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Therapeutic Dose, Duration, and Efficacy of Bee Venom for Treating Clinical Mastitis in Dairy Cow

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Abstract This study was conducted to explore the efficacy of bee venom as a treatment for mastitis and to determine the optimal dosage and treatment period. When 6 mg or 12 mg of bee venom was administered to each experimental quarter of mastitis in dairy cow, the clinical symptoms in the 12 mg quarter were noticeably improved compared to those in the 6 mg quarter. There was no significant difference in the somatic cell count (SCC) in the milk between normal and mastitis quarters between the 6 and 12 mg doses, but there was a steady decrease in the 12 mg-treated quarter ($p = 0.34$). To determine the treatment period, bee venom was administered for 2, 4, and 7 days. After administering 12 mg of bee venom for 7 days, the SCC in the milk was compared before 6 days and after 7 days, and the SCC was significantly decreased to less than 100,000 SC/mL after 7 days ($p = 0.01$). In addition, to investigate the efficacy of bee venom, the minimum inhibitory concentration for *S. aureus*, *E. coli*, and coagulase negative staphylococci was measured, and the results showed that Gram-positive bacteria were more sensitive to bee venom than Gram-negative bacteria, and treatment for Gram-negative bacteria was limited. As a result of this study, it was confirmed that a dose of 12 mg of bee venom and a treatment period of more than 7 days were required to treat mastitis, and that treatment with bee venom alone against Gram-negative bacteria was negative.

Key words mastitis, bee venom, dairy cow, therapeutic effect, efficacy.

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Introduction

Mastitis is one of the important diseases that causes economic losses to dairy farms worldwide through reduced milk production, poor quality, wastage, early culling, and additional treatment and labor costs (16,19,26). Mastitis is normally divided into clinical and subclinical infection, and clinical mastitis is characterized by visual observation of udder swelling, redness, milk containing flakes, crud, and watery milk (31). The major causes of clinical mastitis include *E. coli*, Klebsiella (Gram-negative strain, GN), *Staphylococcus aureus*, and Streptococci (Gram-positive strain, GP) (2,13,17).

The treatment of clinical mastitis necessitates an antibiotic susceptibility test followed by the selection of an appropriate treatment drugs (30,49,50). However, indiscriminate antibiotic usage in farms leads to overuse and subsequent resistance, complicating antibiotic selection and requiring more potent antibiotics (4,7,14,38,43,46). This overreliance poses risks to consumer health and fosters antibiotic resistance, prompting various countries to restrict antibiotic use (12,34,39,42-45). Nonetheless, domestic antibiotic sales for livestock surged by approximately 35% from 765 tons in 2013 to 1,036 tons in 2021 (28). In response, Korea proposed the utilization of bee venom as an alternative approach to curb antibiotic usage in livestock (22).

Bee venom has emerged as a potential solution to reduce antibiotic dependence in mastitis treatment within dairy production (22). Known for its applications in oriental medicine, bee venom has been demonstrated to be effective in treating multiple sclerosis, pain management, and cancer, while also exhibiting anti-inflammatory, antibacterial, and immune-regulating properties (3,24,25,54). Leveraging these attributes, bee venom shows promise for mastitis treatment in dairy farming, potentially decreasing antibiotic reliance, shortening withdrawal periods, enhancing productivity, and positively impacting animal welfare (9,53).

To treat mastitis, there is a method of directly injecting bee venom at specific acupuncture points in the cow's body using live bees, and a method of injecting purified bee venom at acupuncture points using a needle (18,35). However, these methods cause potential allergic reactions, discomfort, and stress of the sting (5,36). To circumvent these issues, a method involving direct injection of bee venom into the udder using a nipple needle was proposed for mastitis patients (22).

Despite the numerous advantages of bee venom as a natural substance for the treatment of mastitis, there is inadequate research on the dosage, mammary tissue response post-administration, and treatment duration in cows with clinical mastitis (24). These gaps emphasize the need for further exploration. Therefore, the purpose of this study was to determine the udder response, establish treatment dosages, and evaluate bee venom efficacy following its administration for mastitis treatment in dairy cows.

