

Effect of melittin on iNOS and NF- κ B expression induced by IL-1 β in C518 cells

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Abstract: Melittin (Mel), a natural detergent, is a major component of bee venom. Mel exhibits favorable clinical effects on the treatment of rheumatoid osteoarthritis, myositis, lumbar muscle strain, and peripheral neurological disorders. Interleukin-1 β (IL-1 β) contributes to the progression of osteoarthritis and is one of the key proinflammatory cytokines. However, the effect of Mel on IL-1 β -induced osteoarthritis has not been reported. We examined the effects of Mel on the expressions of inducible NO synthase (iNOS), nuclear transcription factor κ B (NF- κ B), and I kappa B (I- κ B) in the knee joint cells of C518 rats induced by IL-1 β . Western blot and qPCR results showed that Mel at 0.1 μ g/mL or higher significantly inhibited iNOS expression. Similarly, 1 μ g/mL of Mel prevented IL-1 β -induced I- κ B degradation in the cytoplasm and NF- κ B migration from cytoplasm to nucleus. Mel exerts an inhibitory effect on IL-1 β -induced NF- κ B activation by inhibiting both I- κ B degradation and NF- κ B migration and can potentially be developed as a new anti-osteoarthritis drug. Further research is needed to clarify the detailed mechanism.

Keywords: Melittin (Mel), IL-1 β , NF- κ B, iNOS, anti-inflammatory.

INTRODUCTION

In Oriental medicine, some bee venom components have been generally used to alleviate pain and treat inflammatory diseases, such as tendonitis and rheumatoid arthritis, and other potential venom-related treatments for immune-related diseases, infections and tumor therapies are currently under investigation (Moreno & Giralt, 2015). Melittin (Mel) is a natural detergent and a major peptide constituent of bee venom. Mel accounts for 40%–60% of dry whole honeybee venom and is a 26 amino-acid polypeptide (Bazzo *et al.*, 1988). Mel exhibits various effects, such as antimicrobial, antibacterial, anti-arthritis, antiviral, anti-inflammatory, and anticancer effects and is applied to the treatment of rheumatic and atherosclerosis (Moreno & Giralt, 2015). Although Mel presents abundant biological functions on various organisms except honeybees, studies should still identify their mechanism for these effects.

The efficacy of purified bee venom injection containing a potent anti-inflammatory peptide-Mel on the control of knee or back pain in patients with osteoarthritis is better than that of nabumetone medication and no severe adverse or allergic reaction occurred in patients treated with bee venom (Won *et al.*, 1999). For the experimental rabbit model of osteoarthritis, an intra-articular application of bee venom and hyaluronic acid was used in the treatment of the knee osteoarthritis and no significant difference upon recovery after therapy was observed (Nisbet *et al.*, 2012). Lim *et al.* (2005) showed that bee

venom acupuncture can possibly be used to efficiently cure osteoarthritis of knee joint patients and suggested that bee venom acupuncture is a potential therapy for the osteoarthritis of knee joint. However, studies on the anti-inflammatory effects of Mel on osteoarthritis are limited. Phospholipase A₂ (PLA₂) is responsible for the hydrolysis of arachidonic acid, which is an important precursor of eicosanoids. Eicosanoids are considered to mediate inflammation. Mel and related peptides are anti-inflammatory drugs as they can restrain the enzymatic activity of PLA₂ (Peterson *et al.*, 1998). The findings of Sainigroup showed that Mel binds to secretory PLA₂ and suppresses its enzymatic activity (Saini *et al.*, 1997). These studies are very informative but cannot sufficiently explain how Mel can stop the development of inflammatory molecular mechanisms of osteoarthritis.

