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Therapeutic Effect and Mechanism of Action of Low-molecular-weight Whey Protein Capable of Activating Macrophages in Bovine Mastitis

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Abstract. *Background/Aim: Bovine mastitis is caused by the invasion and propagation of pathogenic microorganisms into the udder and mammary gland tissues of cattle. In this study, the therapeutic effect of a low-molecular-weight whey protein (LMW-WP) on bovine mastitis was evaluated. Materials and Methods: LMW-WP was orally, intraperitoneally, and vaginally administered to bovine with mastitis. The number of somatic cells in milk was measured 24 h before the administration of LMW-WP. The effect of LMW-WP on cytokine production was measured with a microarray that evaluates the expression of cytokines. Results: In the group that received 1,000 mg intraperitoneally, the somatic cell count was reduced to less than 400,000 at the shipment standard value in three of the four udders, indicating 75% efficacy. The group that received 1,000 mg by vaginal administration showed 67% efficacy. It was confirmed that LMW-WP increased the production of cytokines such as IL-5, IL-6, IL-9, IL-12, MCP-1, and VEGF in mouse macrophage cells, but it did not show any antibacterial activity. Conclusion: LMW-WP may be an effective therapeutic agent for bovine mastitis.*

Bovine mastitis is a general term used to describe the inflammation caused by the invasion of pathogenic microorganisms such as bacteria into the udder and mammary gland tissues of cattle. Many abnormalities can appear in the milk as a result, including the degeneration of milk quality, a decrease in the yield, an increase in the number of somatic cells, and an abnormal pH. These can be accompanied by breast swelling, pain, hotness, and redness. As a result, cows affected by mastitis may have reduced milk production and quality and may require expenditure on treatment. Additional drawbacks may arise, such as wasted milk due to shipping restrictions, the renewal of culling, penalties on milk prices due to an increased somatic cell count, and loss of work. The impact of bovine mastitis on dairy management is significant; economic losses are approximately 80 billion yen in Japan each year (1, 2). Over 140 known pathogenic microorganisms cause mastitis, and their diversity makes treatment difficult (3). Currently, antibiotics are the primary treatment modality for mastitis. However, there are concerns about the use of antibiotics in cattle, such as waste milk that has been disposed due to shipping restrictions, the complexity of drug selection, and the emergence of drug-resistant bacteria due to the chronic overuse of antibiotics (4). Various alternatives to antibiotics such as ozone therapy, oral administration of stevia, and intramammary recombinant bovine granulocyte macrophage-colony stimulating factor (rbGM-CSF) have been studied, but no effective treatment has been established thus far (5-7).

Previously, a low-molecular-weight whey protein (LMW-WP) that has been suggested to have a high absorption; functionality has been developed successfully through hydrolysis of a whey protein with immunopotentiating and immunomodulating properties (8, 9). LMW-WP has been reported to induce macrophage phagocytosis and the production of tumor necrosis factor (TNF)-α (10). TNF-α exerts a variety of physiological and pathogenic effects on the activation of a cascade of inflammatory cytokines and tissue dysfunction and destruction (11). Additionally, high

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concentrations of LMW-WP have been reported to induce production of IL-10, an anti-inflammatory cytokine (10). Therefore, the ability of LMW-WP to activate phagocytosis by macrophages and to produce cytokines was hypothesized to be effective against mastitis caused by various bacterial infections. In addition, whey peptides possess antibacterial properties and contain lactoferrin (12, 13), which is involved in host defense mechanisms against various infectious diseases and cancers. In this study, LMW-WP was administered to bovines with mastitis, and changes were evaluated regarding the somatic cell count in milk and comprehensive cytokine production. This study attempted to elucidate the mechanism of action of LMW-WP in bovine mastitis.

Materials and Methods

Preparation of LMW-WP. 50 g of whey protein (PRODIET 80S; Ingredia, France) was dissolved in 500 ml of 100 mM sodium phosphate buffer (SPB) (pH=7.0) and stirred for approximately 20 min to yield a 10% whey protein solution. A protease (Proteax; AMANO Enzyme Inc., Nagoya, Japan) with 5% whey protein by weight in the mixed solution was added, and the solution was reacted at 50˚C for 1 h, and then at 80˚C for 5 min to inactivate the enzyme.

