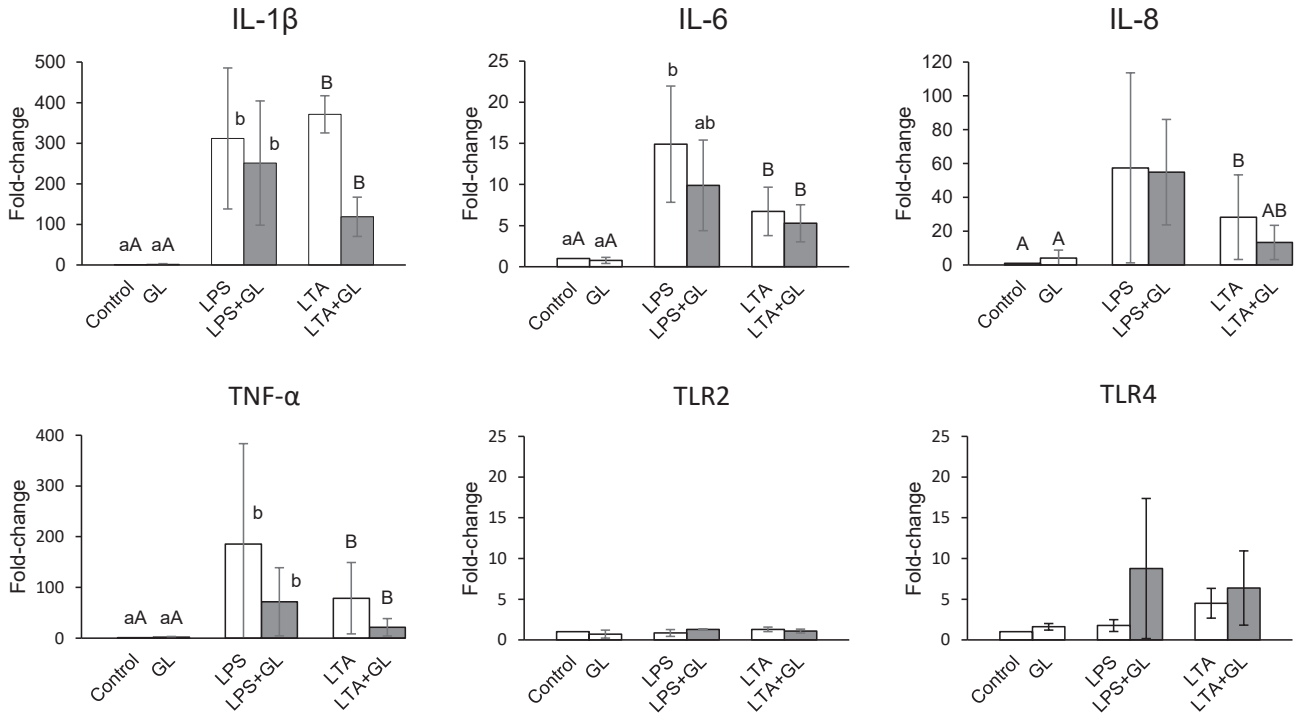

Fig. 2. Gene expression of cytokine and toll-like receptor (TLR) in bMECs stimulated with lipopolysaccharide (LPS) or lipoteichoic acid (LTA).