Materials and Methods

Preparation of bee venom

Korean bee venom used in this experiment was collected and purified from Careside Co., Ltd. Bee venom was purified using the "simple bee venom purification method" proposed by Han et al. (22). American bee venom and melittin were purchased from Sigma-Aldrich, Missouri, USA. All types of bee venom were freeze-dried and stored in a refrigerator, and 50 mg of bee venom was diluted in 50 mL of physiological saline immediately before use. The diluted bee venom was used immediately, and the remaining bee venom was discarded. The quarter receiving 6 mg of bee venom was injected with 6 mL of bee venom dilution, and the quarter receiving 12 mg was injected with 12 mL of bee venom diluted solution into the udder through the nipple. Bee venom was administered according to the plan after sample collection or milking.

Antibacterial susceptibility testing

The antibacterial activity of bee venom was determined by using the spot-on-the-lawn method (32). *Staphylococcus aureus*, *Escherichia coli*, and coagulase negative staphylococci (CNS) isolates served as indicator strains, and they were seeded onto Mueller Hinton agar (Difco Laboratories, Detroit, USA) and overlaid on a plate and left to solidify. Partially purified bee venom forms were serially diluted; 5 μL of each dilution was spotted on the plates. Plates were incubated at 37°C for 18 to 24 h. The minimum inhibitory concentration (MIC) of bee venom and antibiotics was determined by the dilution concentration at which an inhibition zone was formed on the plate. The diluents for MIC measurement were prepared by diluting the following 6 antibacterial agents: ampicillin (1.0-256.0 μg/mL), amoxicillin (8.0-512.0 μg/mL), gentamicin (1.0-256.0 μg/mL), melittin (10.0-5120.0 μg/mL), Korean bee venom (20.0-5120.0 μg/mL), and America bee venom (20.0-10240.0 μg/mL). The bacterial species used for MIC were field strains isolated from dairy cows with mastitis and they included 5 species of *S. aureus*, 5 species of *E. coli*, and 5 species of CNS.

Determination of the treatment dose

Animal

One dairy cow scheduled for culling due to mastitis was included in this study. The cow's front two quarters were normal, and the rear two quarters were affected by clinical mastitis. The

experiment involved administering the bee venom through the teats: 6 mg to a quarter unaffected by mastitis and 6 mg to a quarter affected by mastitis, while the remaining healthy and mastitis-affected quarters received 12 mg. The bee venom was administered twice daily for three days, and the cow was slaughtered on the day after the final treatment. Clinical symptoms and somatic cell counts (SCC) were monitored to determine the therapeutic efficacy of bee venom in the treatment of mastitis. Milk was collected for SCC and bacterial identification before and at 12, 24, 36, 48, 60, and 72 h after bee venom administration.

Histological examination

The entire udder was collected. It was refrigerated, and then, it is transported to the laboratory where the middle part of the teat and the mammary glands were collected and fixed in 4% formalin. The fixed tissues were stained with hematoxylin and eosin and examined under an optical microscope for analysis. In the mammary tissue samples, we observed edema, infiltration of fibroblasts and erythrocytes, infiltration and vacuolization of epithelial cells, and the presence or absence of leukocytes.

Duration and frequency of treatment

Animals

The study focused on 3 dairy farms in the regions of Imsil in Jeonbuk Province, South Korea, each housing between 50 to 100 animals. The subjects of this study were cows diagnosed with clinical mastitis, determined based on clinical symptoms and the California Mastitis Test (CMT) results, indicating positive signs of mastitis.

Treatment groups

The dose determined in the treatment dose determination test was divided into a group of dairy cows with clinical mastitis administered twice a day for 2 days (group A, n = 1), a group administered once a day for 4 days (group B, n = 3), and a group administered once a day for 7 days (group C, n = 4). The milk was collected before and at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 7, and 10 days after bee venom administration. Milk collection was conducted in a separate area from the milking room, and the milk was discarded 2-3 times, placed in two 50 mL tubes, and transported to the laboratory in a refrigerated condition to measure the SCC and identify bacteria.