Clinical trial of mastitis using LMW-WP. The number of somatic cells in milk was measured 24 h before the administration of LMW-WP. The International Federation of Dairy defines mastitis as having a somatic cell count of 400,000/ml or more, and this standard is used in Japan (14). Bovine udders with a somatic cell count of 400,000/ml or more were used as mastitis specimens. The route of an LMW-WP administration was either oral, intraperitoneal, or vaginal. In the oral administration group, three cows (seven udders) were used as samples, and one cow (one udder) was used as the control. A single dose of LMW-WP (100 g) dissolved in water was administered, and the number of somatic cells was measured immediately before administration, as well as 6 h, 12 h, 24 h, and 5 d after administration. The specimens were collected by removing dirt from the nipples during milking, disinfecting the nipples with isopropanol, and collecting the milk by hand-squeezing five times. The measurement of somatic cells was performed by the Tokushima Dairy Agriculture Association. Five days later, the udders for which the number of somatic cells was less than 400,000/ml were judged to be effective. In the intraperitoneal administration group, three cows (five udders) were treated with a dose of 300 mg, two cows (four udders) were treated with 1000 mg, and one cow (three udders) was used as the control. LMW-WP (300 mg or 1000 mg) dissolved in physiological saline was continuously injected intraperitoneally for five days, and the number of somatic cells was counted on days one through six. The udders whose somatic cell counts became less than 400,000/ml at the end of the six-day period were considered to be effective. In the vaginal administration group, four cows (nine udders) were used as samples, and one cow (three udders) was used as the control. The capsule-filled LMW-WP (1000 mg) was continuously injected into the vagina for five days, and the criteria were the same as those in the intraperitoneal administration group. Cows for clinical trials were provided by three farms in the Tokushima Prefecture.

Evaluation of cytokine production ability. RAW 264.7 cells (KAC Co., Ltd., Kyoto, Japan) were cultured in Dulbecco's modified Eagle medium (DMEM) (Wako, Osaka, Japan) for 48 h, and then washed with 1X phosphate-buffered saline (PBS). A serum-free DMEM, 10 mg of whey protein, or 10 mg of LMW-WP was added, and the mixture was incubated at 37˚C for 48 h, followed by supernatant collection. The microarray used was a Quantibody® Mouse Cytokine Array (RayBiotech Life Inc., Norcross, GA, USA). After being washed, the array was scanned using an array scanner GenePix® 4400A (Molecular Devices LLC, Sunnyvale, CA, USA). Using the analysis software Array-Pro Analyzer® Ver.4.5 (Media Cybernetics, Inc., Rockville, MD, USA) the fluorescence intensity value at each spot was calculated from the obtained image (TIFF image, 16-bit format). Microarray measurements and analyses were performed at Filgen, Inc. (Aichi, Japan).

Minimal inhibitory concentration (MIC) measurement by microliquid dilution method. Whey protein (200 mg) and LMW-WP were dissolved in 1 ml of distilled water and sterilized with a 0.2-μm syringe filter. The whey protein solution and the LMW-WP solution were diluted with Nutrient broth medium (NB medium) to prepare a concentration of 98 to 50,000 μg/ml. The diluted solutions were added to a 96-well microplate in 80 μl portions. A coagulase-positive staphylococcal strain (S. aureus subsp. aureus NBRC 12732) that had been cultured at 37˚C for 16 h was added to each well at a concentration of 1×10^6 cells/well. To serve as a blank, 160 μl of the NB medium alone was added to one well. For the control, a mixture of 80 μl of the NB medium and coagulase-positive staphylococci (1×10^6 cells) was prepared. The microplate was incubated at 37˚C for 24 h. As a criterion, the concentration at which no bacterial growth was observed after the incubation period was defined as the MIC value.

Statistical analysis. Data have been expressed as the mean±standard deviation of at least three independent experiments. The statistical significance of the differences between the results was analyzed using the Student's *t*-test. A $p<0.05$ was considered statistically significant.

