

## Effect of fermented *Achyranthes japonica* (Miq.) Nakai extract on osteoarthritis

Hyo Seok Kang, Hee Seop Lee<sup>1</sup>, Heui-Jong Yu, Seon Hyeong Jang<sup>2</sup>, Yoonhee Seo<sup>2,3</sup>,  
Hong Yon Cho<sup>1</sup>, and Soo Young Choe<sup>3,\*</sup>

Food R&D Center, SK Bioland Co. Ltd.

<sup>1</sup>Department of Food and Biotechnology, Korea University

<sup>2</sup>EBO Co. Ltd., Cheongju

<sup>3</sup>School of Biological Sciences, Chungbuk National University

**Abstract** The present study was conducted to evaluate the anti-inflammatory and anti-arthritic effects of fermented *Achyranthes japonica* (Miq.) Nakai extract (FAJE). The FAJE was effective in nitrogen oxide (NO) scavenging in RAW264.7 cells. In the case of experimental Sprague Dawley (SD) rats injected with monosodium iodoacetate (MIA), the levels of TNF- $\alpha$  and IL-1 $\beta$  in blood increased in the osteoarthritis-induced group while decreasing in the group administered with FAJE. In addition, MMP-2 and MMP-9 in cartilage tissues increased in the osteoarthritis-induced group, but decreased in the group treated with FAJE. Cartilage examination indicated that the osteoarthritis-induced group exhibited cartilage erosion and cell degeneration, but in the FAJE administered group the tissue, conditions were recovered and cartilage proteoglycan was increased. Therefore, FAJE clearly showed anti-inflammatory effects and this suggests it is effective for recovery from osteoarthritis induced by MIA.

**Keywords:** *Achyranthes japonica*, osteoarthritis, monosodium iodoacetate

### Introduction

*Achyranthes japonica* (Miq.) Nakai (common name: Oriental chaff flower; Korean name: Soe-mu-reup) is a perennial herb that belongs to the Amaranthaceae. *Achyranthes japonica* (Miq.) Nakai was first reported by Miquel as *Achyranthes bidentata* var. *japonica* Miq.; however, it was raised to a species by Nakai (1-3). Soe-mu-reup means 'cow's knees' and it is the name given to *Achyranthes japonica* (Miq.) Nakai since the shape of its stem resembles cow's knees. In China and India, *Achyranthes bidentata* Blume's roots have been traditionally dried and used for the treatment of arthritis, virus, convulsion, hypertension, and skin disease, as well as for improvement of blood circulation (4,5). In South Korea, *Achyranthes japonica* (Miq.) Nakai's roots have been traditionally dried and used for the treatment of edema and arthritis and to delay women's menstruation. Recent studies have reported anti-inflammatory, pain relief, and antibacterial effects of *Achyranthes japonica* (Miq.) Nakai, as well as its capacity to improve osteoporosis conditions in ovariectomized (OVX) rat (6-10).

Osteoarthritis (OA) is inflammation appearing in joint connection areas and appears more frequently in elderly persons. This disease leads to a gradual loss of articular cartilages with the formation of osteophytes and the patients start to suffer from chronic pain while being restricted on joint functions. At present, there is no appropriate treatment on osteoarthritis and the patients do not recover to normal conditions. Although nonsteroidal anti-inflammatory drugs have been used over the last several years, their treatment effects are still unsatisfactory (11). Recently, research to find out new natural substances that can be safely used over long periods of time has been undertaken. In particular, *Achyranthes japonica* (Miq.) Nakai that has been traditionally used for osteoarthritis patients in South Korea has begun to come to the fore as an alternative to nonsteroidal anti-inflammatory drugs (12). However, available studies on *Achyranthes japonica* (Miq.) Nakai's anti-inflammatory and anti-arthritic effects remain scarce (13).

The purpose of the present study was to identify the anti-inflammatory and anti-osteoarthritic effects of the fermented *Achyranthes japonica* (Miq.) Nakai extract's. Lactic acid fermentation reduced the bitter taste of the *Achyranthes japonica* (Miq.) Nakai's roots and improved flavor. The anti-inflammatory effects were examined using RAW264.7 cells with inflammation induced by LPS and the anti-osteoarthritic effects were examined by evaluating the effects to improve the conditions of osteoarthritis induced by monosodium iodoacetate (MIA) in Sprague-Dawley (SD) rats.