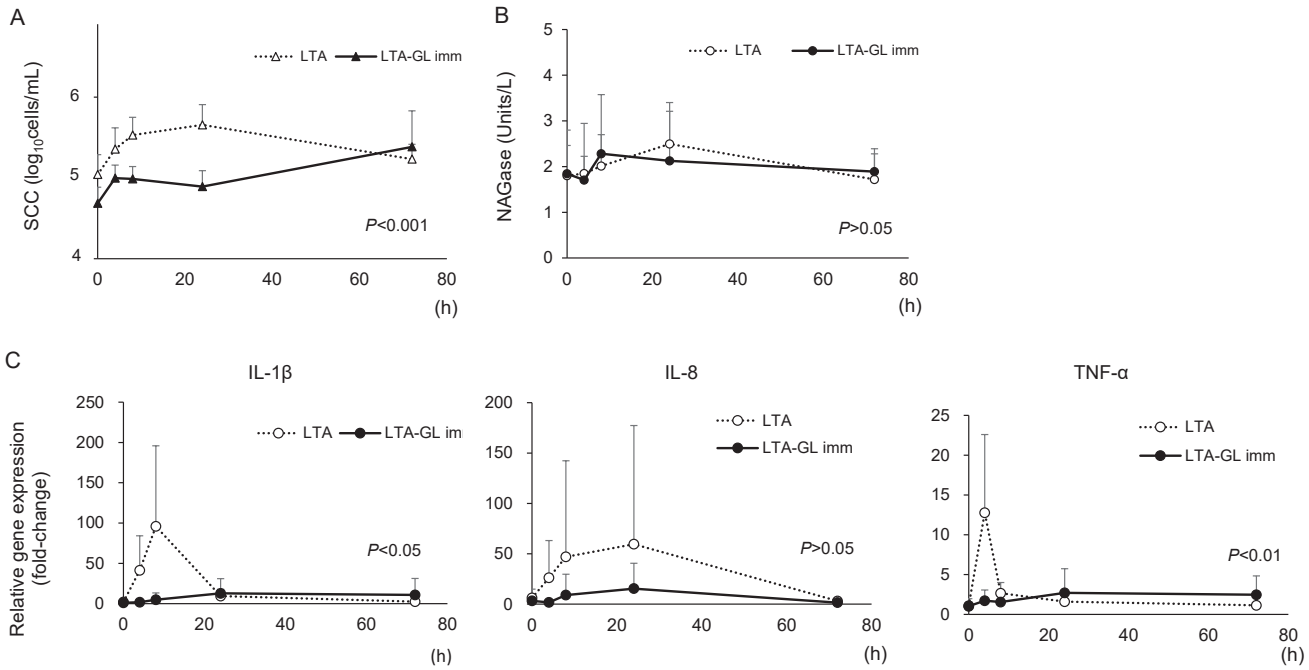

Fig. 3. Somatic cell counts (SCC) (A), NAGase activity (B), and relative gene expression levels of IL-1β, IL-8, and TNF-α (C) in cow milk after intramammary injection of lipoteichoic acid (LTA).

Discussion

Bovine mastitis is caused by bacterial infection, and it is treated primarily with antimicrobial agents (Nobrega et al. 2017). However, anti-inflammatory agents also play an important role in the treatment of bovine mastitis. The increase in antimicrobial resistance in recent years has become an important public health issue worldwide, and it has necessitated reduced and more prudent use of antimicrobial agents, not only in human medicine but also in the veterinary field (World Health Organization 2015). Therefore, it is important to examine the potential uses of anti-inflammatory agents in terms of therapeutic efficacy and whether

they may be effective in reducing the use of antimicrobial agents in the treatment of bovine mastitis. As bovine mastitis is a painful disease (Fitzpatrick et al. 2013), appropriate pain control approaches are needed from the perspective of animal welfare (Petersson Wolfe et al. 2018).

The innate immune response plays an important role in the biophylaxis of bovine mastitis (Nogueira De Souza et al. 2012, Xu et al. 2019). The innate immune response is initiated by the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) on bMECs (Bhattarai et al. 2018b). PAMP recognition activates various intracellular pathways that lead to the secretion of pro-inflammatory cytokines, neutrophil migration, phagocytosis, and the production of reactive oxygen species. This response not only attacks pathogens, but also damages host tissue, leading to the release of endogenous TLR ligands known as damage-associated molecular patterns, which can exacerbate tissue damage (Prince et al. 2011). Therefore, appropriate control of inflammation is important in infection treatment.

