

Doctor's Thesis

The United Graduate School of Veterinary Science

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Evaluation of the efficacy of Ajuga decumbens extract supplement in a rabbit osteoarthritis model and in individuals with knee discomfort
(筋骨草エキスのウサギ軟骨損傷モデルと膝の不調を持つ人への有効性評価)

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Contents

	Page No.
Publications	1
Abstract	2
General introduction	5
References	8

Chapter I.

***Ajuga decumbens* stimulates differentiation of mesenchymal stem cells and regenerates cartilage deficient injury in a rabbit osteoarthritis model**

Abstract	12
1. Introduction	13
2. Materials and Methods	15
3. Results	20
4. Discussion	25
References	28
Figures	33

Chapter II.

Evaluation of the efficacy of *Ajuga decumbens* extract supplement in individuals with knee discomfort associated with physical activity: a randomized, double-blind, placebo-controlled study

Abstract	46
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Contents

1. Introduction	47
2. Materials and Methods	48
3. Results	54
4. Discussion	60
References	64
Figures and Tables	70
Conclusions	85
Acknowledgements	87

Publications

Title : Ajuga decumbens stimulates differentiation of mesenchymal stem cells and regenerates cartilage deficient injury in a rabbit osteoarthritis model

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Abstract

Abstract

The dietary supplement glucosamine hydrochloride (GlcN) has been advocated as a safe and effective option for the management of symptoms of osteoarthritis (OA). Furthermore, the combination of GlcN with other supplements is expected to be more effective in relieving the OA symptoms. Therefore, to achieve a more effective therapy for OA, we investigated the substances having a beneficial effect on OA. *Ajuga decumbens* (AD), a natural herb, has been used long in Japan for pain relief. Recently, AD was reported to have potential for preventing osteoporosis (Ono et al., 2008); however, AD has not been investigated for its effects on damaged cartilage tissue.

In our previous report, we examined the effects of orally administered AD extract (ADE) alone or the combination with GlcN using rabbits with experimentally-induced cartilage injuries, and also compared with the effect of ecdysteroid β -ecdysone (BED), a component of ADE. We revealed that ADE enhanced the regeneration of subchondral bone in this model. Moreover, the combination of ADE with GlcN synergistically increased the regeneration of cartilaginous matrix and subchondral bone. Interestingly, BED exhibited the similar effects on the regeneration of damaged subchondral bone, suggesting that BED is an active component of ADE. Together these observations indicate that the combination of ADE with GlcN synergistically exhibits a therapeutic effect on OA due to the regeneration of the cartilaginous matrix and subchondral bone (Sawada et al., 2014).

In the present study, we concentrated the effective fraction of the extract to give extra ADE (EADE), which included 20-hydroxyecdysone, an active component of ADE. Firstly, we evaluated the effect of EADE on the acceleration of healing in

Abstract

experimental cartilage injury. A model of cartilage injury was surgically created by introduction of three holes, one in the articular cartilage of the medial trochlea and two in the trochlear sulcus of the distal femur. Rabbits used in the experiment were divided into four groups ($n = 3$), namely the control group, the ADE group, the low dosage of EADE (Low EADE) group, and the high dosage of EADE (High EADE) group. ADE and EADE were dissolved in tap water and each dosage was orally administered every day for 3 weeks. After the experimental period, the cartilage matrix was strongly regenerated in the Low and High EADE groups. The average number of osteoclasts per 100 counts of osteoblast in subchondral bone decreased in the High EADE group to a greater extent compared to the control group. EADE also stimulated chondrogenic differentiation of mesenchymal stem cells and induced *in vitro* proteoglycan production. Moreover, EADE significantly attenuated the stimulation of prostaglandin E₂ production by interleukin-1 β in chondrocytes. Therefore, EADE might represent a remarkable improvement on the currently-used ADE.

Secondary, we evaluated the efficacy and safety of the oral administration of EADE supplement to individuals with knee discomfort associated with physical activity. A randomized, double-blind, placebo-controlled study was conducted using 48 subjects. The subjects were randomly allocated to an EADE diet group (oral administration of EADE-containing diet, $n = 24$) and a placebo group ($n = 24$), and the intervention was continued for 12 weeks. Finally, 22 subjects in the placebo group and 22 subjects in the EADE diet group assessed to be eligible for assessment of the efficacy of supplement. The knee function was assessed by changes in the scores of the Japanese Knee Osteoarthritis Measure (JKOM) questionnaire and the