\*Corresponding author: Soo Young Choe, School of Biological Sciences, Chungbuk National University, Cheongju, Chungbuk 28644, Korea

Tel: +82-43-261-2297

Fax: +82-43-260-2298

E-mail: schoe@chungbuk.ac.kr

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## Materials and Methods

### Fabrication of the fermented *Achyranthes japonica* (Miq.) Nakai extract (FAJE)

The *Achyranthes japonica* (Miq.) Nakai's roots used in the present experiment were bought around May 2014 in Andong, Gyeongbuk, and washed with water before use. The extract was extracted from the *Achyranthes japonica* (Miq.) Nakai's roots were added with water amounting to 10 times of the specimen in weight using an extractor (KyungSeo Machines, Incheon, Korea) for 4 h at 95°C. After collecting the extract, the extract was again extracted again for 2 h at 95°C. The extract was then concentrated using a rotary vacuum evaporator R-100 (DooYoung High Technology, Seoul, Korea) and sterilized in a fermenter (Marubishi, Tokyo, Japan). Thereafter, 300 mL of *Lactobacillus fermentum* (KCCM 40401) bought from the Korean Culture Center of Microorganisms were inoculated into the extract and cultured for 24 h at 37°C. The fermentation product was filtered, concentrated to at least 15°Bx, and spray-dried before use.

### Measurement of NO scavenging activity

Nitric oxide (NO) can be detected by a colorimetric assay using the Griess reaction as previously described (14). The difficulties inherent to quantitation of NO can be eliminated by measuring its stable metabolites, in particular, nitrite (NO<sub>2</sub><sup>-</sup>) and nitrate (NO<sub>3</sub><sup>-</sup>). Nitrite is the only stable end-product of the autoxidation of NO in aqueous solution. The Griess reaction was accompanied with formation of a chromophore from the diazotization of sulfanilamide by acidic nitrite followed by coupling with bicyclic amines such as N-1-(naphthyl) ethylenediamine. Briefly, after mixing 100 µL of 0.6 mM NaNO<sub>2</sub>, 800 µL of 0.1 N HCl, and 100 µL of the sample, the mixture was left at room temperature for 1 h for reactions. After mixing 100 µL of the reaction solution and 200 µL 2% of acetic acid into the reactant, Griess reagent (Sigma-Aldrich Co., St. Louis, MO, USA) 40 µL was added to the mixture, the mixture was left at room temperature for 10 minutes for reactions, and the optical density of the reactant was measured at 540 nm. The results were calculated using the following formula.

$$\text{NO scavenging activity (\%)} = \frac{A_{\text{Sample}} - A_{\text{Sample blank}}}{A_{\text{Control}} - A_{\text{Control blank}}} \times 100$$

### Cell culture

RAW264.7 cells bought from ATCC (American Type Culture Collection, Manassas, VA, USA) were used. A culture medium was prepared by adding 100 U/mL penicillin and 100 µg/mL streptomycin to DMEM (Gibco, Grand Island, NY, USA) containing 10% FBS (Gibco) and used during culture in a 5% CO<sub>2</sub> incubator at 37°C.

### Measurement of the effects to suppress NO formation in macrophage cell lines

RAW264.7 cells were seeded into a 24-well plate at a

concentration of 5×10<sup>5</sup> cells/well and cultured for 24 h. After replacing the culture medium with phenol red free RPMI 1640 culture medium (Gibco) 800 µL, FAJE 100 µL, and 1 µg/mL LPS 100 µL were added and the cells were cultured for 24 hours. 100 µL of culture medium and 100 µL of Griess reagent react with each other for 10 minutes at room temperature, the optical density was measured at 540 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

### Experimental animal

A total of 30 male SD rats aged 8 weeks weighing 250-300 g bought from Daehan Bio Link Co., Ltd. (Eumseong, Korea) were used as experimental animals. All animal experiments were conducted using protocols approved by EBO Animal Research Ethics Committee (EBOA201603). Two or three rats were put into each of polycarbonate breeding cages and fed freely with sterilized feed and drinking water; the rats were used in the experiment after undergoing an acclimatization process for one week in an environment with light-dark cycles of 12 h (07:00-19:00), illumination intensity of 200-300 lux, temperature of 22±2 °C, and humidity of 50±5%. In the experiment, the normal group in which osteoarthritis was not induced. The osteoarthritis-induced group, and the groups in which osteoarthritis was induced and 100, 200, or 400 mg/kg of FAJE was administered were organized with six rats per group. FAJE was orally administered at 10 mL/kg for four weeks using sondes.

