Journal of Pharmacological Sciences xxx (xxxx) xxx

Contents lists available at ScienceDirect

Journal of Pharmacological Sciences

journal homepage: www.elsevier.com/locate/jphs

Full Paper

Melittin – A bee venom component – Enhances muscle regeneration factors expression in a mouse model of skeletal muscle contusion

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ARTICLE INFO

Article history:
Received 20 August 2018
Received in revised form
25 March 2019
Accepted 27 March 2019
Available online xxx

Keywords:
Melittin
Sweet bee venom
Muscle contusion
Muscle regeneration
Anti-inflammatory effects

ABSTRACT

Melittin is a major peptide component of sweet bee venom that possesses anti-allergic, anti-inflammatory, anti-arthritis, anti-cancer, and neuroprotective properties. However, the therapeutic effects of melittin on muscle injury have not been elucidated. We investigated the therapeutic effects of melittin on muscle injury in a mouse model of muscle contusion. The biceps femoris muscle of the mice was injured using drop mass method, and the animals were treated with melittin (4, 20, or 100 μg/kg) for 7 days. Melittin significantly increased: locomotor activity in open field test, and treadmill running activity in a dose-dependent manner to level comparable to the positive control, diclofenac (30 mg/kg). Melittin treatment attenuated the pro-inflammatory cytokine MCP-1, TNF-α and IL-6. The expression of muscle regeneration biomarkers, including MyoD (muscle differentiation marker), myogenin, smooth muscle actin, and myosin heavy chain was markedly increased in the injured muscle tissue of melittin-treated mice, as determined by western blotting and quantitative real-time polymerase chain reaction. These results demonstrate that melittin inhibits inflammatory response and improves muscle damage by regenerating muscles in a mouse model of muscle contusion. Taken together, the results of present study suggest that melittin is a promising candidate for the muscle injury treatment.

Introduction

Skeletal muscle is one of the most abundant tissues in our body. It has several functions including control of body movement and body temperature, and serves as a reservoir of nutrients and minerals.[1] Because damage to muscles causes functional problems for our body, proper remedy is important. However, only few treatments have been developed until now.[2] Nonsteroidal anti-inflammatory drugs (NSAIDs) such as naproxen, ibuprofen, and diclofenac are most widely prescribed medicines for acute muscle injury.[3] However, they pose the risk of side effects such as heart attack and stroke.[4] NSAIDs reduce pain and inflammation of injured muscles, however, it is unclear whether NSAIDs have a therapeutic effect on skeletal muscle regeneration.

Despite its toxicity, bee venom has been extensively used as a therapeutic agent for arthritic and rheumatic diseases especially in East Asian countries including Korea, China, and Japan. Recently, studies have reported various effects of bee venom on neuralgia,[5] low back pain,[6] cancer,[7] and arthritis.[8] Moreover, systematic reviews on bee venom pharmacopuncture have reported its effectiveness in musculoskeletal pains[9] and rheumatoid arthritis.[10] Melittin is one of the major bee venom toxins, and is composed of 26 amino acids, and has a property to form channels on plasma membranes.[11] Melittin has been reported to have anti-inflammatory effects in immune cells and hepatocytes.[12] However, the beneficial effects of melittin on muscle regeneration have not been well studied.

In this study, we evaluated the effect of melittin on traumatic muscle injury in a mouse model of muscle contusion. Functional recovery of the damaged muscle and the expression levels of genes related to muscle regeneration were assessed.

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Peer review under responsibility of Japanese Pharmacological Society.

https://doi.org/10.1016/j.jphs.2019.03.009

Please cite this article as: Lee JE et al., Melittin – A bee venom component – Enhances muscle regeneration factors expression in a mouse model of skeletal muscle contusion, Journal of Pharmacological Sciences, https://doi.org/10.1016/j.jphs.2019.03.009

Materials and methods

Reagents and drugs

Melittin (SB10, >99% purity, PubChem CID: 16129627) was obtained from Korea Pharmacopuncture Research Institute (Seoul, Korea) and diluted in phosphate-buffered saline (PBS) for administration into mice. Diclofenac sodium and avertin were purchased from Sigma–Aldrich (St. Louis, MO, USA). ELISA kit was obtained from R&D Systems, (Minneapolis, MN, USA). The antibodies used were as follows; alpha smooth muscle actin (α-SMA) from Thermo Fisher (Waltham, MA, USA); monoclonal anti-myogenin from Sigma–Aldrich; MyoD, β-actin, and secondary antibodies (anti-rabbit or anti-mouse IgG) from Santa Cruz Biotechnology Inc. (Dallas, TX, USA); anti-heavy chain myosin from Abcam (Cambridge, MA, USA); and anti-GAPDH-HRP from Cell Signaling Technology Inc.(Danvers, MA, USA).

