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Research Article

Bee Venom Apitoxin Alleviates Collagen-induced Arthritis in DBA-1J Mice

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ABSTRACT

The anti-inflammatory, antibacterial effect, and anti-aging properties of the venom of honey bee *Apis mellifera* L. have been investigated and exploited for pharmaceutical and cosmetic applications for decades. Apitoxin, a drug based on bee venom, is administered *via* injection and is permitted for use in humans for its antipyretic, analgesic, and anti-inflammatory properties. This study was conducted to expand the scope of apitoxin application to arthritis. We used a DBA-1J mouse model of collagen-induced arthritis (CIA). Apitoxin was injected intradermally into the mice twice a week, and the body weight, arthritis score, and hindlimb paw edema were recorded once a week for 4 weeks in mice with CIA. The body weight of the groups with CIA was significantly lower than that of the group without CIA during the experimental period. Although the arthritis scores of all groups with CIA were higher than those of the group without CIA, the high dose apitoxin-treated group had a significantly improved arthritis score than the vehicle group on days 14 and 28. A significant reduction in edema was observed in the hindlimb paws in the apitoxin-treated group compared with that in the vehicle group. Additionally, inflammatory cytokines, such as IL-6 and IL-1β, and IgG antibodies to type II collagen, were detected in the articular tissue of mice with CIA on day 28. Both IL-6 and anti-type II collagen IgG levels significantly decreased in the apitoxin-treated group compared with those in the vehicle group. Histopathological analysis of the articular tissue also revealed similar improved results in the apitoxin-treated group. In conclusion, these results suggested that apitoxin has potential therapeutic application in patients with arthritis.

Introduction

The venom from the honey bee (*Apis mellifera* L.) is a natural toxin and was used in traditional medicine in ancient Egypt.[1] Recently, several components of bee venom have been proven to have beneficial effects, such as pharmaceutical applications against various diseases.[2] Bee venom contains pharmaceutically active peptides such as melittin, adolapin, phospholipase A2 (PLA2), and apamin.[3] Melittin is the main component of bee venom, accounting for 50% of the total dry weight, and has various medicinal properties, including anti-inflammatory, anti-arthritic, anticancer, antibacterial, and antiviral properties.[4] PLA2 is a mast cell degranulating (MCD) peptide in the bee venom. One study showed that topical administration of PLA2 alleviated atopic dermatitis symptoms and inflammation and modulated IgE concentration.[5] Research on the effect of bee venom on arthritis started with a simple study by Prof. Lee in 2004[6] to confirm that therapeutic enzymes, such as PLA2, play a major role in arthritis.[7]

Osteoarthritis (OA) is a disease that causes life disabilities worldwide.[8] OA is a representative aging

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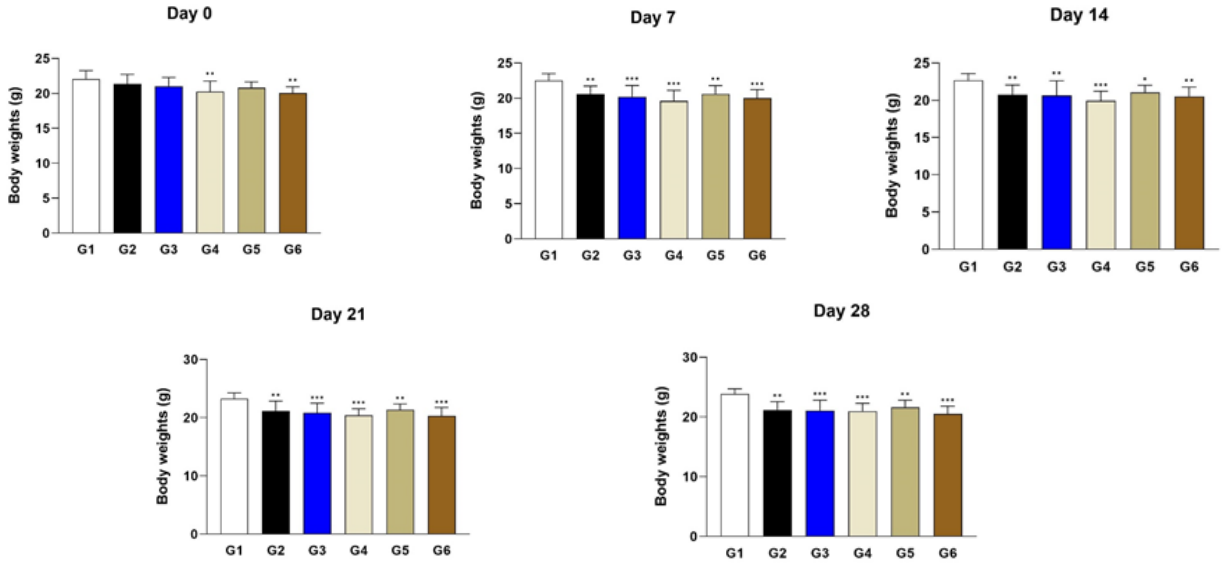


