

# Regulation of Inflammatory Cytokine Production by Bee Venom in Rat Chondrocytes

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Bee venom acupuncture (BVA), as a kind of herbal acupuncture, involved injecting diluted bee venom into acupoints and is used for pain, osteoarthritis and rheumatoid arthritis patients. BVA is growing in popularity, especially in Korea, and is used primarily for pain relief in many kinds of diseases. However, the effect of bee venom anti-inflammatory related action in lipopolysaccharide (LPS) induced chondrocyte stress have not been reported yet. The aim of this study was to investigate the effect of bee venom of cell viability and inflammatory cytokine in rat articular chondrocyte cultures stimulated with lipopolysaccharide. Inflammation was induced in rat chondrocytes by treatment with 10  $\mu\text{g}/\text{mL}$  LPS. The change of cell viability were decreased in chondrocytes after treatment with lipopolysaccharide. The cell viability revealed that BV exerted no significant cytotoxicity in the rat chondrocyte. Bee venom inhibited decreased cell viability in the presence of lipopolysaccharide (10  $\mu\text{g}/\text{mL}$ ) in a dose dependent manner (0.1, 0.5, 1.0 and 5.0  $\mu\text{g}/\text{mL}$ ) at bee venom ( $p < 0.05$ ). Tumor necrosis factor (TNF)- $\alpha$  production in the presence of lipopolysaccharide (1  $\mu\text{g}/\text{mL}$ ) was also inhibited in a dose dependent manner ( $p < 0.05$  from bee venom 0.1  $\mu\text{g}/\text{mL}$ ). Interleukin (IL)-6 production in the presence of lipopolysaccharide (10  $\mu\text{g}/\text{mL}$ ) was inhibited as well ( $p < 0.05$  at bee venom 0.1, 0.5, 1.0 and 5.0  $\mu\text{g}/\text{mL}$ , respectively). Our results demonstrate that bee venom was a anti-inflammatory agent of chondrocytes. Bee venom may exert its anti inflammatory effects through inhibition of TNF- $\alpha$  and IL-6 synthesis, and may then pain relief and reduce the articular destruction.

Key words : Bee venom, Chondrocyte, Inflammatory cytokine

## Introduction

Osteoarthritis (OA) is a degenerative joint disease, considered to be one of the major public health problems<sup>1</sup>. Its prevalence after age 65 is increasing in incidence as a chronic condition widespread, and it is likely to rise further with the obesity epidemic<sup>2,3</sup>. Women are affected more than men<sup>4</sup>, and The most common site in hands, knees, hips, and spine<sup>4,5</sup>. Patients predominant symptoms are joint pain, stiffness and swelling in the early<sup>6,7</sup>. Joint pain typically worsened with weight bearing and functional activity.

Osteoarthritis involved degeneration of articular cartilage as well as erosion of articular cartilage, variable degrees of synovitis, and osteophytes<sup>4,5</sup>. The histological pathology reflects the result of joint inflammation and cartilage erosion. Since the initial stages of OA involve increased cell

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proliferation, proteinases, inflammation cytokines, and other inflammatory mediators by chondrocytes<sup>8</sup>. The relationship between the increased levels of catabolic enzymes and inflammatory mediators such as prostaglandins, nitric oxide, interleukin-1 $\beta$  (IL-1 $\beta$ ), and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) in OA synovial fluids and joint tissue<sup>8,9</sup>.

Current treatment options used to manage OA are fail to reverse the degenerative process of OA. Among the commonly used pharmacologic agents are nonsteroidal anti-inflammatory drugs (NSAIDs), corticosteroids, and hyaluronan preparations<sup>10</sup>. NSAIDs are widely used but their prolonged consumption is associated with serious adverse side effects such as gastrointestinal ulcerations<sup>11</sup>. The need for effective treatment modalities with fewer side effects has prompted OA patients to consider complementary approaches to control pain as well as to improve function and quality of life.

Among the inflammatory mediators associated with inflammatory and degenerative joint diseases, tumor necrosis factor alpha (TNF- $\alpha$ ) and interleukin 6 (IL-6) is well established as a key mediator in the progression of cartilage degeneration. Also there were reported to be upregulated in cartilage of

experimental OA patients and in vivo model<sup>9,12</sup>). High levels of TNF- $\alpha$  are detected in the synovial lining of rheumatic joints and in chondrocytes of osteoarthritic joints<sup>13</sup>). TNF- $\alpha$  promotes further expression of cytokines by synovial cells and chondrocytes, thereby sustaining a renewal of local inflammatory mediators<sup>14</sup>).