Animals

Male C57/BL6 mice (7-week-old, 19–22 g, n = 5–6 per group) were purchased from DBL (Daehan Bio Link, Chung-buk, Korea) and allowed for 7 days to acclimate before the start of the study. Mice were housed at standard conditions (12:12 h light–dark cycle, 23 ± 1°C) with free access to water and food. The University Animal Care and Use Committee at Daejeon University approved all of the animal experimental protocols (DJUARB2017-027). All experimental procedures using animals were performed in accordance with National Institutes of Health guidelines (NIH publication No. 86-23, revised 1985).

Contusion animal model

Mice were anesthetized with avertin (300 mg/kg) by intraperitoneal (i.p.) injection. Muscle contusion was induced by the drop mass method. Briefly, the hind limbs were positioned on a board, and ankles were dorsiflexed to 90°. A 16.28 g (diameter 15.87 mm) stainless steel ball was dropped from the height of 100 cm through a tube (interior diameter of tube was 17 mm) onto an impactor, which had a surface of 28.26 mm^2, resting on the middle of the biceps femoris muscle of the anaesthetized mouse.

Experimental design

Mice were randomly assigned to six groups. Naive group mice were not subjected to muscle damage. The mice from the other group were subjected to hind limb muscle damage. The control group mice (Con) received equivalent volume of vehicle (phosphate buffered saline) *via* intramuscular (i.m.) injection. Diclofenac sodium (30 mg/kg), a well-known nonsteroidal anti-inflammatory drug (NSAID), served as a positive control for the anti-inflammatory effects of treatment.[13] Fifty microliters of melittin (4, 20, or 100 μg/kg; i.m.) were injected in the muscle lesion using 26-gauge needle once a day. The mice were treated with the reagents for 6 consecutive days starting from the day after muscle contusion. After the final treatment, on the next day, mice were sacrificed for plasma and muscle sample collection.

Open field test (OFT)

The tests were performed between 09:00 and 14:00 h in a quiet room. The ambulatory behavior of mice was assessed by OFT. The open field arena (30 cm × 30 cm) was constructed from acrylic sheets. Their behavior was observed and recorded for 10 min using video tracking software (SMART 3.0; Panlab S.I., Barcelona, Spain). Traveled distance was used as the parameter to measure locomotor activity.

Treadmill exercise test

Treadmill exercise test was performed on a 4-lane treadmill (Young Sung biotech Inc., Seoul, Korea). The dimensions of the running belt for each lane were 7 × 30 cm (width × length); each lane was enclosed using transparent acrylic plate wall (16 cm height) and cover. An electric shock field of 7 × 10 cm (width × length) was placed at the end of the running belt. The tests were performed between 09:00 and 14:00 h in a quiet room. Mice were acclimated before each treadmill test using a progressive protocol in which the belt speed was increased from 6 to 10 m/min over the course of 5 min, set at 0°–5° grade and then at 20 m/min at 10°–15° grade for 10 min. Before treadmill testing, mice were deprived access to food for 1 h with ad libitum access to water. Mice were encouraged to run by applying a mild electric shock (0.2 mA). Their behavior was observed for 10 min using video tracking software (SMART 3.0). The traveled distance was used as the parameter to measure the exercise intensity.

Plasma collection

Seven days after the muscle contusion, mice were anaesthetized, and the blood was collected into vacutainer tubes containing EDTA (BD Biosciences, Franklin Lakes, NJ, USA) by heart puncture. The plasma was isolated via centrifugation of blood for 15 min at 3,000 rpm and kept at −80°C until use.

Histological analysis

Biceps femoris muscles were isolated, fixed in 10% formalin, embedded in paraffin, cut at 4 μm thickness and stained with hematoxylin and eosin (H&E) for morphological analysis. Injured muscle lesion areas on stained slides were observed at 400× magnification with bright field microscope (Nikon, Tokyo, Japan).

Enzyme-linked immunosorbent assay (ELISA)

The levels of MCP-1 and IL-6 were measured using commercially available ELISA kit (R&D Systems) according to manufacturer's instructions. The optical density of each well was determined using a microplate reader (Molecular Devices, San Jose, CA, USA) at 450 nm wavelength.