Glycyrrhizin is an anti-inflammatory agent approved in Japan for the treatment of bovine mastitis. However, only a few studies have examined the efficacy of glycyrrhizin in dairy cows or bMECs (Kai et al. 2003, Kurumisawa et al. 2022). Furthermore, all previous studies other than those using dairy cows involved LPS stimulation, thus assuming gram-negative bacterial infection. Therefore, in this study, both LTA and LPS were evaluated in bMECs and LTA was evaluated in a lactating dairy cow. We monitored the expression of several representative cytokines and chemokine as measures of inflammation. Both TNF-α and IL-1β are pro-inflammatory cytokines that play an important role in promoting neutrophil infiltration in the innate immune response during the early stages of infection. IL-6 exerts immunomodulatory effects and plays a role in acute total protein synthesis. IL-8 is a chemokine upregulated in both gram-negative and -positive bacterial infections (Oviedo Boyso et al. 2007). In this study, MAC-T cells stimulated with LTA (a component of gram-positive bacteria) exhibited decreased relative expression of the IL-1β gene in a glycyrrhizin concentration – dependent manner. In addition, the SCC and relative expression of the IL-1β and TNF-α genes were decreased in LTA-stimulated mastitis. These results suggest glycyrrhizin exerts anti-inflammatory effects in gram-positive bovine mastitis. The observations that NAGase activity was not elevated in the present in vivo experiment, SCC was elevated only mildly, and clinical symptoms were mild suggest that the inflammation in the mammary tissue was very mild. In addition, a time lag between infection and the start of treatment is typical in the clinical treatment of naturally occurring mastitis, but in the present study, glycyrrhizin was administered simultaneously with LTA, which does not fully replicate actual treatment scenarios. However, the results of our study indicate that glycyrrhizin exerts at least some anti-inflammatory effect against mild mammary gland inflammation.

Differences in immune responses between MAC-T cells and bMECs have been reported (Strandberg et al. 2005, Günther et al. 2016). The reasons for these differences are not clear, but they may be related to the origin of the cells, the number of passages, or the immortalization process. Although no significant differences were observed in the experiment with bMECs in the present study, a trend toward lower gene expression of cytokines was observed with glycyrrhizin administration, similar to the results obtained with MAC-T cells. In other words, the results obtained with bMECs were not in conflict with those obtained with MAC-T cells, which demonstrated the anti-inflammatory effects of glycyrrhizin. Also, as reported in other studies (Nogueira et al. 2012), LPS stimulation increased pro-inflammatory cytokine gene expression, illustrating the validity of this study.

LPS stimulation reportedly activates both TLR2 and TLR4 (Griesbeck-Zilch et al. 2008, Gilbert et al. 2013, Bhattarai et al. 2018a). Other reports have suggested that TLR2 and TLR4 exhibit a cooperative response to *Staphylococcus aureus* infection (Goldammer et al. 2004, Bhattarai et al. 2018a). However, it has also been reported that LTA alone does not stimulate TLR4 (Bhattarai et al. 2018a, Gilbert et al. 2013). In the present study, neither LTA nor LPS stimulation increased the relative expression of the TLR2 gene, and only slightly increased the relative expression of the TLR4 gene. This may be related to the reported changes in the peak expression of TLR2 and TLR4 over time (Griesbeck-Zilch et al. 2008) and to observations that TLR2 expression in mammary tissue is not elevated 48 h after *S. aureus* challenge (Whelehan et al. 2011). Interestingly, a slight increase in relative expression of the TLR4 gene was observed with glycyrrhizin treatment compared to no glycyrrhizin treatment upon LTA and LPS stimulation. The detailed mechanism of glycyrrhizin's anti-inflammatory effects is not clear. Experiments in LPS-induced murine mastitis and mouse mammary epithelial cells stimulated with LPS have shown that glycyrrhizin inhibits the nuclear factor – kappa B (NF-κB) pathway (Fu et al. 2014). Fu et al. (2014) suggest that inhibition of the NF-κB pathway by glycyrrhizin is due to inhibition of TLR4 translocation to lipid rafts. Other studies have shown that glycyrrhizin inhibits TLR4 homodimerization (Honda et al. 2012). One hypothesis is that the increase in TLR4 gene

expression with glycyrrhizin observed in this study may be related to positive feedback on such mechanisms. In addition to LTA, the muramyl peptide, an elementary constituent of bacterial peptidoglycan, is also involved in pattern recognition in gram-positive bacteria, and TLR2 and nucleotide-binding oligomerization domain 2 cooperate in pattern recognition (Bougarn et al. 2010). The results of the present study were insufficient to form a conclusion regarding the relationship between the anti-inflammatory effects of glycyrrhizin and the expression of TLR genes. Further data are needed, considering the duration and association with multiple PAMPs and PRRs.

In the present study, both in vitro and in vivo experiments showed that glycyrrhizin reduces the expression of proinflammatory cytokine genes in response to LTA-induced inflammation and partially revealed the mechanism of the anti-inflammatory effect of glycyrrhizin on gram-positive bacterial mastitis. Further investigations involving field cases of mastitis with bacterial infections are needed to fully demonstrate the anti-inflammatory and therapeutic effects of glycyrrhizin on bovine mastitis.

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