Bee venom (BV) has been used as treatment against wide variety of ailments including inflammatory diseases for centuries in some Asian countries including Korea<sup>15,16</sup>). It is a complex mixture of substances that are known to induce allergic responses in humans. Nevertheless, BV has been used to treat arthritis, pain, and rheumatoid diseases<sup>17,18</sup>). Bee venom relieve pain and inflammation in osteoarthritis patients or rheumatoid arthritis and stop its progression, but not yet studies have so far failed to prove convincingly that it anti-inflammatory related works in lipopolysaccharide (LPS) induced chondrocyte stress. So our research has focused on the chondrocyte as the cellular inflammation mediator of OA pathogenesis.

This study investigated the effect of bee venom in inflammatory cytokine such as TNF- $\alpha$  and IL-6, which are implicated in osteoarthritis by using rat primary cultured chondrocyte exposed to an LPS-induced stress.

## Materials & Methods

### 1. Preparation of bee venom

A working solution of bee venom was prepared by diluting 1,000 mg of dried bee venom solid (Daihan Pharm, Co. Ltd, Korea). Wherein 0.1 g of BV was added to 99.9 ml of distilled water yielding a bee venom solution of 1 mg/ml. The BV solution was further diluted to 0.1  $\mu$ g/ml to 100  $\mu$ g/ml for use in the experiments.

### 2. Chondrocyte isolation and culture

Cartilage of normal appearance was aseptically prepared from the femoral and tibial articular surfaces of knee joints of 300 g Sprague-Dawley rats. Rats were used after our college ethics committee approved that our protocol fulfilled the guide of animal experience established by the Korean academy of medical sciences. Briefly, EDTA-CMFT(calcium magnesium free tyrode)(0.1%), trypsin (0.15%) and collagenase(2 mg/ml) were treated for total 3 hr. After washing and centrifugation, cells were resuspended in Ham's F12/Dulbecco's modified Eagle's medium with 10% fetal calf serum and antibiotics (penicillin 100 U + streptomycin 50  $\mu$ g/ml). Cells were then washed and seeded in culture flasks or 96-well plate at a density of  $10^4$  cells/cm<sup>2</sup>. The cells were grown at 37°C with 5% CO<sub>2</sub> in air. The

cells from a single animal were obtained for each experiment. The medium was changed every 1 days. The first passage cells were used in all experiments<sup>19</sup>).

### 3. Cell viability

Cell viability was analyzed using a MTT assay. Tetrazolium salts such as MTT are metabolic by mitochondrial dehydrogenases to form a blue formazan dye and are therefore useful for the measurement of cell viability. The cells were gently washed with Hanks' balanced salt solution(HBSS, Sigma Co., USA), and exposed to rat primary chondrocyte. After washing the cells, cultures medium containing 0.5 mg/ml of MTT was added to each well. The cells were incubated for 2 hr at 37°C, the supernatant was removed and the formed formazan crystals in viable cells were solubilized with 110  $\mu$ l of dimethyl sulfoxide. A 100  $\mu$ l aliquot of each sample was then translated to 96 well plates and the absorbance of each well was measured at 550 nm with ELISA reader (Bio-Rad instrument, USA). Data were expressed as a percentage of control measure in the absence of rat primary chondrocyte<sup>20</sup>).

### 4. ELISA assays for TNF- $\alpha$ and IL-6 measure

TNF- $\alpha$  and IL-6 levels in culture media were determined by the quantikine human immunoassay (R&D system Inc., USA). Which employ the quantitative sandwich enzyme linked immunoassay (ELISA) technique. The sensitivity limit of both assays was about 20 pg/ml, the linear assay range was between 100 and 2,000 pg/ml. Aliquots of 100  $\mu$ l from control or LPS-treated cultures were analysed according to the manufactures instruction.

### 5. Statistical analysis

Results were expressed as means  $\pm$  standard deviation(S.D.) of least three separate experiments. The difference between two mean values was analyzed by student t-test. Values of  $p < 0.05$  were considered as significantly different.