Fig. 1: Body weight changes in mice with CIA treated with apitoxin.
Body weights were measured once a week until 28 days after the first apitoxin administration day (day 0). Data are expressed as mean ± SD. G1: normal control; G2: vehicle control; G3: methotrexate 2 mg/kg; G4: Apitoxin low dose; G5: Apitoxin medium dose; G6: Apitoxin high dose; ***/**/* Significant difference at p <0.001/p <0.01/p <0.05 compared with G1.

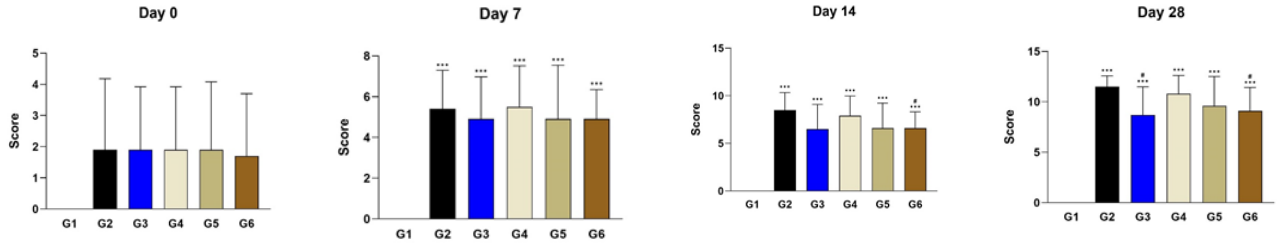


Fig. 2: Arthritis scores in mice with CIA treated with apitoxin.
Arthritis scores were evaluated once a week for 28 days after the first apitoxin administration day (day 0). Data are expressed as mean ± SD. G1: normal control, G2: vehicle control, G3: methotrexate 2 mg/kg, G4: Apitoxin low dose, G5: Apitoxin medium dose, G6: Apitoxin high dose, *** Significant difference at p<0.001 compared with G1. # Significant difference at p<0.05 compared with G2.

disease prevalent in people over 65 years of age, affecting approximately 60% of men and 70% of women.[9,10] The incidence of OA is increasing and is expected to increase to approximately 100,000 cases per year.[11] The increased incidence of OA appears to be due to the extended lifespan of people; in fact, old age, lifestyle, diet, and obesity are more responsible than direct damage to the joint.[8,10-12] OA, characterized by inflammation of the cartilage and synovial fluid, is a chronic degenerative disease.[13] As OA progresses, subchondral bone sclerosis and erosion of bone margin occur. Many pathological causes trigger OA; several pro-inflammatory cytokines and signals of complex mechanisms associated with immune cells are the leading causes of OA.[14] In addition, levels of matrix metalloproteinases, lysozyme, substance-p, and various pro-inflammatory cytokines increase in synovial fluid and serum.[12,15] Drugs used to treat OA include opioids, analgesics, COX-2 inhibitors, and non-steroidal anti-inflammatory drugs (NSAIDs) to alleviate inflammation.[16] The discovery and development of various candidate drug groups is the need of the hour owing to the many side effects, such as cardiovascular and gastrointestinal disorders, of current commercial drugs against OA, which are also harmful to the cartilage.[17]

Apitoxin is a synonym for bee venom, made of dried honey bee venom, and is also the name of the drug administered via injection in Korea. The main component of the drug is melittin, comprising 52% venom peptides and 2% adolapin. This drug has been administered to patients in Korea for its antipyretic, analgesic, and anti-inflammatory effects and to treat diseases other than arthritis. The present study was conducted to expand the scope of apitoxin application to arthritis. We confirmed the potential of apitoxin as a therapeutic agent for arthritis in a mouse model of collagen-induced arthritis (CIA).