Abstract

scores of the Japan Orthopedic Association (JOA) criteria as well as by analyzing the levels of type II collagen synthesis and degradation biomarkers (Procollagen II C-terminal propeptide, Degradation products of C-terminal telopeptide of type II collagen, C-terminus of the piece of type II collagen cleavage product and Matrix metalloproteinase -13). Outcomes were measured at the baseline and 4, 8, and 12 weeks from the start of administration. The subscale II (Joint flexion/stiffness) of the JOA criteria was substantially improved in the EADE diet group compared with the placebo group at 8 and 12 weeks during the intervention. Moreover, in the subgroup analyses using subjects with less knee discomfort, the subscale II (Pain/stiffness) and IV (General activities) scores of JKOM were significantly improved and Total score was substantially improved in the EADE diet group compared with the placebo group. Finally, no adverse effects were identified for the administration of EADE. These observations suggest that the administration of EADE-containing diet is safe and improves the joint function (flexion and stiffness) and general activities in the subjects with mild knee discomfort.

In conclusion, EADE could be a promising candidate as a functional food, which is useful for the joint health. The chondroprotective and anti-inflammatory actions EADE contribute to improve the knee joint function, and general activities and QOL in the subjects with mild knee discomfort.

General Introduction

Osteoarthritis (OA) is a major cause of pain and disability in the aging population; however, the disease pathogenesis remains incompletely understood (Sunita *et al.*, 2012). Because OA is a multifunctional process, characterized by changes in joint structure and function in which mechanical factors play a central role (Martin *et al.*, 2001), treatment of patients with OA is difficult.

The dietary supplement glucosamine hydrochloride (GlcN) has been advocated as a safe and effective option for the management of OA symptoms (Reginster *et al.*, 2001). GlcN possesses potential cartilaginous matrix regeneration properties but not bone matrix regeneration properties (Tamai *et al.*, 2002). In addition, GlcN alone or in combination with other supplements does not reduce pain effectively in patients with OA of the knee (Clegg *et al.*, 2006). Exploratory analyses suggested that the combination of GlcN and chondroitin sulfate is effective in a subgroup of patients with moderate-to-severe knee pain (Clegg *et al.*, 2006). To achieve a more effective therapy for OA, we investigated ingredients that may have a beneficial effect on OA pain and bone regeneration. *Ajuga decumbens* (AD), a natural herb, which has long been used as a medicine for pain relief in Japan, was selected as a candidate. Recently, AD was reported to have potential for preventing osteoporosis (Ono *et al.*, 2008); however, AD has not been investigated for its effects on damaged cartilage tissue.

Active components such as 8-acetylharpagide (Konoshima *et al.*, 2000), cyasterone, and ecdysterone have been identified in AD (Imai *et al.*, 1969); however, the main active component of AD remains unidentified. By referring to previous reports, we focused on phytoecdysones, which are plant-derived ecdysteroids found

in invertebrates and plants (Imai *et al.*, 1969). In mammals, ecdysteroids have been reported to exert a protein anabolic effect (Todorov, *et al.*, 2000). Moreover, the ecdysteroid β -ecdysone (BED) has been reported to have beneficial effects on joints, epiphyseal cartilage, and trabecular bone in ovariectomized rats (Kapur *et al.*, 2010). However, these beneficial effects have not been studied in damaged cartilaginous tissues. In the previous study, we investigated the effect of orally administered AD extract (ADE) and possible synergistic effects of orally administered ADE and GlcN on damaged cartilaginous tissues. Moreover, we investigated the active components in ADE. Additionally, we orally administered BED to an experimental rabbit model with artificially-induced cartilaginous injury and compared the results to a group of rabbits orally administered with GlcN and ADE. After the experimental period, the cartilage matrix was strongly regenerated in the Low and High EADE groups. The average number of osteoclasts per 100 counts of osteoblast in subchondral bone was decreased in the High EADE group to a greater extent than in the control group. Moreover, EADE stimulated chondrogenic differentiation of mesenchymal stem cells and induced *in vitro* proteoglycan production. Finally, EADE significantly attenuated the stimulation of prostaglandin E2 production by interleukin-1 β in chondrocytes (Sawada *et al.*, 2014).

Chapter I, we concentrated the effective fraction of the extract to give extra ADE (EADE), which included 20-hydroxyecdysone, an active component of ADE, to make ADE stronger effect and evaluated the effect of EADE on the acceleration of healing in experimental cartilage injury. Moreover, we evaluated whether the EADE stimulates differentiation of mesenchymal stem cells and have anti-inflammation effect. Because EADE has powerful effect for healing in experimental cartilage injury

and activates osteogenesis in subchondral bone, thus, we hypothesized that EADE changed the chondrogenic differentiation and some anti-inflammation effects were associated with cartilage matrix regeneration (Sawada *et al.*, 2017).