Western blot analysis

We isolated the damaged muscle with operating scissors and stored the tissues in −80°C until use. Frozen biceps femoris muscle tissues were pulverized in chilled mortars, homogenized in protein extraction solution (PRO-PREPTM, Intron Biotechnology, Seoul, Korea), and lysed for 60 min by incubation on ice. The lysates were harvested by centrifugation at 13,000 rpm for 10 min at 4°C and supernatant was recovered. The concentration of protein in the supernatant was determined, and then the supernatant was used for Western blot analysis. The samples were stored at −20°C for further use.

Equal amount of protein (40 μg) were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis on 10% polyacrylamide gels and then transferred to PVDF membranes (Hybond-P, GE Healthcare, Chicago, IL, USA) using a semidry

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transfer system. Blots were blocked for 1 h at 25°C with 5% (w/v) skim milk in Tris-buffered saline solution (TBS) containing 0.1% Tween-20. Subsequently, the membranes were incubated with antibodies against TNF-α, α-SMA, myogenin, MyoD, myosin heavy chain, GAPDH, or β-actin overnight at 4°C. Finally, the membranes were incubated with horseradish peroxidase labeled anti-rabbit or anti-mouse IgG secondary antibodies for 1 h at 25°C. Detection was performed using the ECL western blotting detection system (GE Healthcare, Chicago, IL, USA). After detection, the membranes were washed with TBS-T four times for 5 min each and incubated with stripping buffer (Intron Biotechnology, Seoul, Korea) for 15 min at 25°C to detect the other proteins. Then the membrane was washed with TBS-T four times for 5 min each, blocked with 5% (w/v) skim milk, and immunoblotting was performed.

Quantitative real-time polymerase chain reaction (qRT-PCR)

A quantitative real-time polymerase chain reaction was performed on RNA of biceps femoris muscle tissues obtained from all the experimental groups to investigate the muscle regeneration biomarkers expression. Total RNA was isolated by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and then used for cDNA synthesis, which was conducted with the PrimeScript™RT reagent kit (TaKaRa, Shiga, Japan). The level of mRNA expression was measured with specific primer set. The primer sets used were as follows; alpha 2, smooth muscle, aorta (Acta2) forward primer sequence 5′-CTACGAACTGCCTGACGGG-3′ and reverse primer sequence 5′-GCTGTTATAGGTGGTTTCGTGG-3; myogenin forward primer sequence 5′-ACCCTAACCAGAGACTCCCC-3′ and reverse primer sequence 5′- GTCCCGGCAGGCTGTAATAG-3′; MyoD forward primer sequence 5′-TCGTGTAAATAAGAGTTGCTTGGC-3′ and reverse primer sequence 5′-TCCAACACCTGACTCGCCC-3′. Mouse GAPDH primer set was used as endogenous control (VIC®/MGB Probe, Primer Limited) and was obtained from Applied Biosystems (Foster City, CA, USA). The relative mRNA expression was quantified using a 7500 real-time PCR system (Applied Biosystems) with Power SYBR®Green PCR Master Mix and TaqMan®Gene Expression Master Mix (Applied Biosystems). Relative expression was calculated using ΔΔCt (where Ct is cycle number at threshold) analytical method that includes normalization against GAPDH. The PCR reaction was run for 40 cycles at 95°C (15 s) and 60°C (1 min).

Cell culture

C2C12 cells were purchased from Korean Cell Line Bank (Seoul, Korea). Cells were cultured in Dulbecco's modified eagle's medium (DMEM, Lonza, Walkersville, MD, USA) containing penicillin (100 unit/mL), streptomycin (100 μg/mL), and 10% fetal bovine serum (Lonza), at 37°C in a humidified atmosphere containing 5% CO_2. The cells were seeded on 60 mm culture dishes at a density of 2×10^5 cells/ml overnight. To induce myocyte differentiation, cell culture medium was changed to 2% horse serum containing DMEM. After 16 h incubation with melittin, the cell lysates were harvested for Western blot analysis.

Statistical analysis

Results were analyzed using GraphPad Prism 4 software (Version 4.03, GraphPad software Inc., CA, USA). Data are presented as mean ± SEM. Group comparisons were performed using one-way ANOVA followed by *Post hoc* Tukey test analyses. Differences with $p < 0.05$ were considered statistically significant.

Results

Melittin ameliorates muscle function in muscle contusion mouse model

First, we established a muscle contusion model by drop-mass method. We found that the injuries were relatively constant between experimental animals. To evaluate the functional recovery of skeletal muscle, open field test and treadmill running test were performed. An OFT was performed to measure spontaneous locomotor activity in naive and contused mice. Muscle injury significantly decreased locomotor activity in the control group compared with naive group ($p < 0.05$). Mice in the diclofenac group, which served as a positive control showed increased locomotor activity compared to the control group mice ($p < 0.05$). As shown in Fig. 1A, melittin significantly increased locomotor activity at all three tested doses (4, 20, or 100 μg/kg) in a dose-dependent manner.