Materials and Methods

Animal Experimental Design

Sixty pathogen-free male DBA/1JJmsSlc mice were obtained from SLC (Hamamatsu, Japan). The mice were housed in stainless-steel cages (W 895 × L 795 × H 765 mm).

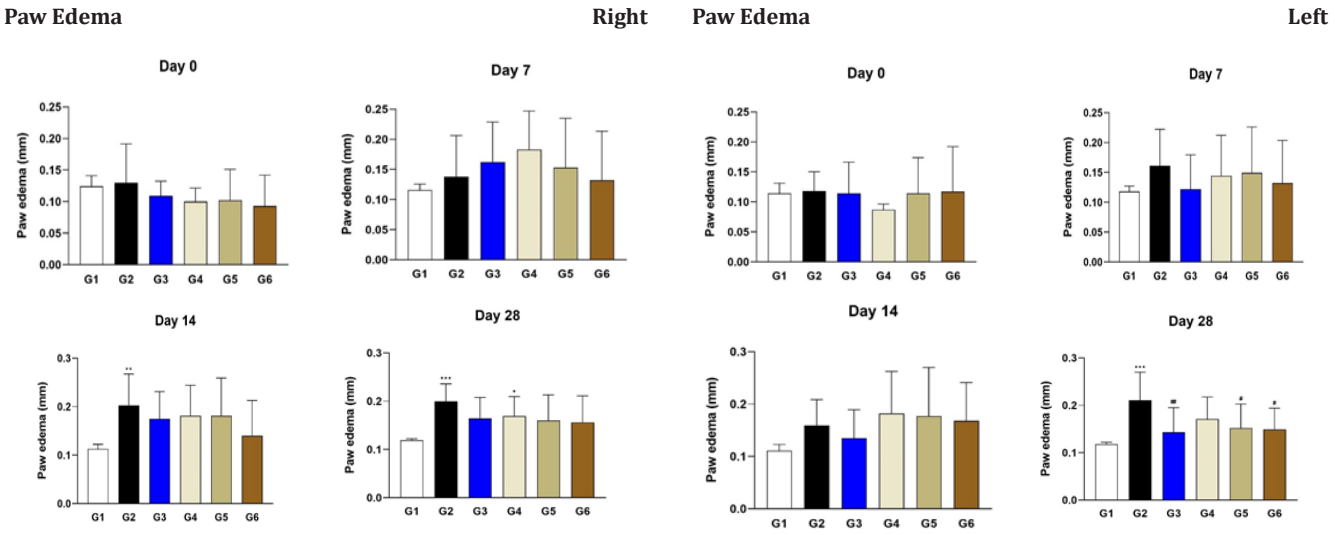


Fig. 3: Paw edema in mice with CIA treated with apitoxin.
(A) Left paw and (B) right paw edema were evaluated once a week for 28 days from the first apitoxin administration day (day 0). Data are expressed as mean ± SD. G1: Normal control, G2: Vehicle control, G3: methotrexate 2 mg/kg, G4: Apitoxin low dose, G5: Apitoxin medium dose, G6: Apitoxin high dose, *** Significant difference at p<0.001 level compared with G1. ##/# Significant difference at p<0.01/p<0.05 level compared with G2.

