



Effect of glycyrrhizin administration followed by symptom-based antimicrobial selection therapy on antimicrobial use in clinical mastitis without systemic symptoms

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ABSTRACT. In bovine mastitis, antimicrobial treatment is often initiated before the causative organism is identified a problem in the prudent use of antimicrobials. In this study, we aimed to reduce the total amount of antimicrobials used in mastitis treatment by administering glycyrrhizin, an anti-inflammatory drug, instead of antimicrobials at the onset of clinical mastitis without systemic symptoms, followed by symptom-based antimicrobial selection therapy (ST), to examine the effect of this treatment strategy on treatment outcomes and antimicrobial use. Comparisons between cases that received antimicrobial treatment starting from the day of diagnosis (blanket antibiotic therapy [BT] group: 33 cases) and cases that received ST starting from the day after the diagnosis (ST group: 57 cases) revealed no difference in the cure rate, milk withholding period, or recurrence rate. However, the ST group had a significantly lower amount of antimicrobials than the BT group. Additionally, a single administration of glycyrrhizin before ST significantly relieved the udder symptoms and reduced the antimicrobial amount when compared with cases without glycyrrhizin administration. Thus, a single administration of glycyrrhizin followed by ST can reduce the total antimicrobial use.

KEYWORDS: bovine mastitis, glycyrrhizin, prudent use of antibiotics, reduction of antimicrobials use, symptom-based antimicrobial selection therapy

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Bovine mastitis is a disease that reduces milk production and milk quality and leads to the use of antimicrobials; as a result, milk shipment is halted (milk withholding period), causing economic losses to the dairy industry [5]. Given that mastitis is mainly caused by bacterial infections, many antimicrobials are used as treatments. In fact, mastitis is the main reason for antimicrobial administration in dairy cows [18].

Reducing antimicrobial use in bovine mastitis requires appropriate selection of antimicrobial use. In culture-based selection therapy, which is an evidence-based mastitis therapy, the treatment strategy differs according to the severity [20]. Thus, bovine mastitis is classified into the following scores: 1 (abnormal milk), 2 (abnormal milk and udder symptoms such as swelling or firmness), and 3 (abnormal milk, udder symptoms, and systemic symptoms). Scores 1 and 2 recommend antimicrobial treatment based on the causative organism, which is usually identified a day after the culture test, whereas score 3 recommends immediate initiation of antimicrobial treatment even when the causative organism is still not known. When the culture result shows an infection by a Gram-positive bacterium or a mixed infection, intramammary antibacterial treatment is selected, but when other results are obtained, such as an infection by a Gram-negative bacterium or no growth of any organism, the mastitis is not treated for the elimination of these organisms. Culture-based selection therapy is disadvantageous especially for mastitis caused by a Gram-positive bacterium or a mixed infection because the start of treatment is delayed by 1 day. However, according to previous studies, the delayed treatment leads to no differences in the recurrence rate, somatic cell count (SCC), milk production, and cow survival [15]. It is also economically beneficial because it reduces the total amount of antimicrobial drugs used and shortens the milk withholding period [14].

However, culture-based selection therapy has been limitedly applied in Japan, and blanket antibiotic therapy (BT), which starts antimicrobial treatment on the first day of diagnosis for all cases, is common for clinical mastitis without systemic symptoms. The

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reason for the early initiation of antimicrobial treatment is that in some cases, the bacteriological test results cannot be obtained rapidly. Therefore, an alternative treatment method is needed when culture results are not available in a timely manner, to achieve prudent use of antimicrobials.