### Preparation of osteoarthritis-induced models by the MIA administration

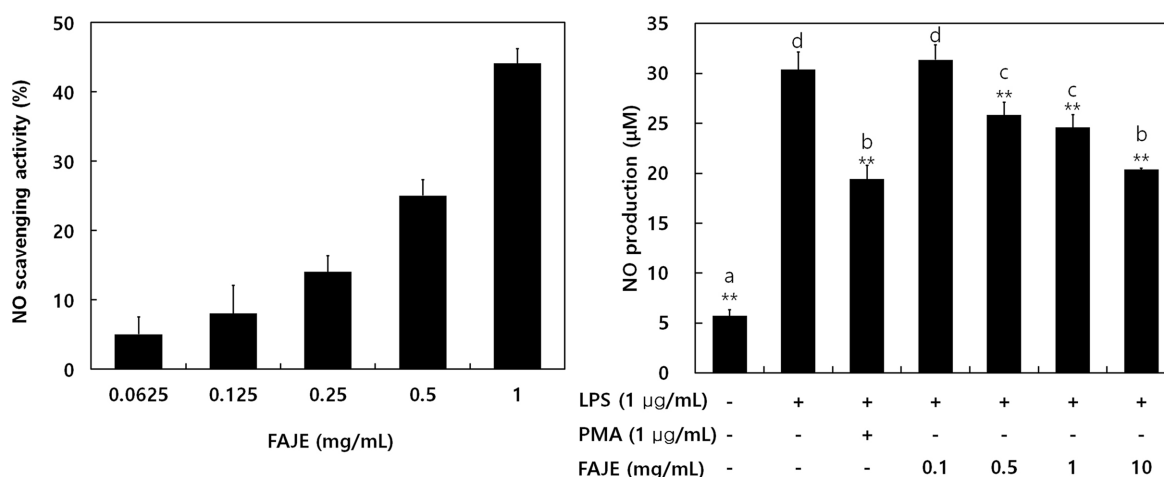
The rats were anesthetized using ether and the hair on the back of the left and right knees and surrounding regions was removed using clippers. After bending the knee joints to 90°, MIA (Sigma-Aldrich Co.) solution was injected into the glenoid cavities after penetrating through the patellar ligaments. The injected MIA solution was made by dissolving 3 mg of MIA per 50 µL of sterilized physiological saline solution and 100 µL of the solution was injected into each of the left and right glenoid cavities once using a 26-gauge needle. The normal group was administered a sterilized physiological saline solution instead of MIA.

### Measurement of inflammatory cytokine expression in the serum

On the day after the final administration of FAJE, the rats were anesthetized using ether to collect blood from the abdominal aorta. The blood was left unattended at room temperature for 15 minutes and subjected to centrifugation at 3,000 rpm for 15 minutes to separate the serum. The production rates of TNF-α and IL-1β in the separated serum were quantified by measuring the optical densities using ELISA kits (Thermo Scientific, Waltham, MA, USA).

### Measurement of the expression of cartilage inflammatory factors MMP-2 and MMP-9

Knee joints were collected from the rats and used for



**Fig. 1. Effect of the fermented *Achyranthes japonica* (Miq.) Nakai extract (FAJE) on the NO scavenging activity and LPS-induced NO production in RAW264.7 cells.** (A) The NO scavenging activity by FAJE. (B) Effect of FAJE on the NO production in LPS-treated RAW264.7 cells. PMA: Phobol 12-myristate 13-acetate. The values are mean±SD,  $n=3$ . Significant difference from LPS only group by Dunnett's t-test: \*\* $p<0.01$ . Values with different letters are significantly different at  $p<0.05$  by Duncan's multiple range test.

measurement of MMP-2 and MMP-9. The collected knee joints were washed with a physiological saline solution and pulverized into small pieces; RIPA buffer was added to the small pieces of the knee joints to extract proteins. The proteins were subjected to centrifugation for 15 minutes at 4°C and 15,000 rpm and the optical density of the supernatant was measured using ELISA kit (Mybiosource, San Diego, CA, USA) to measure the production rates of MMP-2 and MMP-9.