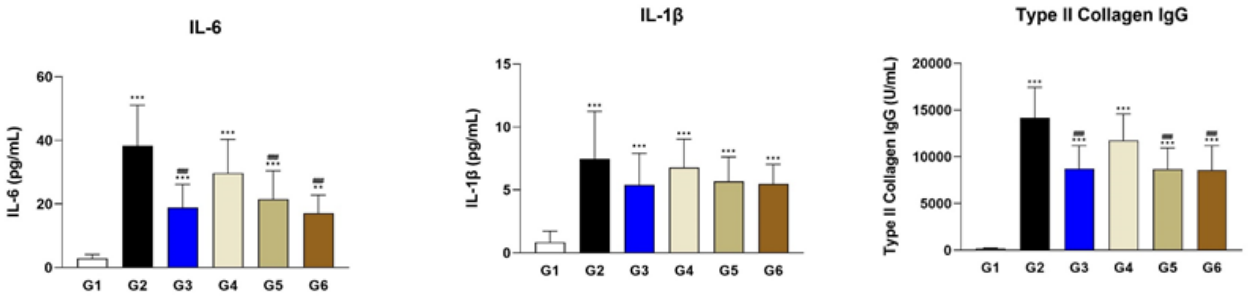


Fig. 4: Enzyme-linked immunosorbent assay (ELISA) of serum from mice with CIA treated with apitoxin.
IL-6, IL-1β, and anti-type II collagen IgG levels were evaluated in euthanized mice on day 28. Data are expressed as mean ± SD. G1: normal control, G2: vehicle control, G3: methotrexate 2 mg/kg, G4: Apitoxin low dose, G5: Apitoxin medium dose, G6: Apitoxin high dose, *** Significant difference at p<0.001, compared with G1. ###/## Significant difference at p<0.001/p<0.01, compared with G2.

Five mice were housed in one cage and placed in an environmentally controlled room maintained at a temperature of 23 ± 3°C, relative humidity of 55 ± 15%, ventilation frequency of 10–20 times/h, lighting time of 12 h/day (8 am to 8 pm), and illuminance of 150–300 lx. Food and water were freely available to the mice. After induction of CIA, when the total average arthritis score of all four limbs of the mice reached 1.5–2.0, they were grouped randomly.

The mice were equally divided into six groups for the experiment: the normal control group (G1), the vehicle-treated group with CIA (G2), the methotrexate-treated (2 mg/kg/twice a week) positive control group with CIA (G3), the low concentration apitoxin-treated group with CIA (G4), the medium concentration apitoxin-treated group with CIA (G5), and the high concentration apitoxin-treated group with CIA (G6).

Apitoxin was administered to the mice in G4, G5, and G6 twice a week for 4 weeks via intradermal injection. After weighing the appropriate amount of apitoxin, it was diluted to the appropriate concentration using saline. G4 received 0.04 mg/kg of apitoxin in the first week, 0.08 mg/kg in the second week, 0.16 mg/kg in the third week, and 0.24 mg/kg in the fourth week. G5 received 0.16 mg/kg of apitoxin in the first week, 0.24 mg/kg in the second week, 0.32 mg/kg in the third week, and 0.64 mg/kg in the fourth week. G6 received 0.32 mg/kg of apitoxin in the first week, 0.64 mg/kg in the second week, 0.96 mg/kg in the third week, and 1.2 mg/kg in the fourth week. Saline was administered to G1 and G2 on each injection day. All animals were closely monitored, and body weight, arthritis score, and paw edema were measured once a week for 4 weeks. On the day of necropsy, all the surviving animals were anesthetized, and the joints were fixed in 10% neutral buffered formalin.

(A) Histopathological Xamination

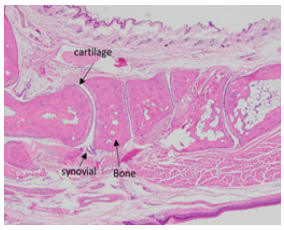
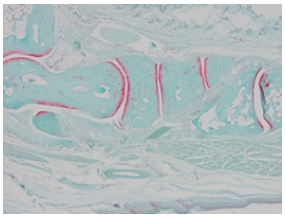
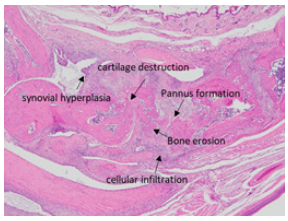
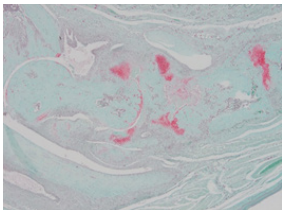
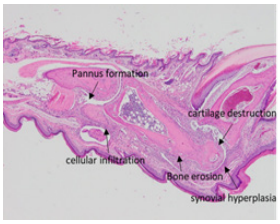
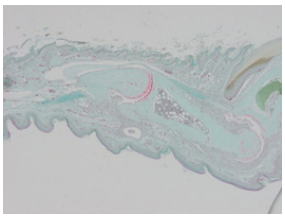
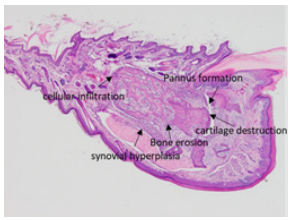
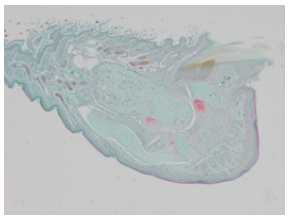
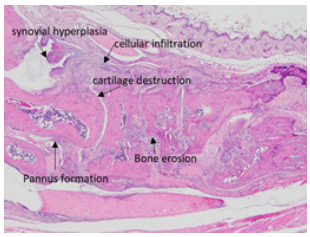
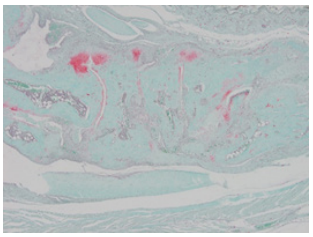
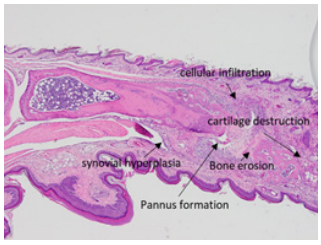
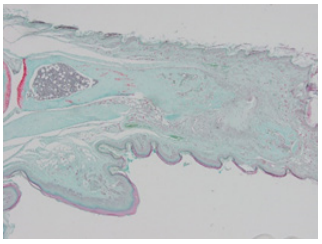
Group	H&E	Safranin O
G1		
G2		
G3		
G4		
G5		
G6		