Somatic cell count

The CMT results were categorized as 3+ (clumps, highly viscous, discarding in portion no longer possible), 2+ (gel), 1+ (traces), and – (negative). Quarters with a CMT ≥ 1+ reaction, and with clinical signs of mastitis, were considered to have clinical mastitis. Milk samples from quarters diagnosed with a CMT ≥ 1+ response were collected aseptically, immediately cooled on ice, and brought to the laboratory for measurement of SCC. Somatic cells were measured using a somatic cell counter (Foss 300®, Foss Electric Ltd, Denmark).

Bacteriological examination

To isolate the causative agents of mastitis, 23 μL of milk was inoculated into a 5% sheep blood agar plate. These plates were then cultured at 37°C for 48 h. Afterwards, the characteristics of bacterial colonies, hemolysis, and gram staining were examined to preliminarily select the bacteria. Further identification of the bacteria was conducted by the National Veterinary Research and Quarantine Service in South Korea.

After culture in 20 mL of Trypticase soy agar at 37°C for 18 h, single colonies were picked in 7 mL of Trypticase soy broth and cultured again in a shaking incubator at 37°C for 16 to 18 h. After culture, the bacterial solution adjusted to 0.5 McFarland using sterilized saline and a turbidity meter were injected under vacuum into the GPI card used for VITEK 1 (bioMerieux SA, Marcy-I'Etoile, France), and after 8 h, the bacteria were identified according to the results.

Statistical analysis

The SCC were measured at 0, 12, 24, 36, 48, 60, and 72 h for each treatment group (normal 6 mg, normal 12 mg, mastitis 6 mg, and mastitis 12 mg). The mean cell counts of the normal 6 mg quarter were compared with those of the normal 12 mg quarter using the Mann-Whitney U test. Similarly, the mean cell counts of the mastitis 6 mg quarter were compared with those of the mastitis 12 mg quarter using the same test. The SCC were also measured daily for 10 days following the initiation of treatment (once a day for 7 days). These time-series measures were obtained from four subjects. Cell counts measured before and after 7 days of treatment were compared using a generalized mixed linear model. The MIC of bee venom and antibiotics were compared using one-way ANOVA stratified by different bacteria. If the ANOVA results indicated statistical significance (p-value less than 0.05), post-hoc analysis was conducted with Tukey HSD adjustment. MIC for bacteria stratified by bee venom and antibiotics were also compared in this manner. All statistical analyses were performed using R version 4.3.1 (R Core Team, Vienna, Austria).

Results

Efficacy of bee venom

The MIC results of bee venom are shown in Table 1. The MICs of domestically-produced and imported bee venom were 56.0 ± 21.9 μg/mL and 112.0 ± 43.8 μg/mL for *S. aureus*, 1024.0 ± 350.5 μg/mL and 2024.0 ± 701.1 μg/mL for *E. coli*, and 56.0 ± 21.9 μg/mL and 112.0 ± 43.8 μg/mL for CNS, respectively. The MIC of melittin was 24.0 ± 8.9 μg/mL for *S. aureus*, 896.0 ± 350.5 μg/mL for *E. coli*, and 20.0 ± 0.0 μg/mL for CNS, respectively. On the other hand, the MICs of ampicillin and gentamycin were 9.6 ± 3.6 μg/mL and 4.4 ± 2.2 μg/mL for *S. aureus*, 230.4 ± 257.4 μg/mL and 121.6 ± 122.7 μg/mL for *E. coli*, and 4.8 ± 1.8 μg/mL and 1.6 ± 0.6 μg/mL for CNS, respectively (Table 1). There was no significant difference between domestically produced bee venom and melittin, but there was a significant difference between domestically produced bee venom and American bee venom ($p < 0.05$). However, MICs of bee venom and melittin were higher than MICs of the tested antibiotics ($p < 0.01$). Additionally, the antibacterial activity of bee venom was better for GP bacteria and CNS than for GN bacteria.