#### Histological evaluation

Other collected knee joints were fixed with 10% formalin. The fixed knee joints were washed with 70% ethanol and decalcified with 5% formic acid for 72 h. Thereafter, the decalcified knee joints were embedded in paraffin and sectioned into 5 µm thickness to check the degrees of inflammatory infiltration and cartilage degeneration in joints' tissues and surrounding tissues through H&E staining and Safranin-O fast green (SOFG) staining, respectively.

#### Statistical processing of data

All data are presented as means and deviations and the statistical analyses were conducted using the SPSS program (IBM Corporation, Armonk, NY, USA). One-way analyses of variation (ANOVA) were conducted. In cases where significance was observed in ANOVA, Dunnett's t-tests were conducted to identify experimental groups with significant differences from the control group; Duncan's multiple range tests were conducted to see whether there were differences between the experimental groups (significance level; both sides 5%).

## Results and Discussion

### Anti-inflammatory effects of the fermented *Achyranthes japonica* (Miq.) Nakai extract (FAJE)

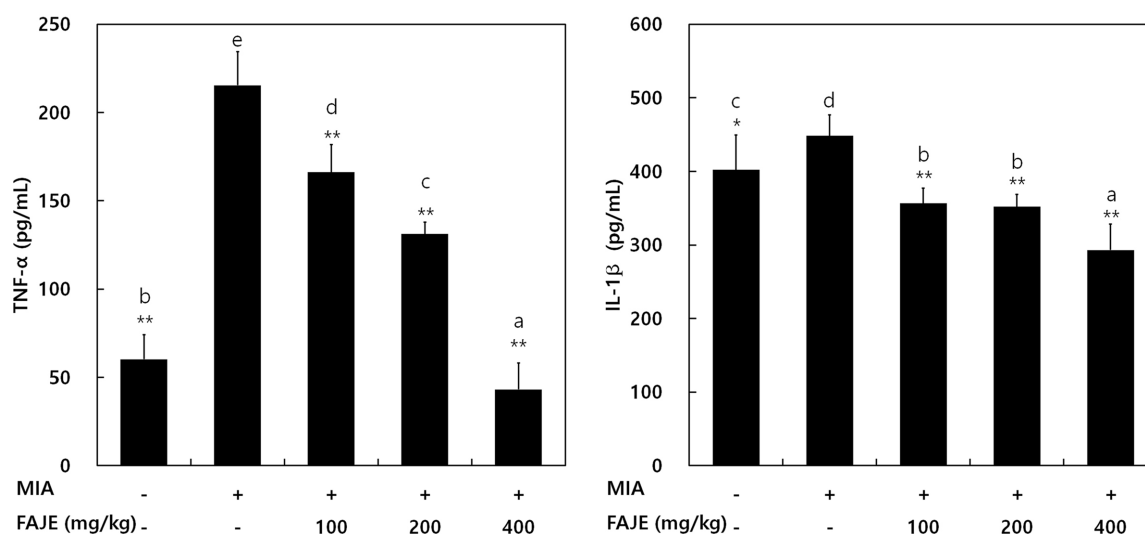
Whereas NO has anti-inflammatory effects under normal

physiological conditions, it starts to act as an inflammation mediator that causes inflammations when abnormally overexpressed (15). NO is over-expressed when RAW264.7 cells, a macrophage cell line, are stimulated (16). We have treated RAW264.7 cells with LPS and FAJE at various concentrations to examine the effects of FAJE to suppress the NO formation.

The NO scavenging activity of the FAJE was evaluated. According to the results, the FAJE at concentrations of 0.0625, 0.125, 0.25, 0.5, and 1 mg/mL showed the NO scavenging activity levels of 5, 8, 14, 25, and 44%, respectively (Fig. 1A). The anti-inflammatory effects of the FAJE were identified by measuring the changes in the NO generation rates. When inflammatory reactions were induced to RAW264.7 cells with LPS. When RAW264.7 cells were treated only with LPS, the NO generation rate became  $30.4\pm 1.7\ \mu\text{M}$ , which was approximately 5 times higher as compared to the NO generation rate when the cells were not treated with LPS ( $5.7\pm 0.6\ \mu\text{M}$ ). In the case of PMA (phobol 12-myristate 13-acetate) that was used as a positive control, NO generation rates decreased to approximately  $19.4\pm 1.4\ \mu\text{M}$ . When the cells treated with LPS were treated with the FAJE at concentrations of 0.1, 0.5, 1, and 10 mg/mL, NO of  $31.4\pm 1.5$ ,  $25.8\pm 1.3$ ,  $24.6\pm 1.3$ , and  $20.4\pm 0.1\ \mu\text{M}$  were generated, respectively, indicating concentration-dependent NO generation inhibiting effects (Fig. 1B).