Fig. 5 (A): Histopathology of articular tissues of mice with CIA treated with apitoxin.
Cellular infiltration, synovial hyperplasia, cartilage destruction, bone erosion, and pannus formation were evaluated in the articular tissues of euthanized mice on day 28. (A) Histopathology analysis, including hematoxylin and eosin (H&E) and safranin O staining. (B) Scoring analysis of H&E staining among all experimental groups. Data are expressed as mean ± SD. G1: normal control, G2: vehicle control, G3: methotrexate 2 mg/kg, G4: Apitoxin low dose, G5: Apitoxin medium dose, G6: Apitoxin high dose, *** Significant difference at $p<0.001$, compared with G1. ###/##/# Significant difference at $p<0.001$/$p<0.01$/$p<0.05$, compared with G2.

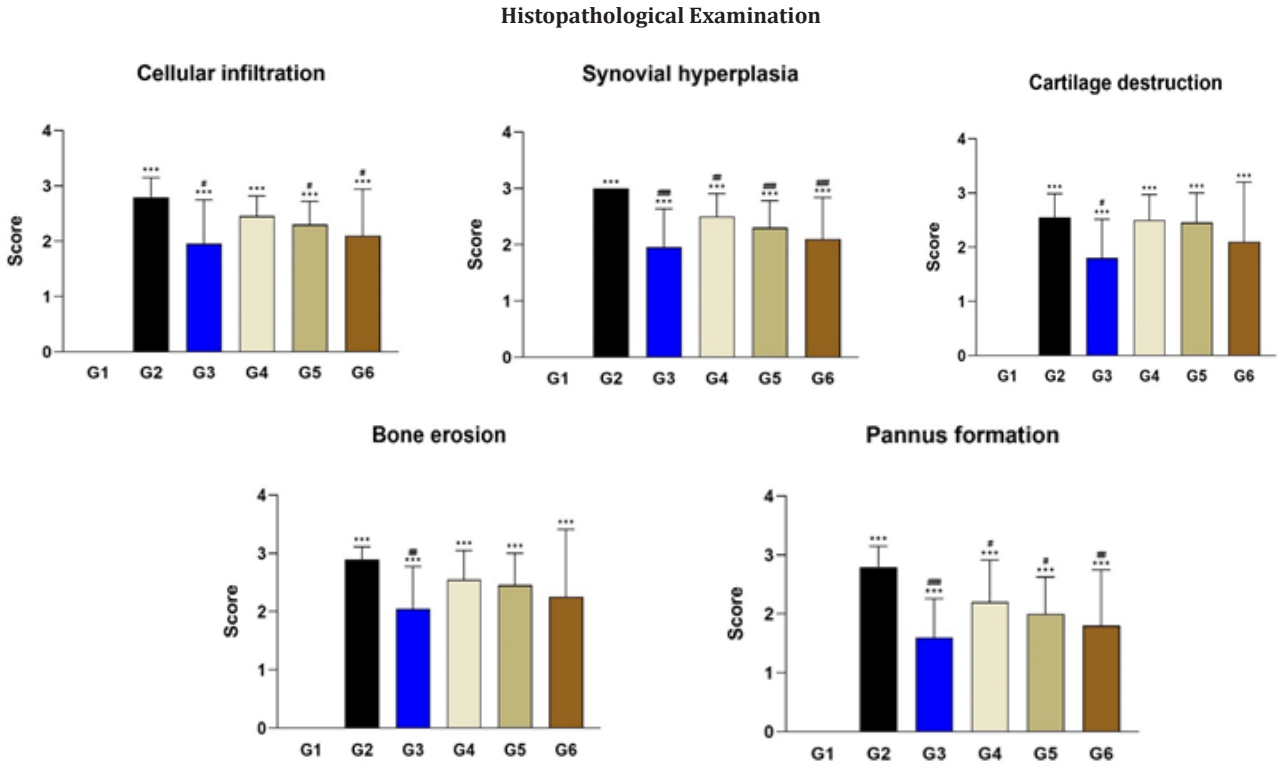


Fig 5 (B)**:** Scoring analysis of H and E staining.

Blood samples were collected from the caudal vena cava. The collected blood was injected into a vacutainer tube containing a blood clot activator. The blood samples were left to coagulate at room temperature for approximately 15–20 minutes. Subsequently, serum samples were collected after centrifugation of blood samples at 12,000 rpm for 2 minutes, and they were kept in a deep freezer (about -70°C). This animal study was approved by the Institutional Animal Care and Use Committee of KNOTUS Co. Ltd., Incheon-si, Korea (Certificate No: IACUC 21-KE-273).

Induction of CIA

A modified mouse model of CIA was used in the present study.[18] Initial immunization for CIA was performed in DBA/1J male mice under light anesthesia via intradermal injection of 100 µL/head of a 1:1 mixture of bovine type II collagen (2 mg/mL, 0.05 mol/L acetic acid; Chondrex, Woodinville, WA, USA) and complete Freund's adjuvant at the base of the tail. The second immunization was on day 21, and the same concentration of bovine type II collagen was administered via intradermal injection along with incomplete Freund's adjuvant at the base of the tail to boost CIA in the mouse model.

Arthritis Score and Paw Edema Measurement