## Results

### 1. Effect of LPS in rat chondrocyte viability

Fig. 1 showed that cell viability was also assessed using the MTT assay (Fig. 1). The effect of cell viability on LPS in the rat chondrocyte was examined as variable LPS time and dose. The dependence of the inhibitory effect of LPS on the LPS concentration (ranging from 0 to 100  $\mu$ g/ml) was examined. Fig. 1A shows that LPS at 0.5  $\mu$ g/ml (24hr) did not inhibit cell viability. However when added at levels over 1  $\mu$ g/

ml, LPS caused significant inhibition of cell viability ( $86.51 \pm 12.84$  % decrease vs. control;  $p < 0.05$ ). As the effect of LPS on cell viability may possibly be influenced by oxidative stress (Fig. 1A). Fig. 1B shows that cell viability occurred at a significantly reduced rate after a 2 hr incubation period with LPS  $10 \mu\text{g/ml}$  ( $85.44 \pm 9.57$  % decrease vs. control;  $p < 0.05$ ). This level of inhibition was maintained over 48 hr incubation period. The dependence of the inhibitory effect of LPS on the LPS time (ranging from 0 to 48 hr) was examined (Fig. 1B). Thus, 24 hr incubation period and  $10 \mu\text{g/ml}$  was used (Fig. 1).

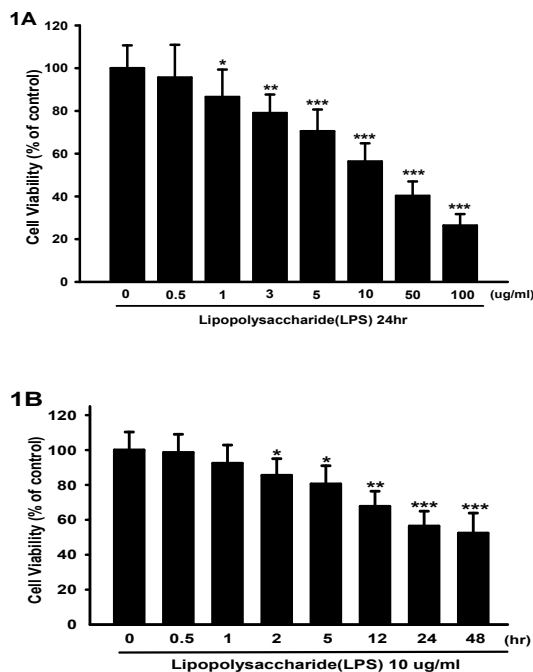


Fig. 1. Dose- and time-dependent effects of LPS on cell viability in rat chondrocyte. (Values are means±S.D. of three independent experiments. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 compared to the control)

2. Effect of bee venom treatment in rat chondrocyte viability

The effect of variable concentration and time bee venom on cell viability by the rat chondrocyte was examined as a function of dose and time. Fig. 2A shows that cell viability incubated with BV at  $0 \mu\text{g/ml}$ ,  $0.01 \mu\text{g/ml}$ ,  $0.1 \mu\text{g/ml}$ ,  $0.5 \mu\text{g/ml}$ ,  $1.0 \mu\text{g/ml}$ ,  $5.0 \mu\text{g/ml}$  for 24 hour were  $97.34 \pm 12.74$  %,  $97.26 \pm 16.43$  %,  $96.86 \pm 13.51$  %,  $99.74 \pm 11.28$  %,  $98.58 \pm 13.57$  % of the control value, respectively. The cell viability revealed that BV exerted no significant cytotoxicity in the rat chondrocyte. However when added at levels over  $10 \mu\text{g/ml}$ , BV caused significant inhibition of cell viability. The inhibitory effect of BV was  $85.64 \pm 10.58$  %,  $35.46 \pm 11.47$  %,  $33.85 \pm 8.32$  % decreased at 10, 100 and 200  $\mu\text{g/ml}$  (Fig. 2A). As the effect of BV on cell viability may possibly be significantly cytotoxicity influenced by high concentration. The cell viability revealed that  $1.0 \mu\text{g/ml}$  BV related incubation time exerted no

significant inhibition effect in rat chondrocyte (Fig. 2B).