### Changes in inflammatory cytokine production

Osteoarthritis is a disease occurring when proteoglycan decreases, leading to the loss of cartilage resulting in osteosclerosis with which the bone below the cartilage grows and becomes thicker to gradually degenerate (17,18), as cartilage matrixes are discharged to the synovia to cause inflammatory reactions (19). Monosodium iodoacetate (MIA) used in the present study is a substance that obstructs the glycolysis occurring in articular cartilage cells when injected into the glenoid cavity. It causes cartilage cell necrosis and a reduction in proteoglycan synthesis, thereby



**Fig. 2.** Effect of the fermented *Acyranthes japonica* (Miq) Nakai extract (FAJE) on inflammatory cytokines in the blood of rats with MIA-induced osteoarthritis (OA). (A) TNF- $\alpha$  (B) IL-1 $\beta$ . The values are mean $\pm$ SD,  $n=6$ . Significant difference from LPS only group by Dunnet's t-test: \*\* $p < .001$ , \* $p < 0.05$ . Values with different letters are significantly different at  $p < 0.05$  by Duncan's multiple range test.

inducing osteoarthritis. In an experiment that induced osteoarthritis in rats, it was reported that the patterns of damage to articular cartilage, functional disorders, and pain appearing in the rats were very similar to those observed in human osteoarthritis (20). Since inflammatory cytokines, such as TNF- $\alpha$  and IL-1 $\beta$ , are produced in large quantities in the synovial membrane in the case of arthritis to hinder the synthesis of cartilage matrixes and to promote the decomposition of cartilage matrixes. These cytokines can be useful targets in arthritis treatment (19,21). TNF- $\alpha$  is secreted at the beginning of immune reactions to stimulate the production of inflammatory cytokines, such as IL-1, IL-6, and IL-8, so that to increase inflammations and to stimulate the formation of MMPs to decompose proteoglycan in joint cartilage matrixes to induce osteoarthritis. IL-1 $\beta$  also stimulates the MMP production to promote decomposition of proteoglycan in joint cartilage matrixes.

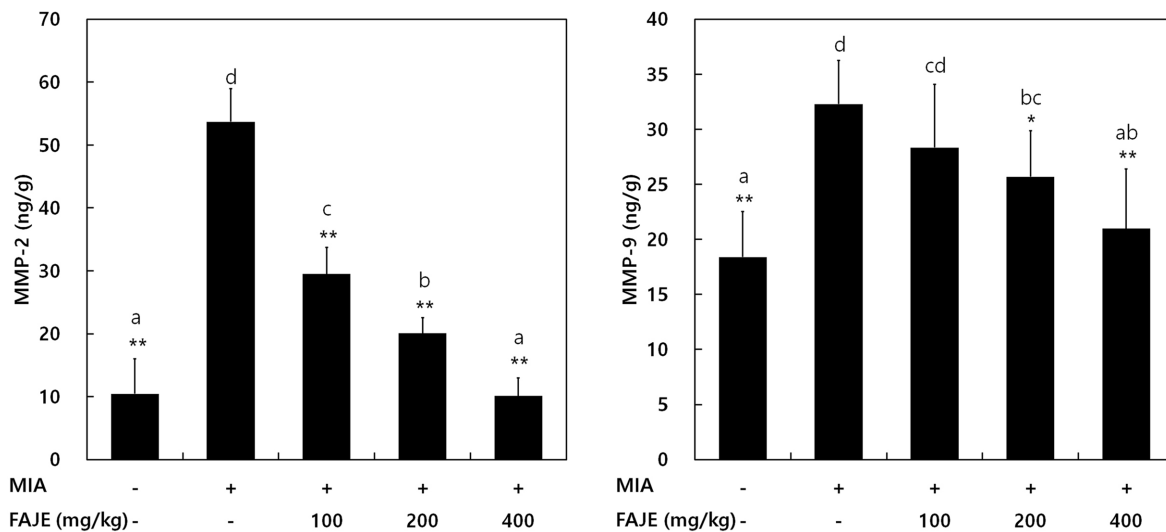
In our experiment, the TNF- $\alpha$  and IL-1 $\beta$  production in blood were examined in the control group in which osteoarthritis was induced by administering MIA. In this control group, the TNF- $\alpha$  and IL-1 $\beta$  production increased as compared to the normal group. TNF- $\alpha$  expressions were  $215.5 \pm 19.2$  pg/mL, i.e. approximately 3.6 times higher than those of the normal group ( $60.3 \pm 13.8$  pg/mL). On the other hand, the groups administered with the FAJE after inducing osteoarthritis showed significant concentration-dependent decreases in the expression. The groups administered with 100, 200, or 400 mg/kg of the FAJE after osteoarthritis was induced showed significant concentration-dependent decreases in the TNF- $\alpha$  expression ( $166.2 \pm 15.6$ ,  $131.1 \pm 6.7$ , and  $43.2 \pm 15.0$  pg/mL, respectively). In particular, the group administered with 400 mg/kg of the FAJE showed a decrease in the TNF- $\alpha$  expression to the level of the normal group (Fig. 2A). Whereas the IL-1 $\beta$  expression of the control group where osteoarthritis was induced was  $448.2 \pm 28.5$  pg/mL, which was higher as compared to that of the normal group ( $402.0 \pm 47.6$  pg/mL), the