Results

Therapeutic effect of LMW-WP on bovine mastitis. In the oral administration group, efficacy of LMW-WP was confirmed five days after administration in one of the seven udders. (Figure 1A). In the 300 mg-intraperitoneal group, efficacy was not confirmed in any of the udders, but in the 1000 mg-intraperitoneal group, efficacy was confirmed in three out of the four udders (75%) (Figure 1B, C). In the vaginal-administration group, efficacy was confirmed in six out of the nine udders (67%) (Figure 1D). In addition, the somatic cell count tended to decrease in all udders of the 1000 mg-intraperitoneal and vaginal-administration groups on day six after administration of LMW-WP, compared with the somatic cell counts before administration. These results confirm that an intraperitoneal or a vaginal administration of 1000 mg of LMW-WP effectively reduces symptoms of bovine mastitis and may be considered as a viable treatment option.

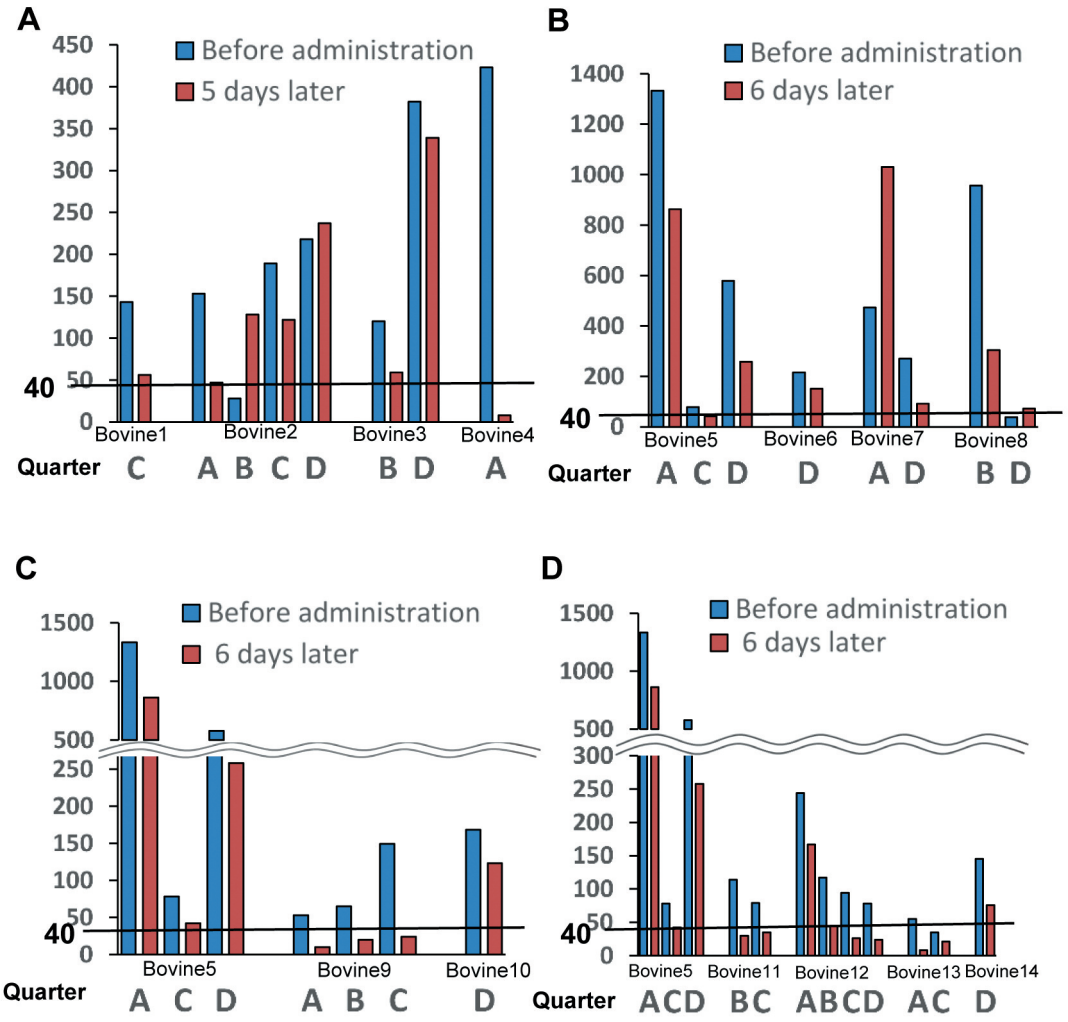

Figure 1. *Number of somatic cells in milk in each udder before and after administration of the LMW-WP. (A) Oral administration group and (B) 300 mg-intraperitoneal administration group and (C) 1000 mg-intraperitoneal administration group and (D) vaginal-administration group. The udders A to D represent the positions of the udders. (A: front left, B: left back, C: front right, D: right back).*