Mastitis is an inflammatory disease, and even mild mastitis has been associated with mastitis-related pain [2]. Therefore, developing appropriate pain-related treatments that can be administered before the start of antimicrobial selection therapy is necessary. In this study, we focused on glycyrrhizin, which is commercially available in Japan as a treatment drug for mastitis. Glycyrrhizin is one of the triterpene saponin components that are extracted from the Chinese herb licorice, and it has many pharmacological properties, such as anti-inflammatory, antimicrobial, immunoregulatory, anticancer, antiulcer, and hepatoprotective properties [1, 6, 8, 23]. In bovine mastitis, a single dose of glycyrrhizin induces an anti-inflammatory activity in coagulase-negative staphylococci (CNS) mastitis [9]; in Japan, it is used as a therapeutic drug for combined use with antibiotics. However, the effect of glycyrrhizin on clinical bovine mastitis remains poorly understood.

Therefore, this study aimed to investigate the effect of administering glycyrrhizin as an alternative to antimicrobials at the onset of clinical mastitis without systemic symptoms, followed by symptom-based antimicrobial selection therapy (ST), on therapeutic outcome and antimicrobial use.

MATERIALS AND METHODS

The study protocol for milk sample collection from lactating dairy cows adhered to the guidelines of the Azabu University Animal Experimentation Committee, and was approved by such committee (No. 201015-1).

Farms

This study was conducted on three commercial dairy farms in Hokkaido Prefecture, Japan. One farm had 40 Holstein cows with tie-stall housing, another had 60 Holstein cows also with tie-stall housing, and the third farm had 120 Holstein cows with free-barn housing. In total, this study included 90 mastitis cases noted between 2019 and 2020.

Treatment protocol

We included cases of clinical mastitis without systemic symptoms and excluded cases of severe mastitis with systemic symptoms. All farms were managed and supervised in the same way by the same veterinarian, and observation of clinical symptoms and treatment strategies of each farmer was unified. A trained farmer scored all eligible cases according to the following: score 1 for those with only abnormal milk and score 2 for those with abnormal milk and udder symptoms [20]. Cases with complications other than mastitis were also excluded. The included cases were subsequently divided randomly into the BT and ST groups. Immediately after study inclusion, the BT group received antimicrobial treatment in the affected quarter. In principle, the treatment, which used β -lactam antibiotics, was repeated thrice every 24 hr, and the treatment duration was changed only when the veterinarian deemed it necessary. In the ST group, the cows were further divided randomly into glycyrrhizin-treated (GL) and no-treatment (NT) groups. On day 0, the GL group was treated with intramammary infusion of 10 mL of Mastrytine[®] (Kyoritsu Seiyaku Corp., Tokyo, Japan) containing 600 mg of glycyrrhizin, while the NT group received no treatment. On day 1, in both subgroups, the same observer on each farm determined the need for antimicrobial treatment according to the clinical symptoms in each case, under the guidance of a veterinarian. The milk properties and udder symptoms were observed as clinical symptoms according to the presence or absence of symptom abnormality and changes in the symptoms in comparison with day 0. Cases with worsening of milk properties or udder symptoms and cases with milk dilution with no symptom changes on day 1 were selected for antimicrobial treatment. In contrast, cases with symptom improvement and cases with no change in symptoms and no milk dilution on day 1 were not selected for antimicrobial treatment. The antimicrobial treatment, which also used β -lactam antibiotics, began on day 1, and was generally repeated thrice every 24 hr; its duration was changed only according to the discretion of the veterinarian. The antimicrobials were used according to the label directions, and no systemic administration of antimicrobials or concomitant use of other anti-inflammatory agents was provided.