Osteoarthritis is a key source of disability, pain and socioeconomic cost worldwide and is a joint disease that occurs not only in the articular cartilage but also in the synovial membrane, subchondral bone, and periarticular soft tissues (Goldring and Goldring, 2007). The epidemiology of the disorder is complicated with biological, biomechanical, and genetic elements (Glynjones *et al.*, 2015). IL-1 β is related to various disorders and is an effective target for therapeutic intervention in some diseases (Kolb *et al.*, 2001). IL-1 β expression is upregulated in inflammatory bone diseases (Barnes & Karin, 1997). However, the effects of Mel on IL-1 β -stimulated osteoarthritis chondrocyte has not been reported. In the present study, we evaluated the role of MEL on the expression of inducible NO synthase (iNOS), nuclear transcription factor κ B (NF- κ B), and I kappa B (I-

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κ B) in C518 cells induced by proinflammatory cytokine IL-1 β . This study showed that Mel strongly inhibited NF- κ B activation and iNOS expression induced by IL-1 β in the C518 cells.

MATERIALS AND METHODS

Cell culture

The cells of C518 rat knee joint (obtained from China Cell Culture Center) were cultured in DMEM medium (Hyclone, Logan, UT) with 10% fetal bovine serum (FBS) (Gibco, USA). Cells were routinely maintained in a humidified environment with 5% CO₂ at 37°C.

Examination of iNOS expression in C518 cells

C518 cells suspended in DMED/F12 medium containing 5% FBS were seeded in 6-well plates at 1 \times 10⁶/mL and cultured for 24 h at 37°C in a humidified environment with 5% CO₂. The medium was then changed to DMED/F12 medium containing IL-1 β (10 ng/mL) (Pepro Tech Inc.) and Mel (0, 0.1, 0.5, 1, and 10 μ g/mL) (Shanghai Aladdin Biochemical Technology Co., Ltd) for 24h. The cells cultured in DMED/F12 Mel without IL-1 β were the control group. The cells were then harvested and lysed in lysis buffer (Solarbio biotech Co., Ltd., China) for 30 min on ice. Lysates were centrifuged at 14 000 \times g at 4°C for 10 min to discard insoluble material. The concentrations of protein were determined using BCA protein kit (Solarbio biotech Co., Ltd., China). Approximately 40 μ g protein samples were loaded on SDS-PAGE and transferred to PVDF membranes. The PVDF membranes were then blocked with 5% nonfat milk in TBST buffer (20 mM Tris-HCl, 120 mM NaCl, and 0.1% Tween) for 1 h (Chevolot *et al.*, 2001). PVDF membranes were incubated with primary antibodies against iNOS (Abcam Inc.) and β -actin (Beijing TransGen Biotech Co., Ltd., China) at 4°C overnight. The blots were exposed to horseradish peroxidase conjugated anti-rabbit or anti-mouse secondary antibody for 2 h at room temperature after washing. ECL luminescence reagent by Sangon Biotech (Shanghai) Co., Ltd. was used to develop images. The expressions of proteins were quantified with Image J software.

RNA isolation and real-time quantitative PCR (qPCR) analysis

After the treatment with DMED/F12 medium containing IL-1 β (10 ng/mL) and Mel (0, 0.1, 0.5, 1 and 10 μ g/mL) for 24 h, C518 cells were collected. Total RNA from C518 cells was extracted using TRIzol reagent according to the instructions approved by the manufacturer (Beijing TransGen Biotech Co., Ltd., China). Subsequently, DNA was synthesized using reverse transcriptase kit from Promega (Beijing) Biotech Co., Ltd., and qPCR was performed on a 7500 Real-Time PCR System (Applied Biosystems) by SYBR Green detection chemistry. The 2^{- $\Delta\Delta$ Ct} method was used to analyze quantitative measurements (Livak & Schmittgen, 2001), and the

expression of β -actin was considered as the internal control gene. The sequences of the primers are shown in table 1. Amplification was carried out under the following conditions: initial denaturation at 95°C for 10 min and then 40 cycles at 94°C for 5 s, 60°C for 15 s, and 72°C for 10 s.