groups administered with 100, 200, or 400 mg/kg of the FAJE after osteoarthritis was induced showed significant concentration-dependent decreases in the IL-1 $\beta$  expression ( $356.5 \pm 20.9$ ,  $351.9 \pm 16.5$ , and  $293.1 \pm 35.0$  pg/mL, respectively) (Fig. 2B).

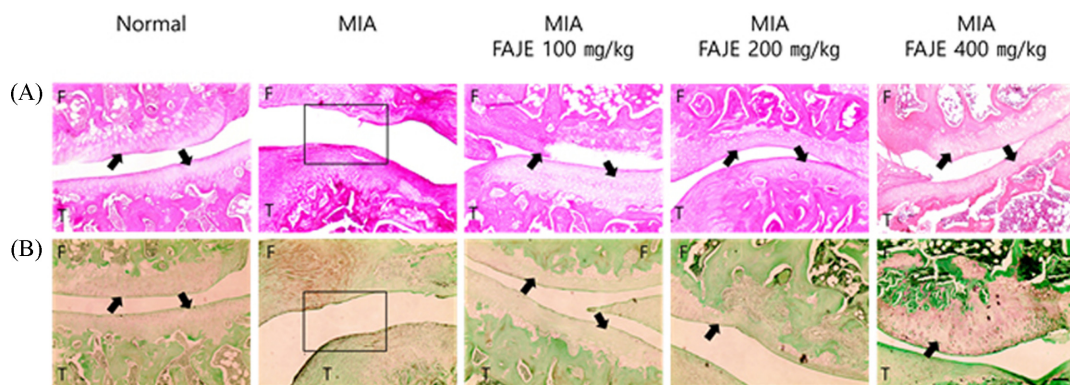
#### Changes in MMP-2, MMP-9 expression

Matrix metalloproteinase (MMP) has been reported to play an important role in decomposing matrixes in osteoarthritis and rheumatoid arthritis (22). Among them, MMP-2 decomposes collagen and activates other MMPs in the cartilage cells where arthritis occurred (23). MMP-9 decomposes collagen, gelatin, proteoglycan, and elastin and thus plays an important role in the destruction of joint cartilage matrixes (18).

In the present experiment, the bone joints collected from the experimental animals were ground and the expression of cartilage inflammatory factors MMP-2 and MMP-9 were measured. The MMP-2 and MMP-9 expression rates increased in the control group in which osteoarthritis was induced compared to the normal group; however, the concentration dependently decreased in the FAJE intake groups. The MMP-2 expression of the control group where osteoarthritis was induced was  $53.7 \pm 5.3$  ng/g, which was approximately 5 times higher as compared to that of the normal group ( $10.5 \pm 5.6$  ng/g). The groups administered with 100, 200, or 400 mg/kg of the FAJE showed significant concentration-dependent decreases ( $29.5 \pm 4.3$ ,  $20.1 \pm 2.5$ , and  $10.11 \pm 2.8$  ng/g, respectively). In particular, the group administered with 400 mg/kg of the FAJE showed a decrease to the level of the normal group (Fig. 3A). The MMP-9 expression of the control group where osteoarthritis was induced amounted to  $32.3 \pm 4.0$  ng/g, which was higher as compared to that of the normal group ( $18.4 \pm 4.2$  ng/g). However, the group administered with 100 mg/kg of the FAJE showed a decreasing tendency, although the decrease did not reach statistical significance; at the same time, the groups administered with 200 or 400 mg/kg