Examination of milk samples

Using sterile techniques, we collected a milk sample from each affected quarter into a sterile culture tube on days 0, 1, 3, and 7. The milk samples were frozen at -20°C and transported to the laboratory for milk property observation (clots, dilution, and abnormal color), bacterial species identification, antimicrobial susceptibility testing, and SCC determination. Each milk sample (10 μL) was inoculated onto 5% sheep blood agar and incubated at 37°C for 24 hr. Cultures that grew at more than 300 CFU/mL were considered as causative organisms. Pure cultures of the colonies were obtained again, and the causative bacterial species were biochemically identified using BD BBL CRYSTAL GP, E/NF (Becton, Dickinson and Co., Franklin Lakes, NJ, USA). Antimicrobial susceptibility testing of the causative bacteria was performed using the simplified disk-diffusion method according to Kurumisawa *et al.*'s method [13]. Briefly, colonies were directly picked up with a sterile cotton swab and spread directly on a testing plate, with 2–3 colonies used for *Staphylococcus* spp., 20–30 colonies for *Streptococcus* spp. and *Enterococcus* spp., 50–100 colonies for *Trueperella pyogenes*, and 1/8–1/4 of the total volume of a single colony for Gram-negative bacteria. Testing plates were prepared with two types of culture media: Mueller–Hinton agar for *Staphylococcus* spp. and Gram-negative bacteria, and 5% sheep blood agar for *Enterococcus* spp., *Streptococcus* spp., and *T. pyogenes*. Antimicrobial disks were placed within minutes after plate inoculation. The incubation conditions were the same for all bacterial species, with aerobic culturing at 37°C for 24 hr. After incubation, the disk zone diameter was measured using a caliper. The susceptibility was determined from the disk zone diameter according to the criteria of Kawai *et*

al. [11]. In addition, the SCC was identified using the DeLaval Cell Counter (DeLaval International AB, Tumba, Sweden) according to Kawai *et al.*'s method [10].

Definition of treatment outcomes

Score 1 and score 2 mastitis cases were determined and recorded by the same observer on each farm, and clinical symptom changes were evaluated on days 0, 1, 3, and 7. As mentioned, the milk properties (clots, dilution, and abnormal color) and udder symptoms (swelling and firmness) were recorded as clinical symptoms according to the presence or absence of the symptoms and symptom changes in comparison with the symptom status on the previous day (improvement, no change, or worsening). Cases were considered cured if milk shipment resumed within 14 days from the last treatment day. Conversely, cases with a blind quarter and disease recurrence (no milk shipment resumption) within 14 days from the last treatment day were regarded as not cured. The cure rate was defined as the ratio of cured cases to all cases in the same group. The number of days from disease onset to milk shipment resumption defined the milk withholding period, excluding the data from noncured cases and recurrent cases. Cases in which milk shipment resumption was not possible because of other diseases before being cured were excluded from the analysis. For cases of recurrence within 14 days from the last treatment day, cases with the same species that was isolated again from the milk sample indicated recurrence, but those with a different species that was isolated from the milk sample indicated reinfection. Furthermore, a bacteriological cure was determined when the causative bacterium isolated in day 0 milk was not detected again in day 7 milk (<300 CFU/mL). We calculated the bacteriological cure rate as the percentage of bacteriologically cured cases in a group, and the symptom exacerbation rate as the percentage of cases in which the clinical symptoms worsened at least once at the observation days 0, 1, 3, and 7.

Evaluation by group comparisons

We first compared the treatment outcomes and antimicrobial use between the BT and ST groups to determine the impact of ST. To verify the existence of cases that should have been treated with antimicrobials but were missed because of ST, we compared the treatment outcomes of cases caused by Gram-positive bacteria between these two subgroups. We only extracted and compared cases caused by Gram-positive bacteria because mastitis cases caused by these bacteria are generally treated with antimicrobial agents. In addition, we compared the GL and NT groups to examine the effects of the single administration of glycyrrhizin without any concomitant medication on day 0 as an initial treatment in ST.

Statistical analysis

In comparing treatment outcomes and antimicrobial use between groups, we used the Mann–Whitney *U* test and Kruskal–Wallis test for assessing their differences, and the χ^2 test for testing independence. The percentage of cases with normal milk in the GL and NT groups was examined by the Cochran Q test and the χ^2 test. For the percentage of cases with improvement, no change, or worsening of udder symptoms in the GL and NT groups, we used the Cochran–Armitage test.