The change of NF- κ B expression induced by IL-1 β in nuclear of C518 cells

After C518 cells (1 \times 10⁶/mL) were treated with DMED/F12 medium containing 10% FBS for 24 h, the cells were cultured in IL-1 β (10 ng/mL)-supplemented medium for 0, 15, 30, 45, 60 and 120 min. The cells were then harvested. The subcellular structure of cytoplasmic and nuclear protein extraction kit (Boster Biological Technology Co., Ltd.) was used to extract nuclear protein, and Western blot analysis was employed to detect the expression of NF- κ B protein induced by IL-1 β with primary anti-NF- κ B p65 (Abcam Inc.). Histone-H protein expression was used as the internal control, and anti-histone H3 mouse monoclonal antibody was purchased from Abbkine, Inc. Western blot analysis was carried out as described above.

Evaluation of the effect of Mel the expression of I- κ B and NF- κ B induced by IL-1 β in cytoplasm and nucleus

After treatment of C518 cells (1 \times 10⁶/mL) with DMED/F12 medium containing 5% FBS for 24 h, the cells were divided into the control, Mel (1 μ g/mL), IL-1 β (10ng/mL), and Mel (1 μ g/mL) + IL-1 β (10ng/mL) groups. First, the groups that were cultured in supplemented Mel (1 μ g/mL)-medium were cultured for 24 h. The groups that were cultured in supplemented IL-1 β (10ng/mL)-medium were stimulated by IL-1 β for 45 min. All cells were collected. Cytoplasmic and nuclear proteins were extracted using the subcellular structure of cytoplasmic and nuclear protein extraction kit (Boster Biological Technology Co., Ltd.). NF- κ B and I- κ B (Cell Signaling Technology, Inc.) were respectively expressed in cytoplasm and nucleus, and expression was measured using Western blot analysis as mentioned above.

STATISTICAL ANALYSES

All experiments were repeated thrice in this study. Image J software was employed for quantitative analysis of protein bands. Data was displayed as mean \pm standard error of means. Statistical analysis was carried out using one-way analysis of variance to compare the control and investigation groups with statistical software package SPSS 11.5. A value of statistical significance was considered to be significant at $p < 0.05$ for all analysis.

RESULTS

Mel down-regulated the expression of iNOS induced by IL-1 β

As shown in fig. 1, compared with IL-1 β (+) + Mel (-)

group with IL-1 β (-) + Mel (-) group, iNOS expression induced by IL-1 β was significantly increased in C518 cells. Compared with IL-1 β (+) + Mel (0.1), IL-1 β (+) + Mel (0.5), IL-1 β (+) + Mel (1.0), IL-1 β (+) + Mel (10) with IL-1 β (+) + Mel (-) group, the expression of iNOS protein induced by IL-1 β in groups treated with Mel was significantly down-regulated. Moreover, when the doses of Mel were 0.5 and 10 μ g/mL, the expression of iNOS protein in the group treated with IL-1 β was as on the level of the untreated group. The mRNA expression of *iNOS* in C518 cells was also examined by qPCR after Mel treatment. Compared with that of IL-1 β (+) + Mel (0.1), IL-1 β (+) + Mel (0.5), IL-1 β (+) + Mel (1.0), IL-1 β (+) + Mel (10) with IL-1 β (+) + Mel (-) group, the mRNA expression of *iNOS* induced by IL-1 β was significantly down-regulated with the increase of Mel dose. (fig. 2), showing that Mel down-regulated the expression of iNOS induced by IL-1 β in C518 cells.