The arthritis score was assessed on the day of the test article administration, just before the administration. Scores were obtained once a week for 4 weeks. Scores ranged from 0 to 4 according to severity: 0, no edema or swelling; 1, minor edema and redness limited to the joint; 2, minor edema and redness along the joint to the carpal or tarsal region; 3, severe edema and redness along the joint to the carpal or tarsal region; 4, diffuse edema and redness with joint stiffness. The sum of scores of all four limbs was calculated; the maximum score would be 16. Paw edema in each mouse was first measured on the day of administration of the test article, followed by once a week for four weeks, using a plethysmometer.

Enzyme-linked Immunosorbent Assay (ELISA) of the Serum

The concentrations of IL-1β, IL-6, and anti-type II collagen IgG in the serum obtained from the centrifugation of blood collected on the day of the necropsy were determined using ELISA.

Histopathological Analysis

Fixed tissues were subjected to general tissue treatment, including sectioning, cutting, dehydration, paraffin embedding, and dissection to prepare specimens for histopathological examination. Hematoxylin and eosin (H and E) and safranin O staining were performed. Histopathological changes were observed under an optical microscope (Olympus BX53; Olympus Corporation, Tokyo, Japan).

Statistical Analysis

The results of this study were assumed to be normally distributed and analyzed using parametric multiple

comparison procedures or non-parametric multiple comparison procedures. When the one-way analysis of variance (ANOVA) results were significant, post-hoc analysis was performed using Dunnett's multiple comparison procedure. In the case of non-parametric multiple comparisons, the Kruskal–Wallis H test was used, and if a significant difference was observed, the Mann–Whitney U test was used for post-hoc analysis. Statistical analysis was performed using Prism 7.04 (GraphPad Software Inc., San Diego, CA, USA), and statistical significance was set at a $p < 0.05$.

Results

Apitoxin Improves Arthritis Symptoms in Mice with CIA

Administration of the test article neither caused death nor any notable clinical signs during the entire study period. The results of body weight measurement (Fig. 1) indicated that the body weights of G4 and G6 were significantly lower than those of G1 on the day (day 0) of initiation of apitoxin administration ($p < 0.01$), and the body weights of the entire CIA induction group were significantly lower than those of G1 from the 7th day (day 7) of the initiation of apitoxin administration till the end of the experiment ($p < 0.001$, $p < 0.01$, or $p < 0.05$).