Determination of the therapeutic dosage

The clinical symptoms after administering 6 mg and 12 mg of bee venom to two normal and two mastitis quarters were as follows: the normal and mastitis-affected quarters that received 6 mg of bee venom exhibited signs of redness and a firm texture in the udder, accompanied by severe swelling. The swelling made it difficult to milk the cow properly. Conversely, normal and mastitis quarters that received 12 mg of bee venom also showed redness and severe swelling, but these quarters were less firm than those that received 6 mg of bee venom. In addition, the 12 mg quarters showed easier milking than the 6 mg quarters because the udder relaxed more quickly and the swelling subsided more quickly.

Table 1. Minimum inhibitory concentration of two types of bee venom, melittin, and three antibiotics against *Staphylococcus aureus* (*S. aureus*), *E. coli*, and coagulase-negative staphylococci (CNS) isolated from mastitis

Items	n	Minimum inhibitory concentration (μg/mL) mean ± SD (range)		
		E. coli	*S. aureus*	CNS
Bee venom (Korea)	5	1024 ± 350.5^{a,A} (640-1280)	56.0 ± 21.9^{b,A} (40-80)	56.0 ± 21.9^{b,A} (40-80)
Bee venom (USA)	5	2048 ± 701.1^{a,B} (1280-2560)	112.0 ± 43.8^{b,B} (80-160)	112.0 ± 43.8^{b,B} (80-160)
Melittin	5	896.0 ± 350.5^{a,A} (640-1280)	24.0 ± 8.9^{b,A} (20-40)	20.0 ± 0.0^{b,A} (20)
Ampicillin	5	230.4 ± 257.4^{a,C} (32-512)	9.6 ± 3.6^{b,C} (8-16)	4.8 ± 1.8^{b,C} (4-8)
Amoxicillin	5	448.0 ± 525.8^{a,D} (64-1024)	14.4 ± 3.6^{b,C} (8-16)	11.2 ± 4.4^{b,D} (8-16)
Gentamycin	5	121.6 ± 122.7^{a,C} (32-256)	4.4 ± 2.2^{b,D} (2-8)	1.6 ± 0.6^{b,C} (1-2)

There is a significant difference between the parameters within rows (A, B, C, D) and the parameters between columns (a, b) ($p < 0.05$).

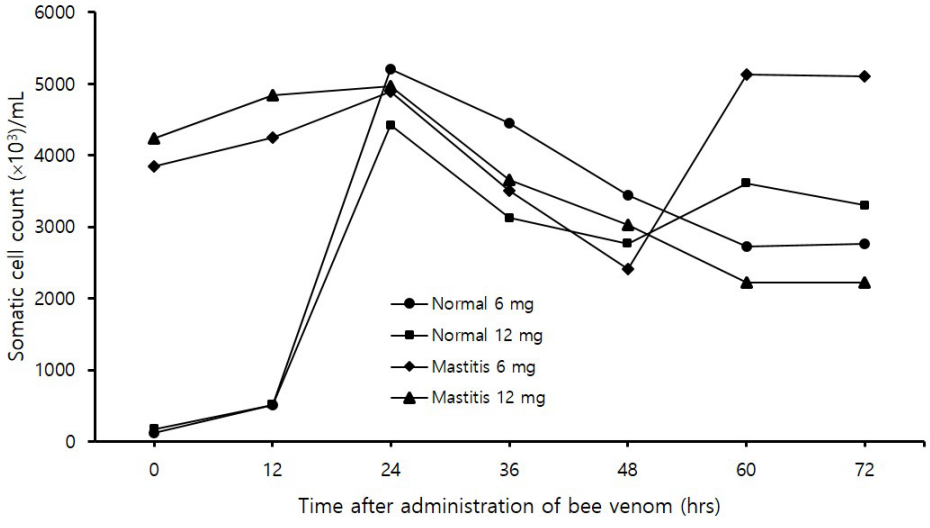

Fig. 1. Somatic cell count according to the time elapsed after bee venom administration.