RESULTS

Comparison of treatment outcomes between the BT and ST groups

Out of 90 cases, 33 (score 1: 15 cases, score 2: 18 cases) were included in the BT group, and 57 (score 1: 27 cases, score 2: 30 cases) were included in the ST group (Table 1).

From antimicrobial susceptibility testing, 1 case in the BT group and 1 case in the ST group showed antimicrobial agent resistance; however, their symptoms improved, so the antimicrobial agents were not changed during the treatment period, and the animals were cured eventually (data not shown). The days in milk (DIM), parity, SCC on day 0, and culture results were not significantly different between the BT and ST groups for both score 1 and score 2 cases. The cure rate was approximately 90% in score 1 cases and over 80% in score 2 cases in both the BT and ST groups. The mean milk withholding period in the BT and ST groups was 7.2 ± 2.5 and 6.3 ± 3.3 days in score 1 cases and 7.3 ± 2.3 and 6.0 ± 3.3 days in score 2 cases, respectively. The recurrence rate was less than 5% in score 1 cases and approximately 10% in score 2 cases in both groups. No significant differences were observed in the cure rate, milk withholding period, and recurrence rate between such groups in score 1 and score 2 cases. Additionally, in both score 1 and score 2 cases, the ST group had a significantly lower percentage of cases that received antimicrobial treatment (41% in score 1 and 57% in score 2; 100% in the BT group), and the mean amount of antimicrobials used per case was significantly lower (approximately 2 vials less in score 1 cases and approximately 1.5 vials less in score 2 cases) than that in the BT group. However, the ST group had a lower bacteriological cure rate (41% vs. >60%) and a higher symptom exacerbation rate (approximately 15% vs. 0%) than the BT group for both score 1 and score 2 cases, although the difference was not significant (Table 1).

Missed cases for antimicrobial treatment in the ST group among cases caused by Gram-positive bacteria

Of the total 90 cases, 52 were caused by Gram-positive bacteria. Cases with antimicrobial use and cases without antimicrobial use in the ST group were examined separately. Thus, the treatment outcomes and antimicrobial usage were compared between the ST group with antimicrobial use, the ST group without antimicrobial use, and the BT group. The DIM, parity, SCC on day 0, and percentages of score 1 and score 2 cases were not significantly different between the three groups. In the BT group and the ST group with antimicrobial use, approximately 70% of the causative organisms were *Streptococcus* spp., whereas in the ST group without antimicrobial use, roughly 70% were CNS. In the ST group with antimicrobial use, the start of antimicrobial treatment was delayed by 1 day compared with that in the BT group, but no significant difference was found in the cure rate (83%), milk withholding period (approximately 7.5

Table 1. Comparisons of the treatment outcomes and antimicrobial use between the blanket antibiotic therapy and symptom-based antimicrobial selection therapy groups

	Score 1			Score 2		
	BT group n=15	ST group n=27	P-value	BT group n=18	ST group n=30	P-value
DIM, mean ± S.D.	212.1 ± 127.3	158.5 ± 89.7	0.17	143.8 ± 122.5	138.0 ± 105.1	0.94
Parity, mean ± S.D.	3.7 ± 1.3	3.3 ± 1.6	0.30	2.8 ± 1.8	2.1 ± 1.3	0.11
Day 0 SCC, 10 ⁴ /mL, mean ± S.D.	475.2 ± 417.0	482.0 ± 481.5	0.86	565.6 ± 809.9	531.8 ± 666.2	0.76
Culture result						
Gram-positive bacteria	47%	56%	0.76	61%	67%	0.57
Gram-negative bacteria	13%	7%		28%	13%	
Yeast spp.	0%	0%		6%	7%	
No growth	40%	37%		6%	13%	
Treatment outcomes						
Cure rate	87%	89%	>0.99	83%	83%	>0.99
Milk withholding period, days, mean ± S.D.	7.2 ± 2.5	6.3 ± 3.3	0.58	7.3 ± 2.3	6.0 ± 3.3	0.38
Recurrence rate	0%	4%	>0.99	11%	7%	>0.99
Bacteriological cure rate	100% (4/4)	41% (7/17)	0.12	64% (7/11)	41% (9/22)	0.38
Symptom exacerbation rate	0%	15%	0.26	0%	13%	0.28
Antimicrobial usage						
Vials used per case, mean ± S.D.	3.4 ± 1.1	1.1 ± 1.5	<0.01	3.2 ± 0.4	1.8 ± 1.7	<0.01
Usage rate, % of cases	100%	41%	<0.01	100%	57%	<0.05
Types, no. of cases						
PCG/KM	12	10		16	13	
CEZ	2	1		2	3	
CXM	1	0		0	0	
PLM	0	0		0	1	