The arthritis assessment (Fig. 2) indicated that arthritis scores of the entire CIA induction group were significantly higher than those of G1 from day 7 till the end of the experiment ($p < 0.001$). The arthritis scores of G6 were significantly lower than those of G2 on the 14th day after the initiation of apitoxin administration ($p < 0.05$), and the arthritis scores of G3 (the methotrexate-treated group) and G6 were significantly lower than those of G2 on the 28th day after the initiation of apitoxin administration ($p < 0.05$).

Paw edema was evaluated in both the left and right hind limbs as shown in Fig. 3, the left hind limb paw edema levels of G2 were significantly higher than those of G1 on day 28 ($p < 0.001$), and the left hindlimb paw edema levels of G3, G5, and G6 were significantly lower than those of G2 on day 28 ($p < 0.01$ or $p < 0.05$).

The right hind limb paw edema levels of G2 were significantly higher than those of G1 on the 14th day ($p < 0.01$) after the initiation of apitoxin administration ($p < 0.05$). The right hind limb paw edema levels of G2 and G4 were significantly higher than those of G1 on day 28 ($p < 0.001$ or $p < 0.05$).

Accordingly, inflammatory cytokines, such as IL-6 and IL-1β, and anti-type II collagen IgG in the serum were analyzed using ELISA (Fig. 4). IL-6, IL-1β, and anti-type II collagen IgG levels in the groups with CIA were significantly higher than those of G1 ($p < 0.001$, $p < 0.01$, or $p < 0.05$), while IL-6 and anti-type II collagen IgG levels in G3, G5, and G6 were significantly lower than those of G2 ($p < 0.001$).

Apitoxin Improves Articular Structures in Mice with CIA

H&E and safranin O staining were performed for the histopathological examination of the joints of mice with CIA (Fig. 5A). H and E staining is the most basic staining method used to determine tissue structure. Safranin O binds to glucosaminoglycan to produce an orange color and is frequently used to stain the articular cartilage. Although the result was negative in safranin O staining, the results of H and E staining suggested the application of apitoxin in treating joint tissues. Cellular infiltration, synovial hyperplasia, cartilage destruction, bone erosion, and pannus formation were evaluated and quantified (Fig. 5B). Histopathological examination revealed that the cellular infiltration levels in all the groups with CIA were significantly higher than those in G2 ($p < 0.001$), and the cellular infiltration levels of G3, G5, and G6 were significantly lower than those of G2 ($p < 0.05$). Synovial hyperplasia and pannus formation levels in all the groups with CIA were significantly higher than those in G2 ($p < 0.001$), and synovial hyperplasia and pannus formation levels of G3 and all the groups administered with apitoxin were significantly lower than those in G2 ($p < 0.001$, $p < 0.01$, or $p < 0.05$). Cartilage destruction and bone erosion levels in all the groups with CIA were significantly higher than those in G1 ($p < 0.001$), and cartilage destruction and bone erosion levels in G3 were significantly lower than those in G2 ($p < 0.01$ or $p < 0.05$).

Discussion

This study aimed to expand the application of apitoxin, dried bee venom, using a mouse model of CIA. Treatment with bee products is called apitherapy; honey, pollen, bee bread, propolis, royal jelly, beeswax, bee venom, and larvae are commonly used. The Latin word *Apis* means bee.[19] Bee products are used in traditional medicine in many countries. Apitherapy products have often been used in natural treatment methods for health maintenance from ancient to recent periods.

Many studies have shown that bee venom has pharmaceutical properties owing to its active peptides, such as melittin, adolapin, phospholipase A2 (PLA2), and apamin.[3] Melittin exhibits anti-inflammatory, anti-arthritis properties, anticancer, antibacterial, and antiviral activities.[4] Topical administration of PLA2, an MCD peptide extracted from bee venom, alleviates atopic dermatitis symptoms and inflammation and modulates IgE concentration.[5] PLA2 is expected to be effective against various types of dermatitis, including atopic dermatitis, due to its anti-inflammatory property.[20] Melittin in bee venom induces cell cycle arrest and apoptosis in various cancer cells and shows beneficial effects in mouse models of liver cirrhosis, atherosclerosis, and Propionibacterium acnes-induced inflammation.[21] Apitoxin is a synonym for bee venom and is the name of the injection drug,

made of dried honey bee venom, used in Korea. The main component of the venom is melittin, which accounts for 52% of venom peptides and 2% of adolapin. This drug has been administered to patients in Korea for its anti-pyretic, analgesic, and anti-inflammatory effects and to treat diseases other than arthritis.