Following OFT, a forced treadmill running test was performed to examine whether injured mice had recovered from injury when treated with melittin. As shown in Fig. 1B, muscle injury greatly decreased the running distance in control group mice compared to that in naive group mice ($p < 0.001$). However, both diclofenac and melittin significantly increased the traveled distance in treadmill running test in a dose dependent manner ($p < 0.001$). Overall, these results indicate that melittin treatment improved the mobility function in muscle contusion model.

Melittin suppresses pro-inflammatory cytokines production in animal model of muscle contusion

To investigate the effect of melittin on inflammatory response in damaged muscle, the production of the pro-inflammatory mediators, MCP-1, TNF-α, and IL-6 were examined. An increased plasma level of MCP-1 was observed in control group mice compared to that in naive group mice ($p < 0.001$). Melittin significantly suppressed MCP-1 level in plasma by more than two-fold, as compared with plasma of control group mice ($p < 0.001$) (Fig. 2A). The level of TNF-α in damaged tissue was decreased by melittin (Fig. 2B). Melittin was also able to suppress the contusion-induced IL-6 in muscle lesion (Fig. 2C). This suggests that melittin has anti-inflammatory effect on muscle injury-induced immune activation.

Melittin increased the expression of muscle regeneration factors

We investigated the protein expressions of muscle regeneration markers including MyoD, myogenin, and α-SMA by Western blot analysis. Although the level of MyoD decreased significantly ($p < 0.01$) in control mice than in naive mice, myogenin and α-SMA levels in injured muscles of control mice were similar to those of naive mice (Fig. 3A–D). Interestingly, melittin treatment markedly increased protein levels of the markers in the damaged muscle compared with the muscles of mice in the control group. Melittin increased the levels of MyoD and myogenin approximately by 1.5-fold and 2-fold, respectively (Fig. 3B and C). The level of α-SMA was also increased by melittin (Fig. 3D). The protein levels of myosin heavy chain (MHC) in injured muscle were increased by melittin in a dose-dependent manner (Fig. 3E). The effect of melittin on MHC expression was confirmed in C2C12 cell culture experiment. When the differentiating C2C12 myocytes were treated with melittin, the expression of MHC was increased in a dose-dependent manner (Fig. 3F).

To examine the transcriptional regulation of the genes by melittin, qRT-PCR was performed to evaluate the mRNA levels of

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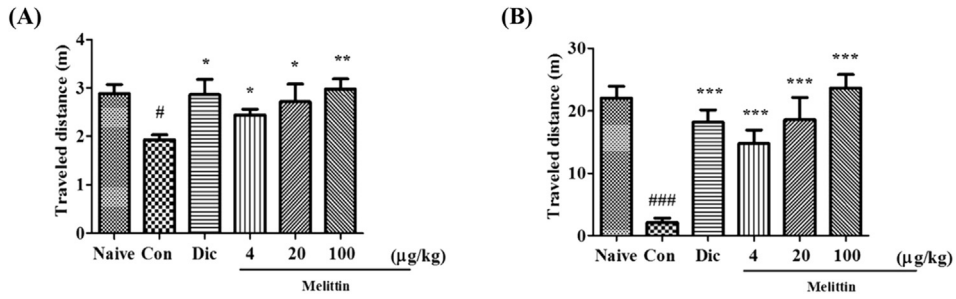

Fig. 1. Effects of melittin on locomotor function of muscle in injured mice. (A) Open field test on day 4 after injury and (B) treadmill running test on day 5 after injury were performed. Data are presented as mean ± SEM. *#p < 0.05, ###p < 0.001* vs. naive group. **p < 0.05, **p < 0.01, ***p < 0.001* vs. Con. Con; control, Dic; diclofenac.