Chapter II, we evaluated the efficacy and safety of the oral administration of EADE supplement to individuals with knee discomfort associated with physical activity. Because ADE has been shown to have anti-inflammatory action on a rat arthritis model (Ono *et al.*, 2008). Besides, ADE promotes the osteoblastic differentiation of cultured osteoblasts (Ono *et al.*, 2008) and the repair of articular cartilage and subchondral bone in a cartilage damaged model (Sawada *et al.*, 2014, 2017). Moreover, EADE has beneficial effect of osteogenesis and anti-inflammatory effect (Sawada *et al.*, 2017). However, these beneficial effects on the joint have not been studied in humans. In this study, we investigated the effect of orally administered EADE on the subjects, who experienced the knee discomfort associated with physical activity but clinically did not need the treatment. Dietary supplement containing EADE was administered to the subjects, and the effect on the knee joint was evaluated using OA scores (the Japanese Knee Osteoarthritis Measure and the Japan Orthopedic Association criteria). In addition, type II collagen degradation (Degradation products of C-terminal telopeptide of type II collagen, C-terminus of the piece of type II collagen cleavage product), synthesis (Procollagen II C-terminal propeptide) markers and matrix metalloproteinase-13 were analyzed to evaluate the effects of EADE on cartilage metabolism. Moreover, to make the effect of EADE more clear, subgroup analyses of the subjects with less knee discomfort (Kellgren–Lawrence grades of 0–I and >75 points of JOA score) were performed. The subscale II (Joint flexion/stiffness) of the JOA criteria was substantially improved

in the EADE diet group compared with the placebo group at 8 and 12 weeks during the intervention. Moreover, in the subgroup analyses using subjects with less knee discomfort, the subscale II (Pain/stiffness) and IV (General activities) scores of JKOM were significantly improved and total score was substantially improved in the EADE diet group compared with the placebo group. No adverse effects were identified for the administration of EADE. In conclusion, these observations suggested that the administration of EADE-containing diet was safe and improved the joint function (flexion and stiffness) and general activities in the subjects with mild knee discomfort. (Sawada *et al.*, 2016).

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Chapter I.

***Ajuga decumbens* stimulates differentiation of mesenchymal stem cells and regenerates cartilage deficient injury in a rabbit osteoarthritis model**

Abstract

In our previous study, *Ajuga decumbens* extract (ADE) showed potential to decrease the number of osteoclasts in subchondral bone damage and had a synergistic effect with glucosamine on cartilaginous injury in a rabbit osteoarthritis model. In the present study, we concentrated the effective fraction of the extract to give extra ADE (EADE), which included 20-hydroxyecdysone, an active component of ADE, and evaluated the effect of EADE on the acceleration of healing in experimental cartilage injury. A model of cartilage injury was surgically created by introduction of three holes, one in the articular cartilage of the medial trochlea and two in the trochlear sulcus of the distal femur. Rabbits used in the experiment were divided into four groups ($n = 3$), namely the control group, the ADE group, the low dosage of EADE (Low EADE) group, and the high dosage of EADE (High EADE) group. ADE and EADE were dissolved in tap water and each dosage was orally administered every day for 3 weeks. After the experimental period, the cartilage matrix was strongly regenerated in the Low and High EADE groups. The average number of osteoclasts per 100 counts of osteoblast in subchondral bone was decreased in the High EADE group to a greater extent than in the control group. Moreover, EADE stimulated chondrogenic differentiation of mesenchymal stem cells and induced *in vitro* proteoglycan production. Finally, EADE significantly attenuated the stimulation of prostaglandin E2 production by interleukin-1 β in chondrocytes.

Therefore, EADE may represent a remarkable improvement on the currently-used ADE.