DIM: The days in milk. SCC: somatic cell count. Cure rate: percentage of cases in which milk shipment resumption was possible within 14 days from the last treatment day. Bacteriological cure rate: percentage of cases in which the same bacterial genus that was detected in day 0 milk was detected again in day 7 milk at <300 CFU/mL. Only the bacteriological cure rate had a limited study population. Symptom exacerbation rate: percentage of cases in which the clinical symptoms worsened at least once between the observation days. PCG/KM: composite of benzyl penicillin and kanamycin. CEZ: cefazolin. CXM: cefuroxime. PLM: pirlimycin. The Mann–Whitney *U* test was used to examine differences between groups, and the χ^2 test was used to examine independence as statistical analyses.

days), or recurrence rate (approximately 5%) between these two groups. The cure rate (91%) and recurrence rate (9%) were similar between all three groups, but the ST group without antimicrobial use had a significantly shorter milk withholding period (approximately 2.6 days) and a lower amount of antimicrobials used (0%). However, the bacteriological cure rate was significantly lower in the ST group (both with and without antimicrobial use; 9–36%) than in the BT group (83%) (Table 2).

Effect of single-dose glycyrrhizin monotherapy on day 0

Of the 57 cases in the ST group, 32 (score 1: 12 cases, score 2: 20 cases) were included in the GL group, and 25 (score 1: 15 cases, score 2: 10 cases) were included in the NT group. The DIM, parity, SCC on day 0, and culture results were not significantly different between the two groups in both score 1 and score 2 cases. The cure rate (80–90%), milk withholding period (approximately 5 and 7 days in the GL and NT groups, respectively), and recurrence rate (0–10%) also showed no significant differences between such groups in both score 1 and score 2 cases. Furthermore, in both score 1 and score 2 cases, the GL group had a significantly lower percentage of antimicrobial-treated cases (by approximately 40%) and a lower amount of antimicrobials used (by >1 vial) than the NT group (Table 3). The percentage of cases with normal milk changed significantly from day 0 to day 7 in both the GL group ($P<0.05$) and the NT group ($P<0.01$), but no significant differences were observed either day between these two groups (Fig. 1). In comparing changes in udder symptoms from day 0 to day 1, the percentage of cases with improvement, no change, or worsening of symptoms differed significantly between the GL and NT groups ($P<0.05$); in particular, the percentage of cases with symptom improvement on day 1 was 50% in the GL group but only 20% in the NT group (Fig. 2).

DISCUSSION

In this study, we administered glycyrrhizin, an anti-inflammatory drug, on the first day of diagnosis and then ST on the following day to examine the effect of this treatment strategy on treatment outcomes and antimicrobial use.