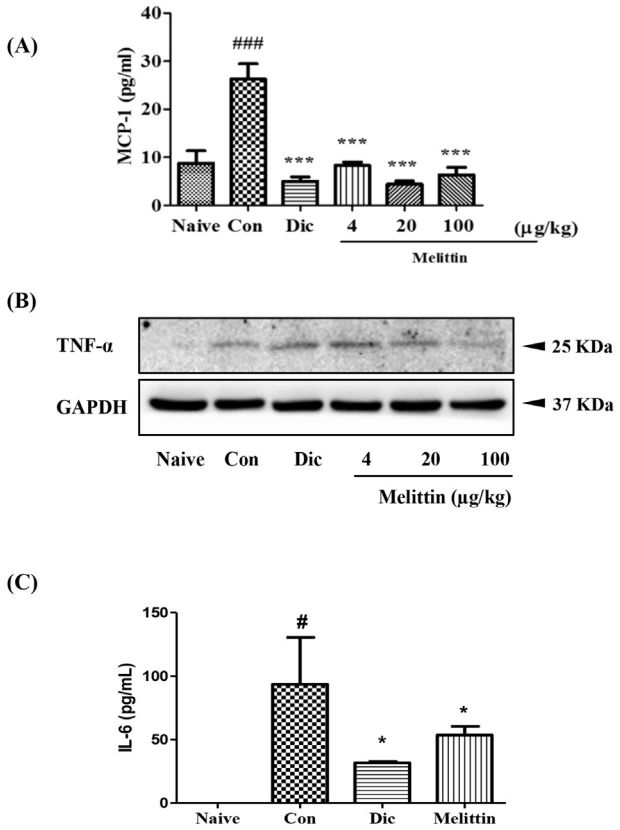

Fig. 2. Effects of melittin on the productions of pro-inflammatory cytokines. After injury, the mice were administered melittin (4, 20, or 100 μg/kg) or diclofenac (30 mg/kg) for 6 consecutive days. The next day, (A) blood plasma was obtained and the levels of MCP-1 was measured by ELISA and (B) the level of TNF-α in damaged skeletal muscle was measured by Western blot analysis. (C) The mice were administered melittin (4 μg/kg) or diclofenac (30 mg/kg) prior to the muscle damage. The damaged skeletal muscle was obtained for 6 h after injury and subjected to ELISA. Data are presented as mean ± SEM. *#p < 0.05, ###p < 0.001* vs. naive group. **p < 0.05, ***p < 0.001* vs. Con. Con; control, Dic; diclofenac.

MyoD, myogenin, and α-SMA. In accordance with the protein level, melittin treatment increased mRNA expression of participating genes in a dose-dependent manner (Fig. 4). At the highest concentration, 100 μg/kg, melittin treatment significantly increased the mRNA levels of MyoD, myogenin, and α-SMA approximately by 3.8-, 1.2-, and 2.5-fold, respectively, in injured muscle compared to that in control group (Fig. 4). These findings suggest that melittin actively participated in muscle repair upon injury by increasing the expressions of MyoD, myogenin, and α-SMA muscle repair markers.

Melittin improved histological changes of damaged tissue

To investigate the effects of melittin on morphological changes in muscle tissue, muscle sections were stained with hematoxylin and eosin (H&E). Large lymphocyte infiltration was observed in the muscle lesion in the control group mice compared to the muscle lesion in the naive group mice (Fig. 5A and B, yellow arrowhead). Melittin treatment dramatically reduced the lymphocyte infiltration. Interestingly, the number of central nucleated cells were increased in melittin-treated mice (Fig. 5B, red arrowhead). Moreover, melittin increased the number of fused myoblasts in the damaged skeletal muscle and recovered muscle fiber structure. These results indicated that melittin ameliorates the changes in muscle histology from contusion.

Discussion

The present study showed that melittin, a major peptide component of sweet bee venom, can facilitate the recovery of muscle injury by accelerating the regeneration of muscle fibers in contused mice. Previous studies have reported that muscle injury induces pain, and damages the normal muscle fibers.[14] Therefore, we performed behavioral and molecular biological analysis to investigate whether melittin administration accelerates muscle function recovery and production of muscle fiber regenerating factors after physical trauma.

Muscle degeneration and inflammation occur in first few days after injury. When the damage due to injury is serious enough to affect muscle function, body movement can be limited. In an attempt to provide experiment-based evidence, for the effect of melittin, an open field test and treadmill running test were performed in this study. It was found that melittin effectively acted at the site of injury and markedly increased the spontaneous locomotor and running activity in mice. This indicates that melittin could improve the recovery of muscle function from traumatic injury.

Inflammation is an essential step during muscle regeneration after damage. However, chronic or hyperactive inflammation may retard the healing process or may result in secondary damage to the skeletal muscle. Therefore, control of inflammation can facilitate in the recovery of muscle damage. MCP-1, TNF-α, and IL-6 are pro-inflammatory cytokines secreted upon muscle degeneration.[15] Elevated MCP-1 level induce muscle pain.[16] In this study, we measured the plasma level of pro-inflammatory cytokines MCP-1, TNF-α, and IL-6. Control mice receiving vehicle showed more than two-fold increase in MCP-1 level compared with naive mice after injury. Melittin treatment suppressed the secretion of MCP-1 in the plasma. Melittin perhaps attenuated the MCP-1 secretion induced upon muscle injury by ameliorating the inflammatory responses, resulting in an analgesic effect.[17]