**Fig. 3.** Effect of treatment of the fermented *Acyranthes japonica* (Miq) Nakai extract (FAJE) in the cartilage of the rats with MIA-induced OA on MMPs. (A) MMP-2 (B) MMP-9. The values are mean $\pm$ SD,  $n=6$ . Significant difference from LPS only group by Dunnett's t-test: \*\* $p<0.01$ , \* $p<0.05$ . Values with different letters are significantly different at  $p<0.05$  by Duncan's multiple range test.



**Fig. 4.** Effect of the fermented *Acyranthes japonica* (Miq) Nakai extract (FAJE) in the cartilage of rats with MIA-induced OA. (A) H&E staining. (B) Safranin-O fast green (SOFG) staining. T: Tibia, F: Femur, Arrow: Cartilage, Square: Degenerated cartilage. Magnification:  $\times 100$ . Scale bar: 50  $\mu$ m

showed significant decreases in the MMP-9 expression (Fig. 3B).

#### The effects of the FAJE on cartilage tissues to which osteoarthritis was induced

Hematoxylin & eosin (H&E) staining and Safranin O fast green staining were conducted for histological analysis. During H&E staining, whereas inflammatory cell infiltration, degeneration, and cartilage losses were identified in the osteoarthritis-induced group due to the MIA administration, considerable recoveries of cartilage conditions were observed in the FAJE sample-administered groups as the concentration of the administered sample increased. In the normal group, cartilage was eroded and cell degeneration occurred in the control group where osteoarthritis was induced, the FAJE-administered groups showed recoveries from inflammatory cell infiltration, cell degeneration, and cartilage conditions to the conditions of the normal group according to increases in sample concentrations (Fig. 4A).

In addition, to identify the degrees of cartilage tissue

degeneration, proteoglycan layers were stained with Safranin O. Whereas the losses of cartilage layers and proteoglycan were severe in the osteoarthritis-induced group, recoveries of cartilage layers that had been lost due to the MIA administration and recoveries of proteoglycan contents were observed in all groups administered with the FAJE in different concentrations. Rich proteoglycan contents that led to the staining of cartilage layers into red were observed in the normal group administered with saline. However, decreases in proteoglycan contents could be identified alongside with losses of cartilage in the control group where osteoarthritis was induced. Although cartilage damage was observed in the group administered with 100 mg/kg of the FAJE sample, cartilage tissues considerably recovered and proteoglycan contents increased as compared to the osteoarthritis-induced group. Although some damage to the cartilage was observed in the group administered with 200 mg/kg of the FAJE sample, a relatively smaller-scale damage to cartilage was observed and increases in proteoglycan contents were identified as compared to the group administered with 100 mg/kg of the FAJE. In the

group administered with 400 mg/kg of the FAJE, almost no damage to cartilage was observed and rich proteoglycan contents were observed so that a recovery of the conditions to a level close to that of the normal group could be identified (Fig. 4B).

Through the present experiment, we have identified that the FAJE has anti-inflammatory effects and contributes to improving osteoarthritis conditions by suppressing the expression of inflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$  and MMP-2 and MMP-9 which are a major cause of proteoglycan decomposition in osteoarthritis.

## Conclusion

The anti-inflammatory and anti-arthritis effects of the fermented *Achyranthes japonica* (Miq.) Nakai extract (FAJE) was evaluated in this study. Our experiments showed that the FAJE was effective *in vitro* (LPS-treated RAW264.7 cells) and *in vivo* (OA-induced SD rats) study. FAJE clearly decreased the NO levels in *in vitro* study, and the inflammation markers (TNF- $\alpha$ , IL-1 $\beta$ , MMP-2 and MMP-9) in *in vivo* study. Moreover, the damages on the cartilage were recovered and the proteoglycans were increased by FAJE. These overall results strongly suggest that the FAJE has anti-inflammatory effects and contributes to improving osteoarthritis conditions by suppressing the expression of inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$ , MMP-2 and MMP-9 which are a major cause of proteoglycan decomposition in osteoarthritis.

## Disclosure

The authors declare no conflict of interest.

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