Immunization with type II collagen induces inflammatory arthritis in animals such as rabbits, rats, and mice.[22] We chose DBA-1J mice for this study because they are sensitive to bovine type II collagen, which is known as an intrinsic material for arthritis, and extensive historical databases of this species exist, which can be used to interpret and evaluate the study results.[18] In this study, we used the CIA model to evaluate the potential of apitoxin in OA treatment. The body weights of mice with CIA were lower than those of G1 (Fig. 1), while the arthritis scores of G6 were significantly lower than those of G2 14 days after the initiation of apitoxin administration ($p < 0.05$; Fig. 2). The arthritis scores of G3 (the methotrexate-treated group) and G6 were significantly lower than those of G2 28 days after the initiation of apitoxin administration ($p < 0.05$). These results indicate a similar prognosis as found in previous studies of OA treatment with bee venom.[6,7,23-26] Prof. Anderson suggested that paw edema in animals caused by adjuvant-induced infection is similar to the arthritis-induced edema caused by increased lysosomal enzyme activity in the articular cartilage.[27] As shown in Fig. 3, the left hindlimb paw edema levels of G2 were significantly higher than those of G1 on day 28 ($p < 0.001$), and the left hindlimb paw edema levels of G3, G5, and G6 were significantly lower than those of G2 on day 28 ($p < 0.01$ or $p < 0.05$), although the right hindlimb paw edema levels of the entire apitoxin-treated group were not significantly decreased compared with those of G2. These results suggest that apitoxin is a potential therapeutic agent for arthritis.

IL-6 and IL-1β mainly participate in inflammatory signaling pathways associated with the pathological process of OA in the peri-articular phase, supporting connective tissue breakdown and synovitis.[28] Thus, we evaluated the presence of these cytokines in the serum of mice with CIA (Fig. 4). IL-6, IL-1β, and anti-type II collagen IgG levels in all the groups with CIA were significantly higher than those in G1 ($p < 0.001$, $p < 0.01$, or $p < 0.05$, respectively), while IL-6 and anti-type II collagen IgG levels in G3, G5, and G6 were significantly lower than those in G2 ($p < 0.001$).

The findings of the histopathological analysis were similar to the previous results. IL-6 and IL-1β play a role in the structural decomposition of cartilage proteoglycans and connective tissues.[29] They also enhance the activity of lysosomal enzymes, leading to the disruption of extracellular activity. Additionally, they disrupt cellular structures and play a role in tissue damage in OA. As shown in Fig. 5, although the result was negative with safranin O staining, the results of H and E staining suggested the application of apitoxin in treating joint tissues. The cellular infiltration levels of G3, G5, and G6 were significantly lower than those of G2 ($p < 0.05$). Synovial hyperplasia and pannus formation levels of G3 and all the groups administered with apitoxin were significantly lower than those of G2 ($p < 0.001$, $p < 0.01$, or $p < 0.05$). Unexpectedly, cartilage destruction and bone erosion did not improve in the apitoxin-treated group.

In the present study, we used the mouse model of CIA, which exhibited clinical symptoms and histological status similar to those in patients with arthritis.[30] Cartilage defects, joint malformations, gait abnormalities, and bone erosion are serious hazards in aging people. In general, arthritis treatment involves using NSAIDs and steroidal and immunosuppressive drugs to alleviate pain and inflammation, despite various side effects. Therefore, researchers continue to search for new drugs. Recently, approximately 80% of people worldwide opted for alternative treatments such as plant-based drugs to treat arthritis-related pain.[29] Our results on the effects of apitoxin treatment in mice with CIA suggested positive outcomes, which shows a possibility for the application of apitoxin as a drug in patients with arthritis. Further investigations are necessary to perform clinical trials targeting patients with arthritis.

Acknowledgement

This animal study was approved by the Institutional Animal Care and use Committee of KNOTUS Co. Ltd., Incheon-si, Korea (Certificate No: IACUC 21-KE-273)

Conflict of interest

The authors have no conflicts of interest in this study.

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