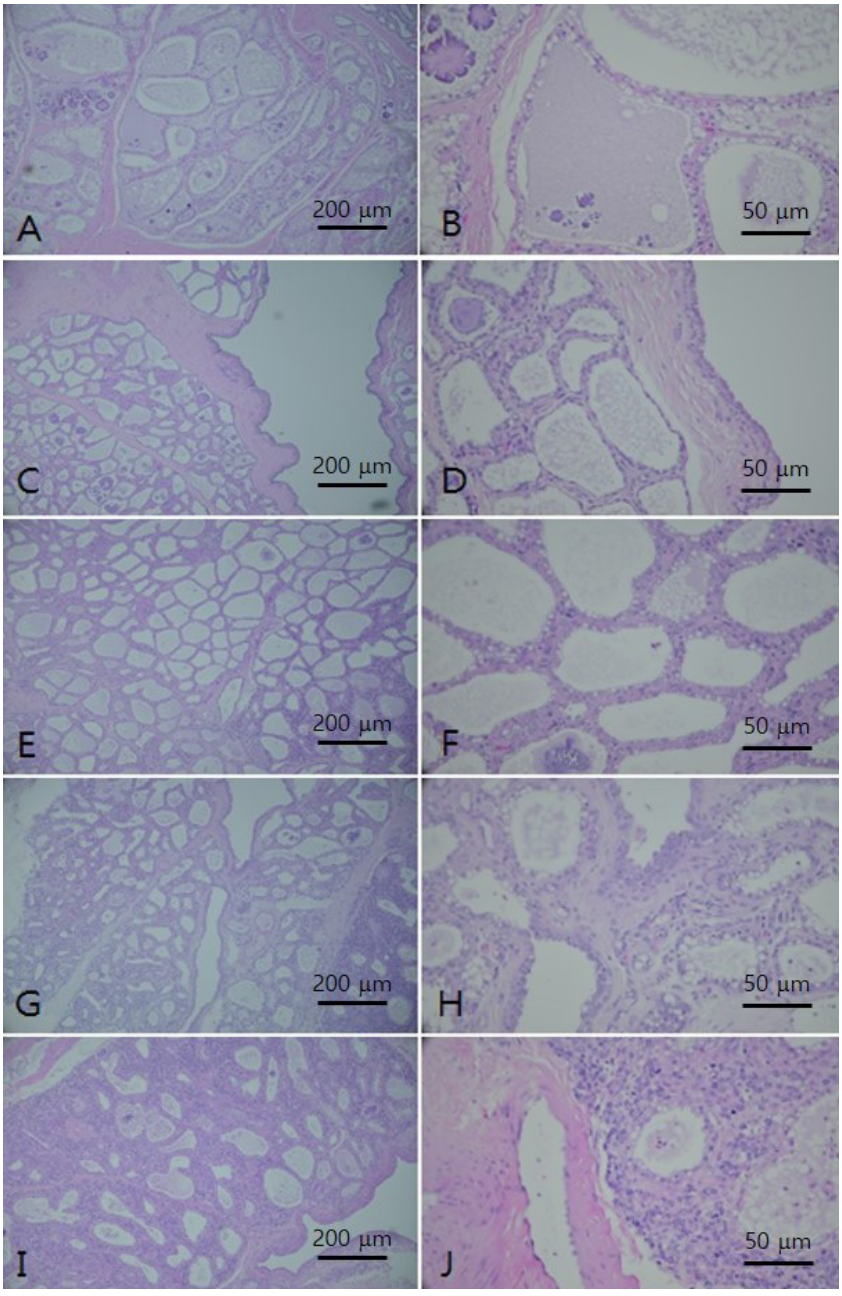

Fig. 2. Histological findings of mammary glands after administration of bee venom to normal and mastitis-affected quarters. (A, B) Normal, (C, D) 3 days after administering 6 mg of bee venom to a normal quarter, (E, F) 3 days after administering 12 mg of bee venom to a normal quarter, (G, H) 3 days after administering 6 mg of bee venom to a mastitis-affected quarter, (I, J) 3 days after administering 12 mg of bee venom to a mastitis-affected quarter. (C-J) All mammary tissues administered bee venom were hypertrophied, and plasma cells were widely distributed in the mammary tissues. (D, F, H) Infiltration and vacuolization of plasma cells were observed. Additionally, a large number of plasma cells and polymorphonuclear leukocytes were observed. (H) Severe destruction of mammary gland tissue, vacuolization of mammary tissue, and more polymorphonuclear leukocytes were observed. The images were obtained at 100 (A, C, E, G, I) and 400 magnifications (B, D, F, H, J). The scale bars represent 200 μm for images at 100 magnification and 50 μm for images at 400 magnification. Section of the mammary gland stained with hematoxylin-eosin.