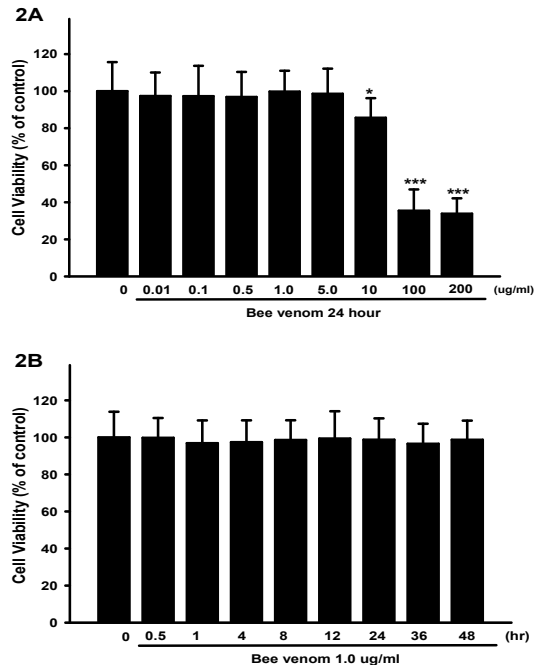


Fig. 2. Dose- and time-dependent effects of BV on cell viability in rat chondrocyte. (Values are means±S.D. of three independent experiments. \*P < 0.05, \*\*\*P < 0.001 compared to the control)

3. Effect of LPS and bee venom treatment on cell viability in rat chondrocyte proliferation

The effect of bee venom and LPS on cell viability by the rat chondrocyte was examined as a function of dose. Fig. 3 shows that cell proliferation occurred at a significantly increased rate after a 24 hr incubation period with 0.1 and 0.5  $\mu\text{g/ml}$  BV. This level of inhibition was maintained over 48 hr incubation period. BV caused significant cell proliferation when added over 24 hr. Thus, a 24 hr incubation period was used (Fig. 3).

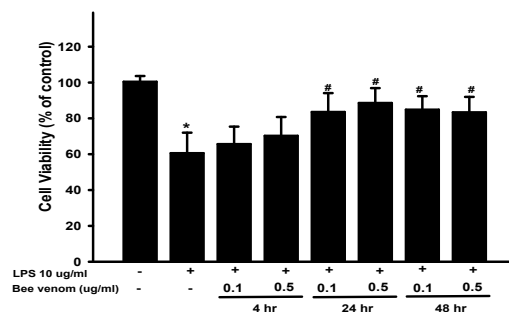


Fig. 3. Effect of LPS and BV treatment on cell viability in rat chondrocyte. (Values are means±S.D. of three independent experiments. \*P < 0.05 compared to the control. #P < 0.05 compared to the LPS-treated group)

4. Effect of bee venom on TNF-α synthesis

This results showed that the TNF- $\alpha$  levels by BV in the rat chondrocyte. The level of TNF- $\alpha$  was markedly increased to  $238.50 \pm 18.32$  pg/ml following treatment with  $10 \mu\text{g/ml}$  LPS for 24 h, while decreased to  $180.54 \pm 13.51$  pg/ml,  $124.48 \pm 11.28$  pg/ml,  $90.35 \pm 13.57$  pg/ml, and  $82.34 \pm 10.58$  pg/ml in cells treated with BV at  $0.01 \mu\text{g/ml}$ ,  $0.1 \mu\text{g/ml}$ ,  $0.5 \mu\text{g/ml}$ ,  $1.0 \mu\text{g/ml}$ ,  $5.0 \mu\text{g/ml}$ , respectively (Fig. 4).

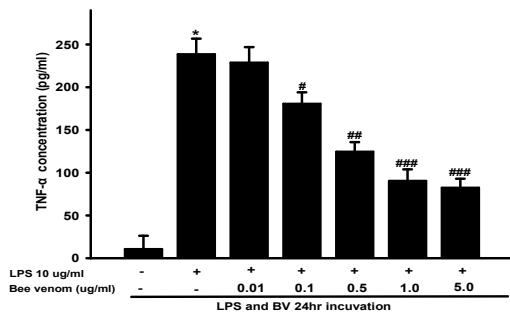


Fig. 4. Effect of bee venom dose dependent on TNF- $\alpha$  production in rat chondrocyte. (Values are means $\pm$ S.D. of three independent experiments. \*P < 0.05 compared to the control. #P < 0.05, ##P < 0.01, ###P < 0.001 compared to the LPS-treated group)

#### 5. Effect of bee venom on IL-6 synthesis

This results showed that the IL-6 levels by BV in the rat chondrocyte. The level of TNF- $\alpha$  was markedly increased to  $238.50 \pm 18.32$  pg/ml following treatment with  $10 \mu\text{g/ml}$  LPS for 24 h, while decreased to  $180.54 \pm 13.51$  pg/ml,  $124.48 \pm 11.28$  pg/ml,  $90.35 \pm 13.57$  pg/ml, and  $82.34 \pm 10.58$  pg/ml in cells treated with BV at  $0.01 \mu\text{g/ml}$ ,  $0.1 \mu\text{g/ml}$ ,  $0.5 \mu\text{g/ml}$ ,  $1.0 \mu\text{g/ml}$ ,  $5.0 \mu\text{g/ml}$ , respectively (Fig. 5).