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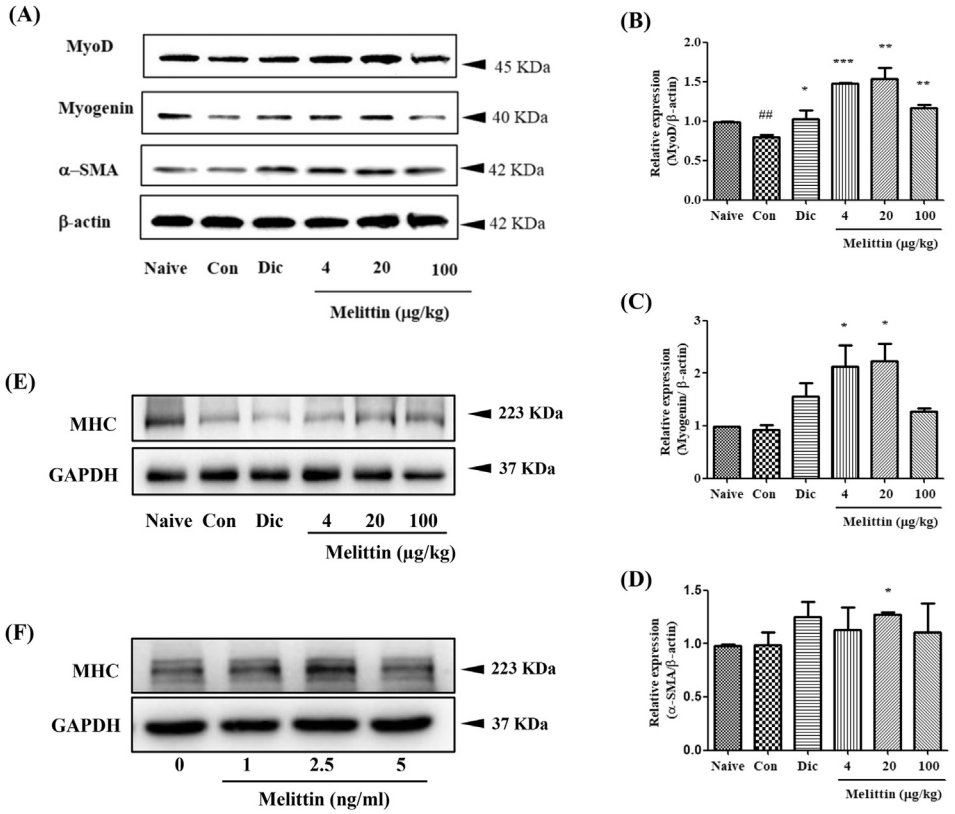

Fig. 3. Effects of melittin on skeletal muscle regenerating factors in the damaged muscle. After injury, the mice were administered melittin (4, 20, or 100 μg/kg) for 6 consecutive days. The next day, injured muscle lesion was isolated from mice. (A) The protein levels of MyoD, myogenin, and α-SMA in the damaged skeletal muscle were measured by Western blot analysis. The relative levels of (B) MyoD, (C) myogenin, and (D) α-SMA were calculated from the band intensities. The level of MHC was measured by Western blot analysis in (E) the damaged skeletal muscle or (F) differentiated C2C12 myocytes. Data are presented as mean ± SEM from three independent experiments. *##P < 0.01* vs. naive group. **p < 0.05, **p < 0.01, ***p < 0.001* vs. Con. Con; control, Dic; diclofenac.