Comprehensive analysis of the cytokine production ability of LMW-WP. The cytokine production ability of LMW-WP was measured with a microarray to evaluate the expression of cytokines other than TNF-α and IL-10, which have been previously studied (10). The results showed that LMW-WP, in comparison with whey protein, increased the production of the following cytokines: IL-5 (1.53 times), IL-6 (1.77 times), IL-9 (1.49 times), IL-12 (1.45 times), MCP-1 (15.03 times), and VEGF (2.06 times) (Figure 2).

Antibacterial activity of LMW-WP. One potential mechanism of action of LMW-WP against bovine mastitis is as follows: LMW-WP may contain an antimicrobial peptide, and the action of the antimicrobial peptide may reduce the bactericidal effect of the causative bacteria. However, the inhibitory effect of the bacteria could not be confirmed up to the maximum concentration of 50,000 μg/ml (Table I). This result indicated that antimicrobial peptides were not present in the LMW-WP.

Discussion

In this clinical trial for bovine mastitis, the first oral route of LMW-WP administration was simple and did not require the assistance of a veterinarian, but efficacy was confirmed in only one udder (14%). One reason for the failure of this treatment method to reduce the number of somatic cells in milk is the fact that the digestive system of bovine ruminants is more complex than that of other organisms. Bovines have four stomachs; the largest of these is called the rumen (lumen), which has a

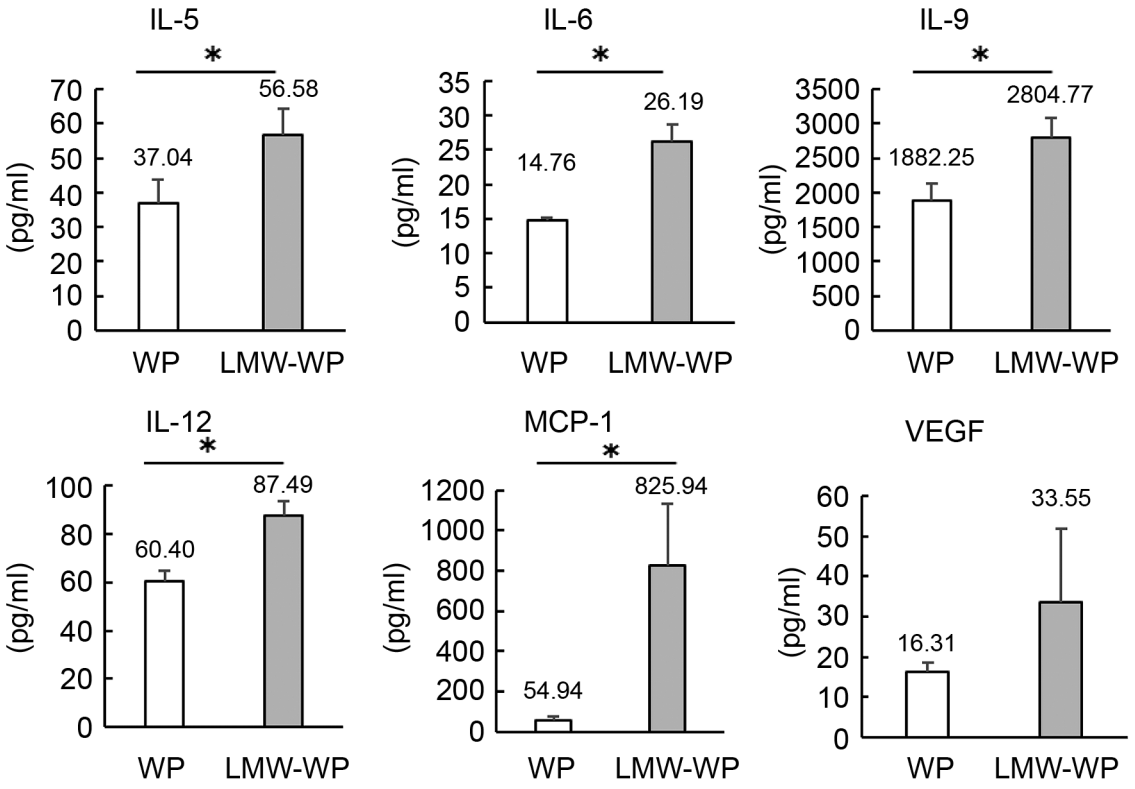

Figure 2. *Cytokine production by macrophages stimulated with whey protein and LMW-WP. Data have been presented as mean±SD (*p<0.05).*