Comparison of treatment outcomes and antimicrobial use between the ST and BT groups revealed that ST can reduce antimicrobial use without adversely affecting the treatment outcomes. Thus, in BT, unnecessary antimicrobial treatment was being used. Furthermore, the cure rate and recurrence rate were not significantly different between the BT and ST groups among cases caused by Gram-positive

Table 2. Comparisons of the treatment outcomes between the blanket antibiotic therapy and symptom-based antimicrobial selection therapy groups in cases caused by Gram-positive bacteria to examine missed cases for antimicrobial prescriptions

	BT group n=18	ST group		P-value
		Antimicrobial treatment		
		+	-	
	n=23	n=11		
Day 0 mastitis severity				
Score 1	39%	43%	45%	0.93
Score 2	61%	57%	55%	
DIM, mean ± S.D.	171.9 ± 129.1	157.9 ± 91.2	133.7 ± 86.7	0.67
Parity, mean ± S.D.	2.4 ± 1.3	2.5 ± 1.4	2.2 ± 1.6	0.61
Day 0 SCC, 10 ⁴ /mL, mean ± S.D.	579.0 ± 839.0	567.7 ± 752.9	376.3 ± 255.6	0.95
Culture result				
<i>Streptococcus</i> spp.	67%	65%	36%	
Coagulase-negative staphylococci	11%	26%	64%	
<i>Staphylococcus aureus</i>	6%	9%	0%	
<i>Trueperella pyogenes</i>	17%	0%	0%	
Treatment outcomes				
Cure rate	83%	83%	91%	0.84
Milk withholding period, days, mean ± S.D.	7.5 ± 2.3 ^a	7.5 ± 1.7 ^a	2.6 ± 1.4 ^b	a–b <0.001
Recurrence rate	6%	4%	9%	0.88
Bacteriological cure rate	83% ^a (10/12)	36% ^b (8/22)	9% ^b (1/11)	a–b =0.001
Antimicrobial usage				
Vials used per case, mean ± S.D.	3.7 ± 1.6 ^a	3.0 ± 0.8 ^a	0.0 ^b	a–b <0.001
Usage rate, % of cases	100%	100%	0%	
Types, no. of cases				
PCG/KM	18	19	0	
CEZ	0	3	0	
PLM	0	1	0	

DIM: The days in milk. SCC: somatic cell counts. Cure rate: percentage of cases in which milk shipment resumption was possible within 14 days from the last treatment day. Bacteriological cure rate: percentage of cases in which the same bacterial genus that was detected in day 0 milk was detected again in day 7 milk at <300 CFU/mL. Only the bacteriological cure rate had a limited study population. PCG/KM: composite of benzyl penicillin and kanamycin. CEZ: cefazolin. PLM: pirlimycin. The Kruskal-Wallis test was used to examine differences between groups, and the χ^2 test was used to examine independence as statistical analyses.

bacteria; hence, ST may be appropriate for identifying cases requiring antimicrobial agents. The results were similar for both score 1 and score 2 cases, suggesting that ST is applicable to all cases of bovine clinical mastitis without systemic symptoms. However, given that the ST group consisted of the GL and NT groups, the possibility that glycyrrhizin administration may have affected the results should be considered. Nevertheless, the lack of difference in the treatment outcomes between the GL and NT groups suggested that glycyrrhizin administration did not affect the treatment outcome of the ST group. Moreover, considering that glycyrrhizin administration appeared to have improved the udder symptoms, we cannot rule out the possibility that this anti-inflammatory drug had influenced the decrease in antimicrobial use in the ST group.

Regarding ST, selecting antimicrobial treatment solely according to the symptoms without basing on bacteriological examination may have several risks. One undeniable risk is that it may have adverse effects on the long-term recurrence rates over 14 days after the last treatment (not followed in this study), and milk production performance after cure. In this study, the ST group had a higher rate of symptom exacerbation and a lower rate of bacteriological cure, although the difference was not significant. Given that this study only investigated short-term results, further research is needed to elucidate the long-term results. Another risk is that if treatment is selected according to the symptoms only, using a specific treatment strategy for each causative organism would be impossible. Without bacteriological examination, we cannot distinguish between fungal and algal mastitis, which cannot be treated with antimicrobial agents, and *Mycoplasma* spp. and *Staphylococcus aureus* mastitis, which differ in treatment and control strategies from other environmental mastitis [3, 12]. In addition, without antimicrobial susceptibility testing of the causative organism, we cannot select an effective and appropriate antimicrobial agent; this issue should be avoided if we want to achieve prudent use of antimicrobials. Therefore, in the antimicrobial treatment of mastitis, confirming the causative organism and antimicrobial susceptibility by bacteriological examination is necessary.