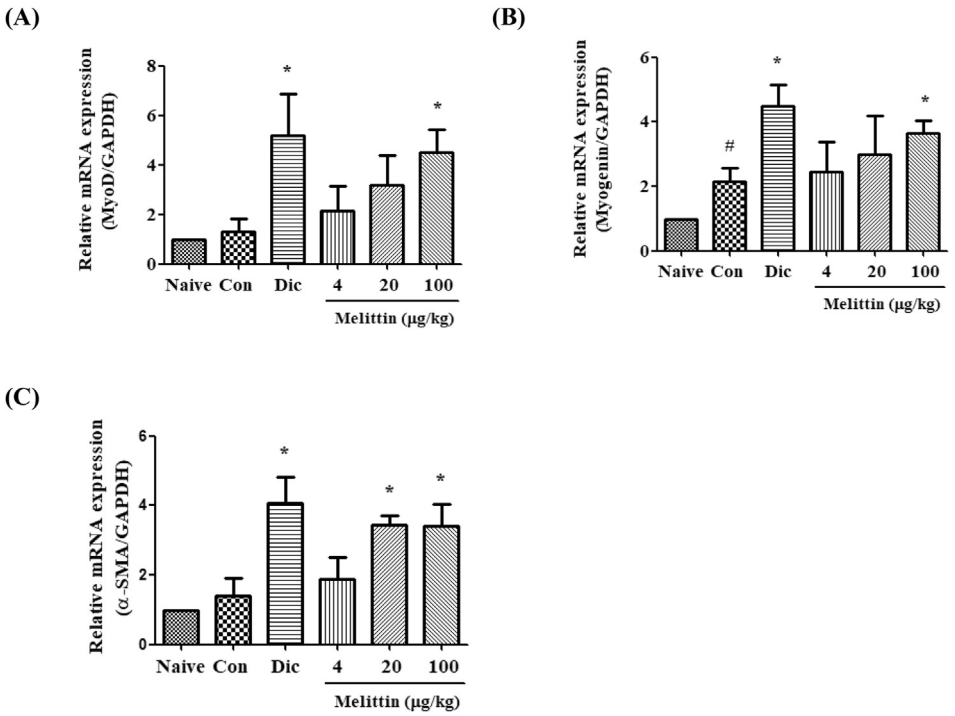

Fig. 4. Effects of melittin on expressions of skeletal muscle regenerating factors. After injury, the mice were administered melittin (4, 20, or 100 μg/kg) for 6 consecutive days. The next day, injured muscle lesion was collected from mice for isolating RNAs. The mRNA levels of (A) MyoD, (B) myogenin, and (C) α-SMA in the damaged skeletal muscle were measured by real time-PCR. Data are presented as mean ± SEM from three independent experiments. *#P < 0.05* vs. naive group. **p < 0.05* vs. Con. Con; control, Dic; diclofenac.

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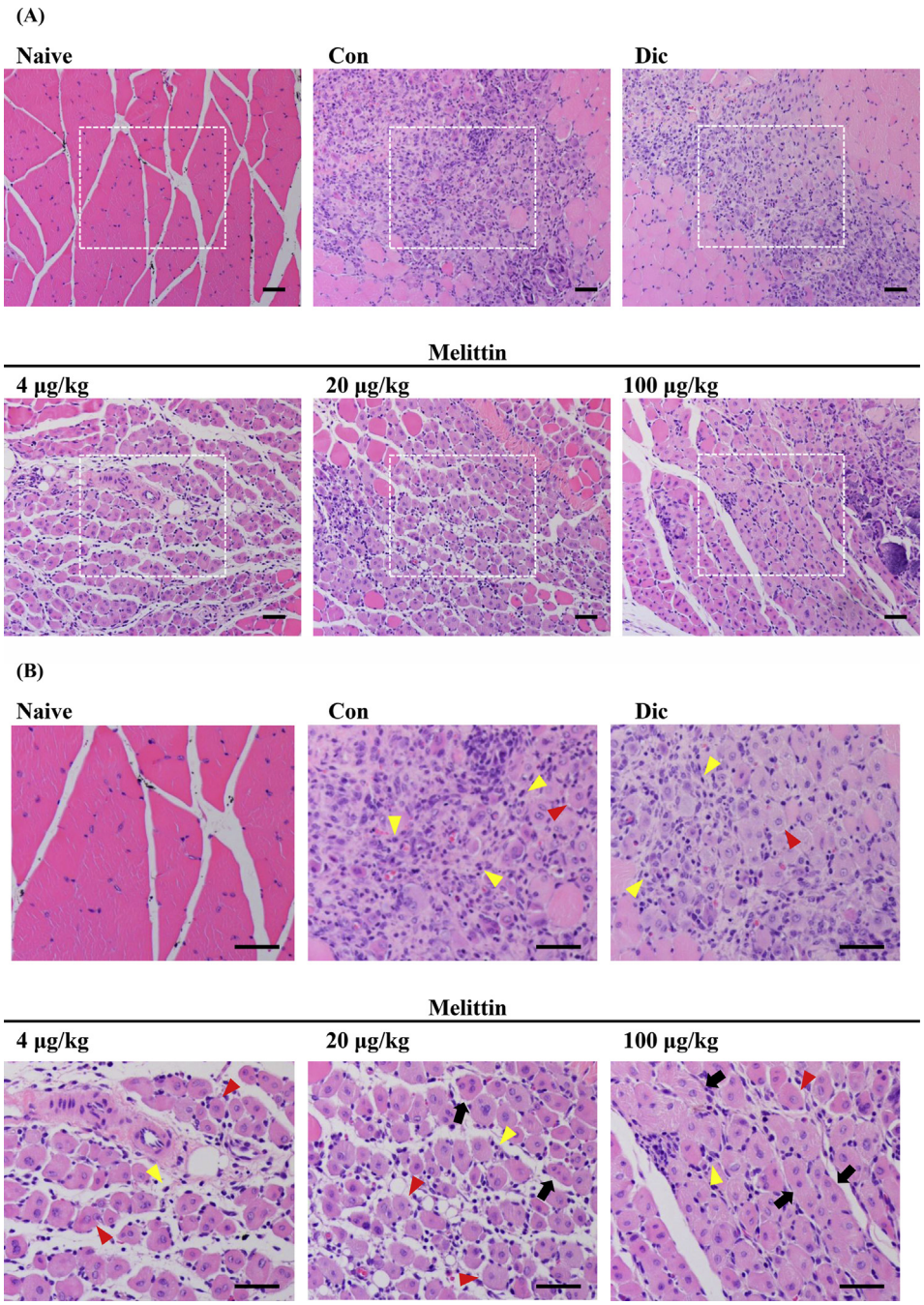