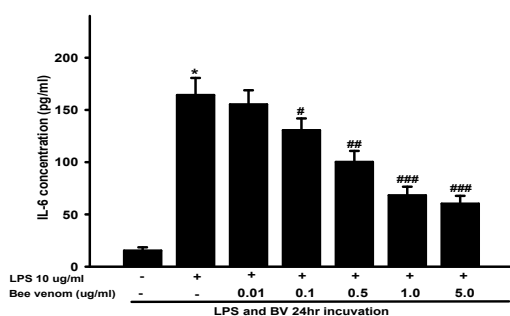


Fig. 5. Effect of bee venom dose dependent on IL-6 production in rat chondrocyte. (Values are means $\pm$ S.D. of three independent experiments. \*P < 0.05 compared to the control. #P < 0.05, ##P < 0.01, ###P < 0.001 compared to the LPS-treated group)

## Discussion

Pain, the predominant symptom in OA, is multidimensional in its nature and mediated through a variety of factors such as inflammation, articular structures and nerve

sensitization<sup>21,22</sup>. Inflammation is a complex biological response involving numerous cellular mediator. Also it is increasingly recognized as contributing to the pain, symptoms, and progression of disease<sup>23</sup>. Many chronic joint pain conditions have been shown to originate from peri-articular inflammation<sup>24</sup>. BV therapy is one of the most traditional complementary and alternative therapeutic methods that have been used to be effective in the treatment of many diseases<sup>25</sup>. The present study investigated the effect of bee venom in pro-inflammatory cytokine (TNF- $\alpha$  and IL-6), which are implicated in osteoarthritis by using rat chondrocyte exposed to an LPS-induced stress.

In the present study, cell proliferation occurred at a significantly decreased on the LPS concentration and time. Cell proliferation occurred at a significantly increased rate after a 24 hr incubation period with BV. Chondrocyte survival is essential for ensuring ongoing homeostatic maintenance of cartilage and for bringing about repair to damaged cartilage<sup>26</sup>. Cytokines acting individually or in networks, profoundly influence cellular responses in joint tissues, modifying both catabolic and anabolic activities<sup>8</sup>. Inflammatory cytokines released locally during inflammation modulate nociceptive processing via multiple mechanisms<sup>27,28</sup>. Inflammatory cytokines can directly sensitize nociceptors and increase neuronal sensitivity to heat, mechanical and chemical stimuli<sup>29</sup>. BV have been initially produces a local hyperalgesia and inflammatory response, but at the same time it activates primary afferents to induce both an antinociceptive and anti-inflammatory effect<sup>25,29</sup>. We also found that treatment with variable BV concentration significantly reduced secretion of inflammatory cytokines in response to stimulation with LPS in rat chondrocyte (Fig. 4, 5). The decrease in cyclooxygenase-2 and phospholipase A2 expression and the decrease in the levels of tumor necrosis factor  $\alpha$ , interleukin-1, interleukin-6, nitric oxide and oxygen reactive species are suggested to be the anti-inflammatory activities of BV in the treatment of arthritis<sup>30,31</sup>. BVA administered i.p. into the patients inhibited cytokine production from both T-cells and macrophages<sup>32</sup>.

On the other hand, the controversial effects of BV have also been described with respect to the experimental model. reported the best results for inhibition of edema when BV was administered prior to arthritis induction<sup>33</sup>.

Overall these results imply that the inhibition of inflammatory cytokines are powerful modulators of the expression of many receptors involved in pain and analgesia. These results suggest the pharmacological activities of bee venom on anti-inflammatory process include the inhibition of pro-inflammatory cytokines (TNF- $\alpha$ , and IL-6) production.

These observations provide a scientific rationale for the pain-reducing and anti-inflammatory effects of bee venom observed in osteoarthritis patients.

## Conclusion

In this study, changes in chondrocyte phenotype and function were determined following treatment with LPS induced cell stress and bee venom mediators that contribute to sustaining the inflammatory processes associated with arthritis. The anti-inflammatory effects of bee venom have not been investigated previously in rat articular chondrocyte. The inhibition of inflammatory cytokine (TNF- $\alpha$ , IL-6) critical for structural and functional maintain of cartilage was regulated by bee venom. Inflammatory mediators can therefore improve chondrocyte function and contribute to the recede of cartilage degeneration through anti-inflammatory signaling events.

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