In comparing the treatment outcomes and antimicrobial use between the GL and NT groups, results showed that glycyrrhizin administration can reduce antimicrobial use without adversely affecting the treatment outcomes. The udder symptoms from day 0 to day 1 had improved significantly more in the GL group than in the NT group, while the percentage of cases with normal milk showed no significant difference between the two groups. Thus, the anti-inflammatory effect of glycyrrhizin administered on day 0 may have

led to the earlier recovery of udder symptoms. The anti-inflammatory effects of glycyrrhizin may have resulted from the suppression of proinflammatory cytokine expression, suppression of the arachidonic acid cascade by phospholipase A2 inhibition, and antioxidant effects [19, 24]. They have also been reported to be caused by the drug's interaction with cell membranes rather than blockade of specific intracellular targets [4, 7, 21]. Yunhe *et al.* observed that the preadministration of glycyrrhizin reduced the histopathological

Table 3. Comparisons of the treatment outcomes and antimicrobial use between the glycyrrhizin-treated and no-treatment groups

	Score 1			Score 2		
	GL group n=12	NT group n=15	P-value	GL group n=20	NT group n=10	P-value
DIM, mean ± S.D.	175.1 ± 88.3	145.2 ± 88.5	0.44	143.9 ± 93.1	126.8 ± 124.0	0.38
Parity, mean ± S.D.	3.5 ± 1.7	3.1 ± 1.6	0.60	2.4 ± 1.5	1.5 ± 0.5	0.12
Day 0 SCC, 10 ⁴ /mL	447.7 ± 385.3	507.1 ± 539.9	0.62	454.8 ± 479.7	685.7 ± 914.1	0.66
Culture result						
Gram-positive bacteria	42%	67%	0.41	60%	80%	0.27
Gram-negative bacteria	8%	7%		10%	20%	
Yeast spp.	0%	0%		10%	0%	
No growth	50%	27%		20%	0%	
Treatment outcomes						
Cure rate	83%	93%	0.83	85%	80%	>0.99
Milk withholding period, days, mean ± S.D.	5.3 ± 3.8	7.0 ± 2.6	0.23	5.1 ± 2.3	7.6 ± 4.3	0.1
Recurrence rate	0%	7%	>0.99	5%	10%	>0.99
Antimicrobial usage						
Vials used per case, mean ± S.D.	0.4 ± 1.0	1.7 ± 1.6	0.02	1.4 ± 1.8	2.7 ± 0.9	0.02
Usage rate, % of cases	17%	60%	0.01	40%	90%	0.02
Types, no. of cases						
PCG/KM	2	8		5	8	
CEZ	0	1		2	1	
PLM	0	0		1	0	

DIM: The days in milk. SCC: somatic cell counts. Cure rate: percentage of cases in which milk shipment resumption was possible within 14 days from the last treatment day. PCG/KM: composite of benzyl penicillin and kanamycin. CEZ: cefazolin. PLM: pirlimycin. The Mann-Whitney *U* test was used to examine differences between groups, and the χ^2 test was used to examine independence as statistical analyses.