Table I. *Antibacterial activity of LMW-WP.*

	Fungal species	Sample concentration (μg/ml)										
		50,000	25,000	12,500	6250	3125	1563	781	391	195	98	0
WP	S.A.	+	+	+	+	+	+	+	+	+	+	
LMW-WP	S.A.	+	+	+	+	+	+	+	+	+	+	
Control	S.A.											+
Blank												–

S.A.: *Staphylococcus aureus*; Control: mixed solution of the NB medium and bacterial solution; blank: NB medium only. +: Growth of the test bacteria was observed. –: No growth of the test bacteria was observed.

capacity of 180 liters in the adult cattle (15). Many microorganisms coexist in the rumen, and by the action of these microorganisms, feed such as grass is fermented and converted into a nutritionally useful form. Therefore, it is likely that LMW-WP, the active ingredient, was degraded by microorganisms coexisting in the rumen before it could exert any therapeutic effects. In order to improve the bioavailability of LMW-WP, administration was performed intraperitoneally. As a result, no effect was obtained at 300 mg, but the therapeutic effect was confirmed at 1000 mg, suggesting that the dosage of LMW-WP that is effective for mastitis may be 1000 mg or more. However, an intraperitoneal administration has to be performed by a veterinarian, which presents a challenge for administering this treatment to animals on farms. Therefore, an intravaginal administration was examined as a method that can be administered by non-veterinary doctors and deliver a high bioavailability. In the intravaginal group, a 67% therapeutic effect was observed after 1000 mg was administered continuously for five days. This result suggests that LMW-WP is absorbed by the mucous membrane in the vagina.

Next, in order to elucidate the mechanism of action of LMW-WP on bovine mastitis, the cytokine production ability of LMW-WP was evaluated. Enhanced production of the following cytokines was confirmed: IL-5 (16), which selectively promotes the proliferation and differentiation of eosinophils, prolongs survival, and promotes the migration of inflammation-related molecules; IL-6 (17), which is involved in immune responses, hematopoiesis, acute phase response, and B cell differentiation and IL-12 (18), which suppresses the growth of bacteria at the initial stage of infection by activating macrophages. In particular, MCP-1, the monocyte chemotactic factor, showed an approximate 15-fold increase in the production ability. MCP-1 is extensively involved in the enhancement of the phagocytic activity of macrophages, which suggests that one of the mechanisms of action of LMW-WP is suppression of bacterial growth *via* an activation of the immune system.

Activation of macrophages by LMW-WP has been hypothesized to induce two types of macrophage differentiation. First, it induces differentiation into the M1-type macrophages at the site of infection in the udder tissue and exhibits an antibacterial activity against pathogenic bacteria. Next, since the LMW-WP's ability to produce VEGF was confirmed, the induction of differentiation into the M2-type macrophages with anti-inflammatory and immunosuppressive functions terminated the tissue damage reaction and repaired the tissue (19). These are two probable reasons why the LMW-WP was able to decrease the number of somatic cells in milk.

Another possible mechanism of action of the LMW-WP on bovine mastitis under consideration involved the antimicrobial peptides contained in the LMW-WP. However, the results of the antimicrobial test showed that the antimicrobial peptides were not involved.

In conclusion, LMW-WP may be an effective therapeutic agent for bovine mastitis because LMW-WP exhibits a growth inhibitory effect on *Staphylococcus aureus* and Streptococcus, the causative bacteria of bovine mastitis, by inducing the phagocytic activity of macrophages and production of various cytokines. In the future, the application of LMW-WP may prove to be an effective treatment modality for various cancers and infectious diseases.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

T.T. and Y.U.designed the study. T.T, K.M., H.Y., and A.S. carried out all experiments and drafted the manuscript. H.U., K.T., A.H., A.G., K.G., and K.G gave technical support and conceptual advice. As the principal investigator, Y.U. supervised the study. All Authors read and approved the final manuscript.

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