Fig. 5. Effect of melittin on morphological recovery after muscle injury. After injury, the mice were administered melittin (4, 20, or 100 μg/kg) for 6 consecutive days. The next day, injured muscle lesion was collected from mice. (A) The paraffin sections of the muscle tissues were stained with hematoxylin and eosin and observed under a bright field microscope (magnification 400×). White dash lines indicate the areas that were magnified and presented in (B). Yellow arrowheads indicate infiltrated lymphocytes. Red arrowheads indicate central nucleated cells. Black arrows indicate fused myoblasts. Con; control, Dic; diclofenac. Bar scale = 50 μm.

In this study, melittin increased the expression of MyoD, myogenin, and α-SMA at both transcriptional and translational levels. The regeneration of damaged skeletal muscle is mediated by the transcriptional expression of myogenic factors, such as MyoD.[18] The expressed MyoD is accumulated in satellite cells, inducing the production of myosin heavy chain. Myogenin and α-SMA are also important myogenic factors for skeletal muscle regeneration. It is important to mention that melittin markedly induced the expression of associated genes, as assessed by qRT-PCR, suggesting that transcriptional factors were generated upon injury in mice.[19] Notably, all muscle regeneration biomarkers were highly expressed in biceps femoris muscle of melittin-treated mice compared to that of control mice after injury. The formation of new myofibers during muscle regeneration could have led to the healing process after muscle injury.[20] Histological studies confirmed the recovery of injured muscle in naive and other groups. Due to lower production of myofibers, muscle regeneration was delayed in the control mice.[21] In contrast, melittin treatment actively facilitated the recovery of damaged muscles through the formation of new myofibers. These results indicate that melittin may facilitate recovery from muscle contusion by up-regulating muscle regeneration factors.

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Safety is one the main concerns in the clinical application of bee venom, which may cause allergic reactions such as anaphylaxis.[22] Melittin has been suggested as one of the bee venom components causing anaphylaxis. However, studies demonstrate that melittin can be safely used. In toxicological studies, LD50 of melittin was higher than 30.0 mg/kg in Sprague–Dawley (SD) rats.[23] In another toxicological study of 13-weeks, repeated intramuscular injection of melittin did not show any significant toxicity up to 0.28 mg/kg, which is much higher than the clinically used dose.[24] In the present study, we did not observe any toxicological effect of melittin in animals during the experiment even at the highest concentration, 0.1 mg/kg. Considering these results it is highly appeared that the clinical use of melittin is relatively safe as compared to crude bee venom or bee sting itself. Purified melittin has another advantage, in that it is easy to control the treatment dose in practical use.

Considerable efforts have been made to develop new anti-inflammatory drugs in the recent years. In the present study, using open field test and treadmill running, we showed that melittin not only showed better functional performance, but also suppressed the inflammatory cytokines expression. The data provides evidence of beneficial effect of melittin on muscle regeneration after tissue injury possibly rendered by its anti-inflammatory action. In conclusion, our data suggest that melittin possesses potential to regulate the skeletal muscle regenerating factors; however, further pre-clinical and clinical studies are needed to prove the therapeutic effects of melittin on skeletal muscle contusion.

Conflict of interests

The authors declare that they have no conflicts of interests.

Acknowledgements

This research was supported by the Daejeon University fund (2015).

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Please cite this article as: Lee JE et al., Melittin – A bee venom component – Enhances muscle regeneration factors expression in a mouse model of skeletal muscle contusion, Journal of Pharmacological Sciences, https://doi.org/10.1016/j.jphs.2019.03.009