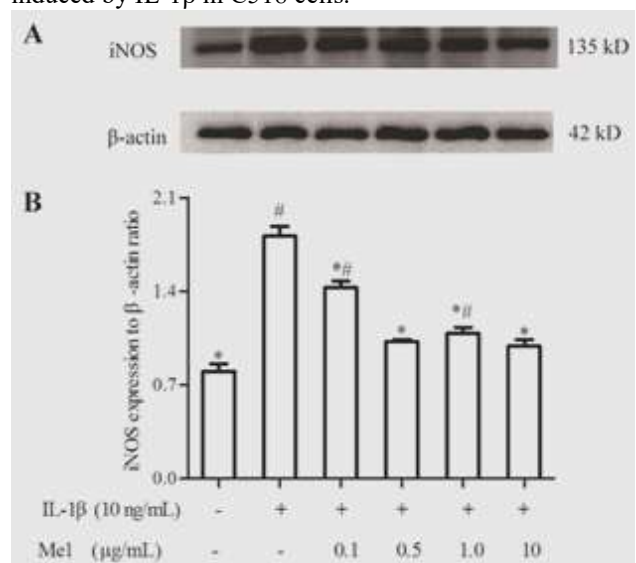


Fig. 1: Mel down-regulated iNOS expression induced by IL-1 β for 24 h. A, B: Western blot analysis of iNOS induced by IL-1 β C518 cells, respectively. The values are expressed as mean \pm S.E.M (n = 3). *, significant differences as compared with the IL-1 β (+) + Mel (-) group at $P < 0.05$; #, significant differences as compared with the IL-1 β (-) + Mel (-) group at $P < 0.05$.

Effect of IL-1 β on NF- κ B expression in nucleus

In order to investigate the time effect of IL-1 β on NF- κ B expression in the nucleus, we measured the level of NF- κ B expression in the nucleus after IL-1 β treatment for 0, 15, 30, 45, 60 and 120 min, respectively (fig. 3). After IL-1 β treatment for 45 min, the expression level of NF- κ B significantly increased and reached the highest level. The NF- κ B expression was nearly the same when IL-1 β treatment was performed for 0, 15, 60 and 120 min. Hence, IL-1 β treatment for 45 min was used in the next experiment to explore the effect of Mel on the activation of NF- κ B induced by IL-1 β in C518 cells.

Mel inhibited IL-1 β -stimulated nuclear translocation of NF- κ B in C518 cells

The level of I- κ B and NF- κ B in cytoplasm was not drastically changed before and after Mel treatment, as shown in fig. 4 (A-a and A-b). When C518 cells were treated with IL-1 β , compared that of IL-1 β (+) + Mel (-) group with IL-1 β (-) + Mel (-) group, the level of I- κ B in cytoplasm was drastically decreased. However, the level of NF- κ B was significantly increased. When C518 cells were first treated with IL-1 β for 15 min and then treated with Mel, I- κ B expression was significantly higher than in the group treated only with IL- β . Opposite results were found for NF- κ B expression. The group treated with both IL-1 β and Mel showed lower expression of NF- κ B than that of IL-1 β -treatment group. Different expression levels of I- κ B and NF- κ B were observed between the non-treated and IL-1 β -treatment groups, thereby suggesting that Mel inhibited the degradation of I- κ B induced by IL-1 β in C518 cells.

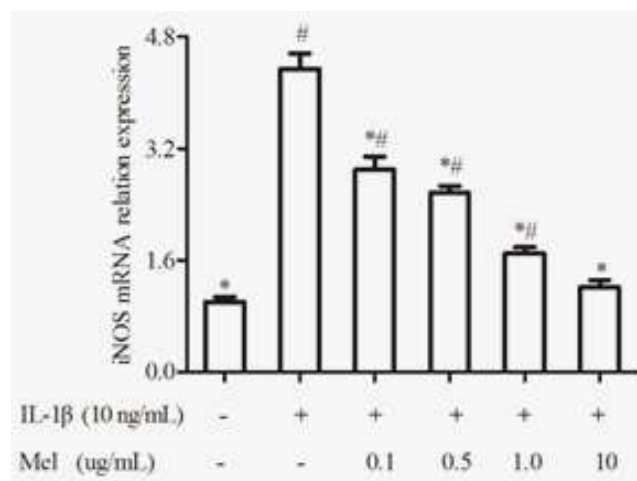


Fig. 2: Mel decreased the mRNA expression of *iNOS* induced by IL-1 β in C518 cells by qPCR. The values are expressed as mean \pm S.E.M (n = 3). The values are expressed as mean \pm S.E.M (n = 3). *, Significant differences as compared with the IL-1 β (+) + Mel (-) group at $P < 0.05$; #, Significant differences as compared with the IL-1 β (-) + Mel (-) group at $P < 0.05$.