The results of measuring the SCC after administering bee venom to normal and mastitis quarters are shown in Fig. 1. When 6 mg and 12 mg of bee venom were administered in the normal quarter, the SCC increased from less than 200,000 SC/mL before administration to more than 5 million SC/mL after the third administration. The SCC gradually decreased from the 4th to 6th

administration of bee venom, but it remained above 2 million SC/mL even after the 6th administration. After administering 6 mg and 12 mg of bee venom to the mastitis quarter, the SCC increased from less than 5 million SC/mL before administration to more than 5 million SC/mL after the third administration. The SCC gradually decreased until the 4th to 5th administration of bee venom. After the sixth administration of 12 mg of bee venom, the SCC decreased to less than 3 million SC/mL, while the SCC in the 6 mg administration quarter increased again to more than 5 million SC/mL. After the 6th administration of bee venom, the SCC was highest in the 6 mg administration mastitis quarter, followed by the 12 mg administration normal quarter, 6 mg administration normal quarter, and 12 mg administration mastitis quarter. There was no significant difference in the SCC between normal and mastitis quarters that received 6 mg and 12 mg doses, but there was a steady decrease in 12 mg-treated quarter ($p = 0.34$).

The histological findings of the mammary glands that received bee venom are shown in Fig. 2. All mammary tissues that received bee venom were hypertrophied, and plasma cells were widely distributed in the mammary tissues. When bee venom was administered to the normal quarter, tissue destruction was not severe, but infiltration and vacuolization of plasma cells were observed. Histological findings of mastitis-affected mammary glands showed destruction of the mammary glands, and extensive vacuolization was observed in the mammary cells that received 6 mg of bee venom. Additionally, a large number of plasma cells and polymorphonuclear leukocytes were observed. Meanwhile, in the quarter that was administered 12 mg of bee venom, severe destruction of the mammary gland tissue, vacuolization of mammary tissue, and more polymorphonuclear leukocytes were observed than in the quarter that was administered 6 mg of bee venom.

Histopathological findings showed that tissue destruction and the appearance of polymorphonuclear leukocytes were more severe in the 12 mg dose quarter than in the 6 mg dose quarter. However, based on the clinical symptoms and decrease in the SCC, the recommended dose for treatment was selected as 12 mg of bee venom.

Determination of the treatment duration and frequency

We investigated changes in SCC in dairy cows with clinical mastitis that were administered 12 mg of bee venom twice a day for 2 days (group A), once a day for 4 days (group B), and once a day for 7 days (group C) (Fig. 3). In all groups that were administered 12 mg of bee venom, the SCC increased until the third administration, but it rapidly decreased upon the fourth administration. The SCC in groups A and B gradually decreased, but it remained above 1 million SC/mL. In group C, the SCC decreased to less than 300,000 SC/mL on the 6th day and to normal levels on the 7th day. As a result, the number of treatments was once a day and the treatment period was 7 days.

Group A was diagnosed with nonspecific mastitis in which bacteria could not be isolated. In addition, the clinical symptoms of Group A improved, but the SCC did not return to normal; thus, it was determined that the patient was not