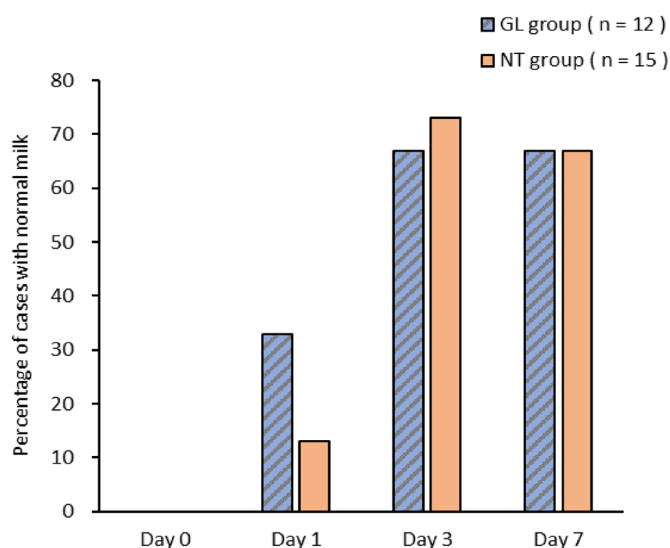


Fig. 1. Percentage of cases with normal milk in score 1 cases from day 0 to day 7 in the glycyrrhizin-treated (GL) and no-treatment (NT) groups. The percentage of cases with normal milk changed significantly from day 0 to day 7 in both the GL group ($P < 0.05$) and the NT group ($P < 0.01$). Comparison of these groups showed no significant difference on either day.

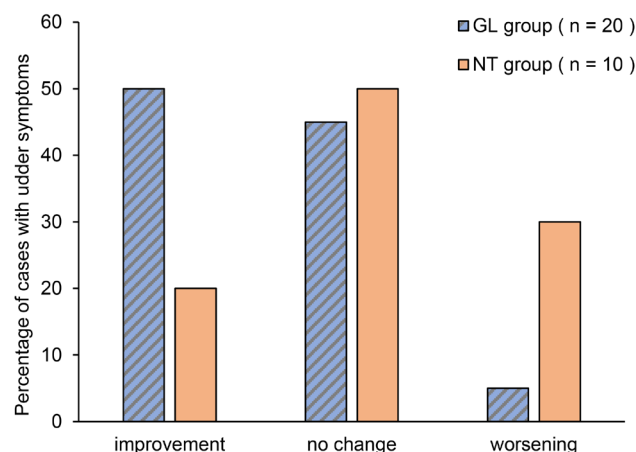


Fig. 2. Percentage of cases with worsening, no change, or improvement of udder symptoms on day 1 in comparison with that on day 0 in score 2 cases. The percentage of cases with improvement, no change, or worsening of symptoms differed significantly between the glycyrrhizin-treated (GL) and no-treatment (NT) groups ($P < 0.05$).

changes in mice's mammary tissue with lipopolysaccharide-induced mastitis and suppressed the expression of tumor necrosis factor- α , interleukin-1 β , and interleukin-6 [4]. Although the clinical effects of anti-inflammatory drugs on clinical mastitis without systemic symptom are still insufficiently investigated, the administration of the nonsteroidal anti-inflammatory drug meloxicam in addition to antimicrobial therapy induced a lower SCC, increased the probability of a bacteriological cure, diminished the likelihood of culling, and improved the conception rate compared with the administration of antibiotics alone in cows [16, 17, 22]. In lipopolysaccharide-induced mastitis, meloxicam can relieve udder pain and reduce udder edema and the body temperature [2]. Considering these findings in combination with the reduction of udder symptoms after glycyrrhizin administration demonstrated in the current study, glycyrrhizin has the potential to exert anti-inflammatory and analgesic effects on mastitis.

In conclusion, the administration of glycyrrhizin alone before the start of antimicrobial treatment significantly relieved the udder symptoms on the following day and reduced the amount of antimicrobials required. In addition, ST for clinical mastitis without systemic symptoms may be useful as a new treatment selection strategy to reduce antimicrobial use. A single administration of glycyrrhizin at the start of treatment followed by ST on the next day may reduce the amount of antimicrobial agents needed.

CONFLICT OF INTEREST. Authors declare no Conflict of Interests for this article.

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