As shown in fig. 4 (B-a, B-b), the expression levels of I- κ B and NF- κ B in the nucleus of C518 cells were decreased, but the difference was not significant in IL-1 β (-) + Mel (+) group compared with IL-1 β (-) + Mel (-) group. The IL-1 β (+) + Mel (-) and non-treated group showed non-significantly different expressions of I- κ B and NF- κ B induced by IL-1 β in the nucleus, but an increasing trend was observed. In contrast with that of the IL-1 β (+) + Mel (-) treatment group, I- κ B and NF- κ B expressions were drastically down-regulated in the group treated with IL-1 β and Mel, indicating that Mel treatment significantly prevented the IL-1 β -induced the activation of NF- κ B.

DISCUSSION

Bee venom is regularly used for the certain forms of arthritis and rheumatism therapy in some countries (Mraz, 2015). Considering the growing number of users and the commercial development of bee venom and related supplements as anti-inflammatory treatment, exploring the comprehensive protection of bee venom extract-Mel is urgently needed. Mel is the main toxic component in the venom of honey bee (*Apis mellifera*) and has been used to treat inflammation, especially osteoarthritis. In this study, we aimed to investigate the effects of Mel on the expression of iNOS, NF- κ B, and I- κ B stimulated by IL-1 β in a rat knee joint cells using C518 cells.

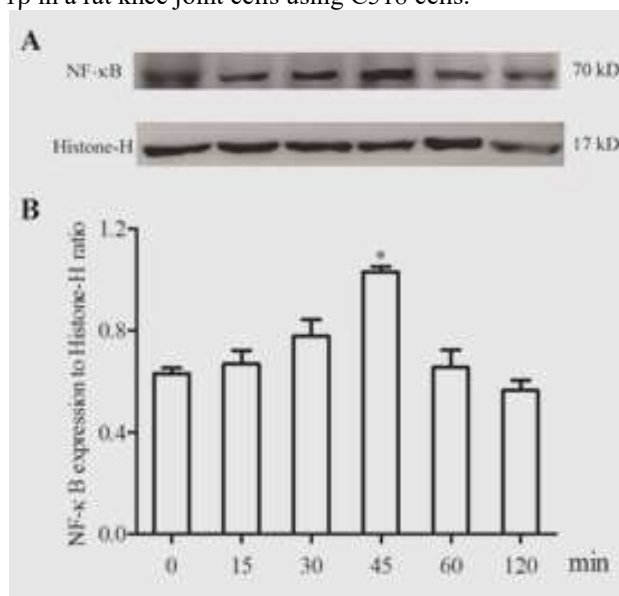


Fig. 3: The effect of IL-1 β on the expression of NF- κ B in nucleus of C518 cells at different time point. A, B: Western blot analysis of NF- κ B in nucleus of C518 cells, respectively. The values are expressed as mean \pm S.E.M (n = 3). *, significant differences as compared with the group treated with IL-1 β for 0 min at $P < 0.05$.

Catabolic events, which includes the release of catabolic cytokines, such as IL-1 β , IL-6, and TNF- α , are responsible for cartilage matrix degradation (Goldring, 1999). IL-1 β , an iNOS-inducing cytokine, can stimulate the expression of iNOS (Adams *et al.*, 2002). In human muscle biopsies, the linear relevance between the IL-1 β content and iNOS expression also agrees with the correlation of IL-1 β with the regulation of iNOS expression in skeletal muscle (Adams *et al.*, 2002). Our results first displayed that Mel significantly suppressed iNOS expression and inhibited the action of NF- κ B induced by IL-1 β in rat knee joint cells. When IL-1 β stimulated C518 cells, iNOS was overexpressed in C518 cells. Following the exposure to Mel, all concentrations of Mel measured in this study down-regulated the expression of iNOS protein and *iNOS* gene in C518 cells in a dose-dependent manner, thereby indicating that iNOS

expression induced by IL-1 β could be suppressed by Mel. These findings were in agreement with the effect of echinocystic acid, which attenuated the expression of iNOS induced by IL-1 β in human osteoarthritis chondrocyte (Ma *et al.*, 2016), thereby suggesting that Mel may be a potential candidate in the treatment of osteoarthritis.