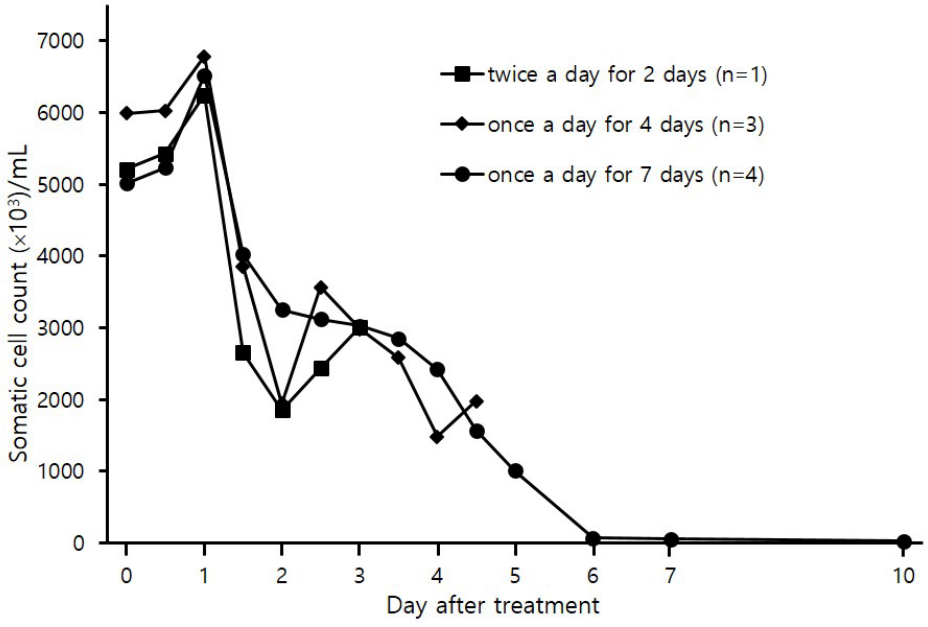

Fig. 3. Changes in the somatic cell count after treatment of dairy cows with mastitis treated with 12 mg of bee venom. Group A: a group administered twice a day for 2 days (n = 1). Group B: a group administered once a day for 4 days (n = 3). Group C: a group administered once a day for 7 days (n = 4).

completely cured. Group B showed different treatment responses depending on the causative bacteria. The causative agent of mastitis in cow No. 1 was *Serratia marcescens*, *E. coli* in cow No. 2, and *Citrobacter koseri* in cow No. 3. The SCC was decreased to less than 300,000 SC/mL on the 13th day after treatment only in cow No. 3. The bacteria identified in group C was *E. coli*, and the SCC on the 3rd day after the last administration was decreased to less than 200,000 SC/mL. After administering 12 mg of bee venom for 7 days, the SCC was compared before 6 days and after 7 days, and the SCC was significantly decreased to less than 100,000 SC/mL after 7 days ($p = 0.01$). Based on the results of the preliminary experiment, the administration dose of bee venom was determined to be 12 mg, the administration frequency to be once per day, and the administration period to be 1 week.

Discussion

Despite many years of efforts to control mastitis, this type of infection remains one of the leading causes of economic losses to the global dairy industry (33). Losses due to mastitis include many costs, such as diagnosis of the source of infection, veterinary services, medication, farrowing, wasted milk, reduced milk production, early culling and mortality (16,19,26). In addition, the use of antibiotics to treat mastitis has a significant impact on human health and it is also an important factor in animal welfare (33,53).

Bee venom has been used to treat human diseases for a long time, and current reports show that it is a natural physiologically active substance that is effective in anti-inflammatory, antibacterial, and pain management, and for the treatment of chronic inflammatory diseases or incurable diseases (3,24,25,29,54). Bee acupuncture therapy is being utilized not only in human medicine but also in the promotion of livestock production (23), and for the prevention and treatment of diseases in livestock (1,11,54). It involves the insertion of needles at specific points on the body or acupoints for preventive and therapeutic purposes, applied on the skin or muscles. It is known to have excellent effects, particularly in enhancing the immunity of newborn pigs and calves (21,40), treating diarrhea in animals (11,18,35), managing agalactia in sows (21), and improving mastitis and metritis in cows (22). An advantage is its ability to enable livestock farmers to directly treat animal diseases themselves (11). Furthermore, a significant issue in animal agriculture is the overuse of antibiotics leading to antibiotic resistance, resulting not only in reduced effectiveness but also in the accumulation of residual antibiotics in animal products that can affect human health (4,7,14,43,46). Hence, for the production of safe, high-quality animal products, acupuncture has recently gained considerable attention as an alternative method.

Generally, bee venom is used by directly injecting it or bee stings into acupoints or affected areas. In cattle with mastitis, bee venom is injected into the Yangmyeong acupoints, but the treatment is limited due to injections and stress reactions. In this study, a method of injecting 0.2 mg of bee venom into the Yangmyeong acupoint of 19 cows with mastitis was selected in a preliminary experiment, but due to the stress of cows, further treatment was not possible from the second session. Therefore, a method of injecting bee venom into the udder was proposed to overcome these limitations (22). Therefore, in order to facilitate the treatment of mastitis, direct intramammary administration was chosen as the administration route.

As a result of comparing the efficacy of Korean bee venom and American bee venom, domestic bee venom had a lower MIC than imported bee venom (Table 1). This result was consistent with the report by Kim et al. (29), which showed that domestic bee venom was more effective than American bee venom. In addition, the efficacy of domestic bee venom showed similar results to that of melittin, and it was confirmed in a previous preliminary experiment that the concentration of melittin in domestic bee venom was higher than that of American bee venom (data not shown). As a result, the findings were identical to the report, which showed that the antibacterial activity of bee venom is closely related to the content of melittin (10,37,51). Bee venom contains many different types, including peptides such as melittin and apamin, enzymes such as phospholipase A2 and hyaluronidase, non-peptides such as biologically active amines, and amino acids such as histamine and epinephrine (6,11). Among them, melittin, which is the main compound and accounts for 40-60% of the dry weight of bee venom, is the substance that exerts the main efficacy (8,15,41). Additionally, it has been reported that the higher the melittin content in bee venom, the better the efficacy (37). The content of melittin varies greatly depending on the season and region where bee venom is collected (47). Therefore, it seems necessary to further standardize bee venom when trying to use it as a natural substance that can replace antibiotics. In this study, the bee venom showed more robust antibacterial activity against GP bacteria than GN bacteria, while the bee venom and melittin also have antibacterial activity at higher concentrations than the antibiotics. This result was consistent with the report from previous researchers (19,37,51).

There are reports related to the dosage of bee venom in humans, including the therapeutic dosage using acupuncture points and oral dosage of bee venom in humans (27,48,49).

In animals, there are reports related to providing drinking water and acupuncture point treatment (20,29,52), but reports of doses administered directly into the mammary gland affected by mastitis are extremely rare (22). According to one report, treatment of quarters affected by mastitis with 3, 6, 12, and 24 mg of bee venom showed that 12 and 24 mg were more effective than 3 and 6 mg. However, it was reported that the therapeutic effects of 12 mg and 24 mg were similar, and 12 mg was selected as the therapeutic dose (22). In this study, when 6 mg and 12 mg were administered, redness and swelling were more severe in the 12 mg group than in the 6 mg quarter, but udder flexibility was better in the 12 mg quarter. Histological findings also showed that the quarter that received 12 mg showed more pronounced mammary gland phagocytosis, and a greater presence of polymorphonuclear leukocytes than the quarter that received 6 mg (Fig. 2), but the decrease in SCC was evident (Fig. 1); thus, 12 mg was set as the therapeutic dose.

The criteria for determining effectiveness in the treatment of mastitis are disappearance of clinical symptoms and reduction of SCC to less than 200,000 SC/mL (22). In this study, to examine the treatment period and number of daily administrations of bee venom for the treatment of mastitis, SCCs were measured after bee venom was administered for 2, 4, and 7 days. In the group that was administered for 7 days, the SCC continued to decrease until the 5th day and it decreased to less than 200,000 SC/mL on the 7th day (Fig. 3). In the group treated within 4 days, the SCC did not decrease below 200,000 SC/mL by the 4th day. Considering the results of this study, it is judged that treatment for at least 7 days is necessary to treat clinical mastitis, and administration once a day is advisable because administration twice a day may affect the mammary tissue. It is also necessary to vary the treatment period depending on the causative agent of mastitis. The results were similar to those in reports that observed changes in SCC after treating mastitis for 7 days and that treatment results vary depending on the causative agent of mastitis and SCC at diagnosis (22).

In conclusion, it was confirmed that a dose of 12 mg of bee venom and a treatment period of more than 7 days were required to treat mastitis, and that treatment with bee venom alone against GN bacteria was negative.

Conflicts of Interest

The authors have no conflicting interests.

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