The transcription factor NF- κ B plays an important role in the regulation of a diverse range of cellular genes, particularly those related to inflammatory and immune responses (Kretz-Remy *et al.* 1996). NF- κ B is also involved in the regulation of viral promoters, such as the human immunodeficiency virus long terminal repeat (Kretz-Remy *et al.*, 1996). The content fluctuation of NF- κ B further leads to oxidative stresses and physiological and physical changes and its supplementary functions, such as regulation of cell proliferation, differentiation, survival, and apoptosis (Oeckinghaus *et al.*, 2011; Oeckinghaus *et al.*, 2006). In the cytoplasm, NF- κ B is predominantly bound to an inhibitory I- κ B protein (Finco and Baldwin, 1995). In response to a wide variety of extracellular stimuli, the NF- κ B complexes, which are inactive, are dissociated; thus, free NF- κ B dimers are allowed to transport to the nucleus, and transcription of genes including I- κ B regulatory component are activated (Kretz-Remy *et al.*, 1996). The I- κ B protein is phosphorylated when signaling pathways are activated by inducement, such as cytokines IL-1 β , TNF α , or oxidative stress. The phosphorylated I- κ B is degraded to allow NF- κ B dimers to transfer to the nucleus, and NF- κ B interacts with κ B site, thereby leading to a series of changes, including the transport of cytokines into cell nucleus (Lorenzi *et al.*, 2013). Phosphorylation and subsequent degradation of I- κ B are necessary for the activation and transition into the nucleus of NF- κ B (Baldwin, 1996). In the present study, NF- κ B expression is highest in the nucleus when C518 cells were stimulated with IL-1 β for 45 min, among the time points tested. C518 cells were cultured in Mel-medium for 24 h and then exposed to IL-1 β for 45 min. Mel not only inhibited the expression of NF- κ B in the cytoplasm, but also effectively suppressed the metastases of NF- κ B in the nucleus. No significant change of NF- κ B and I- κ B expression in C518 cells treated only with Mel was found compared with the control group. The data presented here demonstrated that concentrations at which Mel displayed inflammatory activity did not cause significant cytotoxicity to C518 cells, thereby suggesting that Mel can down-regulate the activation of NF- κ B. The findings of McIntyre *et al.* (2003) showed that inhibitors of NF- κ B can be used for anti-degradative and anti-inflammatory effects on osteoarthritis animal models. Gerlag *et al.* (2000) also showed that catabolic activity that can inhibit NF- κ B may be a promising therapeutic agent for osteoarthritis therapy. Therefore, Mel may be a candidate agent for the treatment of osteoarthritis.

Table 1: Primer sequences used in qPCR

Gene	Primer (5'→3')
<i>iNOS</i>	F-CTCACTGTGGCTGTGGTACCTA
	R-GGGTCTTCGGGCTTCAGGTTA
β -actin	F- CCT GAC CGA GCG TGG CTA CAGC
	R- AGC CTC AGG GCA TCG GAAC

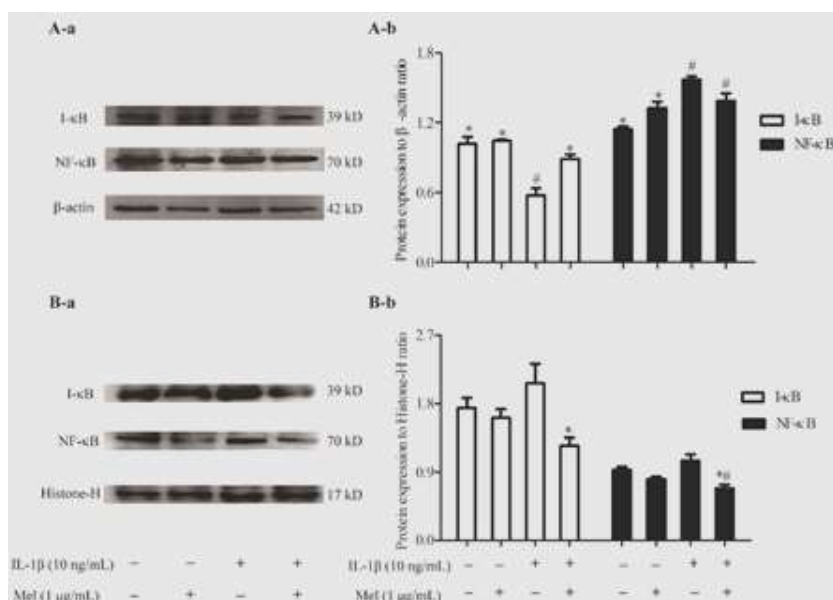


Fig. 4: The effect of Mel on the expression of I-κB and NF-κB induced by IL-1β in cytoplasm and nucleus of C518 cells. A-a, A-b: Western blot analysis of I-κB and NF-κB in cytoplasm of C518 cells, respectively; B-a, B-b, Western blot analysis of I-κB and NF-κB in nucleus of C518 cells, respectively. The values are expressed as mean ± S.E.M (n = 3). *, Significant differences as compared with the IL-1β (+) + Mel (-) group at $P < 0.05$; #, Significant differences as compared with the IL-1β (-) + Mel (-) group at $P < 0.05$.

Park *et al.* demonstrated that a considerable mechanism underlying the anti-arthritis effect of BV was inactivation of NF-κB by the suppression of I-κB release and p50 translocation induced by lipopolysaccharide (LPS) (Park *et al.*, 2004). In addition, they explained that the inactivation of c-Jun N-terminal kinase (JNK) pathway is related to the anti-arthritis and anti-inflammatory effects of Mel by NF-κB inactivation (Park *et al.*, 2008). Mel exerts the anti-inflammatory effect not only by suppressing LPS-induced *iNOS* expression but also by inhibiting LPS-induced activation of NF-κB by blocking I-κB-α degradation and phosphorylation of JNK and Akt in BV2 microglia (Moon *et al.*, 2007). Acute liver injury mice model induced by d-galactosamine (GalN)/LPS-induced were treated with Mel, and the results displayed that Mel attenuated the increase of inflammatory cytokines, and the activation of NF-κB induced by GalN/LPS was prevented (Park *et al.*, 2012). The result of this study was in line with the finding of the two above-mentioned studies that Mel could inhibit the activation of NF-κB.

Therapeutic applications of the anti-inflammatory effects of Mel is widely employed, such as skin inflammation,

atherosclerosis, neuroinflammation, liver inflammation, and arthritis (Lee & Bae, 2016). Mel treatment significantly reduced the number of infiltrated inflammatory cells, which were abundant in a unilateral ureteral obstruction model (An *et al.*, 2016). Mel markedly attenuated the expression of various inflammatory cytokines in heat-killed *Propionibacterium acnes*-treated THP-1 monocytic cells (Lee *et al.*, 2014b) and provided the prevention of indomethacin-induced gastrointestinal inflammation (Rahmy *et al.*, 2013). Mel treatment diminished inflammation and stimulated the signaling for cell survival in the spleen and lung in an amyotrophic lateral sclerosis mice model (Lee *et al.*, 2014a). Atherosclerosis development is suppressed by pro-inflammatory cytokines and adhesion molecules in high-fat/LPS-treated mice treated with Mel (Kim *et al.*, 2011). The results of the current study present the potential application of Mel to the treatment of osteoarthritis.

CONCLUSION

In conclusion, our results presented the first evidence on the effect of Mel on the expressions of *iNOS*, I-κB, and

NF- κ B in C518 rat knee joint cells induced by IL-1 β . We showed that Mel significantly suppressed the iNOS expression and prevented I- κ B degradation in cytoplasm and NF- κ B migration from cytoplasm to nucleus IL-1 β -induced in C518 cell. Detailed molecular mechanisms, for instance, cytokines, genomic, and proteomic responses underlying the IL-1 β -induced in C518 cells, remain to be expounded. Further scientific exploration is needed to measure the animal and clinical efficacy and safety of Mel in osteoarthritis. Our observations provide a basis for further studies on the anti-inflammatory efficacy of Mel and for the development of Mel as a potential anti-inflammatory supplement against osteoarthritis.

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