1. Introduction

Osteoarthritis (OA) is a chronic degenerative joint disorder causing pain, stiffness, and limitations in the range of motion. OA is a major public health problem in the elderly, particularly OA of the knee, which is one of the most common forms of OA and estimated to affect up to 25 million people in Japan (Yoshimura *et al.*, 2009). OA is caused by articular cartilage degeneration, include fibrillation of the articular surface, and a decrease in the size and aggregation of proteoglycan (PG) (Buckwalter *et al.*, 2010, Buckwalter *et al.*, 1994, Martin *et al.*, 2001). These alterations are principally the result of changes in chondrocyte function due to aging that decrease the ability of the cells to maintain the tissue, including a decrease in synthetic activity (Martin *et al.*, 2001). The dietary supplement D-glucosamine hydrochloride (GlcN) has been used as a safe and effective treatment for the management of OA symptoms (Reginster *et al.*, 2001). GlcN decreases chemical mediators in chondrocytes (Nakamura *et al.*, 2004), brings about cartilaginous matrix regeneration in an OA model, and restores the articular surface at the site of injury through its effects on PG and collagen (Tamai *et al.*, 2002, Naito *et al.*, 2010). Conversely, GlcN alone, or in combination with other supplements, does not effectively reduce pain in patients with OA of the knee (Clegg *et al.*, 2006). However, tentative analyses have suggested that the combination of GlcN and chondroitin sulfate is effective in a subgroup of patients with moderate-to-severe knee pain (Clegg *et al.*, 2006). To develop a more effective supplement for the treatment of OA,

we investigated a compound that may have a beneficial effect on this disease.

Ajuga decumbens (AD), a naturally-occurring herb, which has long been used as a medicine for pain relief in Japan, was selected as a candidate that could be an effective supplement in the treatment of OA. In a previous study, AD extract (ADE) showed potential in the prevention of osteoporosis (Ono *et al.*, 2008), decreasing the number of osteoclasts following subchondral bone damage and having a synergistic effect with GlcN on cartilaginous injury in a rabbit OA model (Sawada *et al.*, 2014). 20-hydroxyecdysone, an active component of ADE, has also been shown to decrease the number of osteoclasts following subchondral bone damage in cartilaginous injury (Sawada *et al.*, 2014). As 20-hydroxyecdysone has beneficial effects on epiphyseal cartilage tissue and trabecular bone in ovariectomized rats (Kapur *et al.*, 2010), ADE was effective when used for subchondral bone regeneration. However, the mechanism of regeneration within the cartilage matrix induced by ADE is unclear, and its effect on such regeneration was somewhat insufficient in the previous study. The role of chondrocytes seems to be the most important factor with respect to repair in cartilage degradation (Mardones *et al.*, 2015). The chondrocytes are present in hyaline cartilage and develop from the highly regulated differentiation of mesenchymal stem cells (MSCs), mesodermal-derived stem cells present in several *fet al* and adult tissues (Mardones *et al.*, 2015). MSCs have recently been applied to the treatment of OA in clinical trials because of their regeneration potential and anti-inflammatory effects (Mardones *et al.*, 2015). Several studies have demonstrated the therapeutic effects on OA resulting from transplantation of chondrogenic-differentiated MSCs (Mardones *et al.*, 2015). Although transplanted native MSCs can be problematic because of their multipotent

differentiation activity, the use of pre-differentiated MSCs enhances the speed of defect healing (Ham *et al.*, 2015). Furthermore, some compounds affect the differentiation of MSCs to chondrocytes and also have therapeutic effects on joint injury (Johnson *et al.*, 2012).

In the present study, to increase the efficacy of ADE in the treatment of OA, we concentrated the effective fraction of the extract—which includes 20-hydroxyecdysone—to form extra ADE (EADE), and evaluated its effect on cartilage in the cartilage injury rabbit model. In addition, to more thoroughly investigate the mechanisms of the regeneration process in cartilage injury, the ability of EADE to induce differentiation of MSCs and its anti-inflammatory effect in chondrocytes was assessed.

2. Materials and Methods

2.1. Preparation of ADE and EADE

The whole dried AD plant was refluxed with aqueous ethanol and AD was extracted to give ADE. EADE was obtained from the extract using semi-polarity resins with the concentrated fraction having >1 wt% concentration of 20-hydroxyecdysone. The ADE and EADE were purchased from Matsuura Yakugyo Co., Ltd. (Aichi, Japan).

2.2. Animal model

The animal model was established using our previously published method (Osaki *et al.*, 2012, Sawada *et al.*, 2014). Twelve-week-old clinically healthy Japanese Albino female rabbits were purchased from Shimizu Laboratory Supplies

Co., Ltd. (Kyoto, Japan) with a body weight of approximately 2.0 kg, and they were acclimated for 1 week in the laboratory environment. The use of the animals and the procedures followed were approved by the Animal Research Committee of Tottori University.

2.3. Experimental procedures

An analgesic (xylazine hydrochloride, 10 mg/kg) was administered as premedication. After sedation, induction of anesthesia was performed in an anesthetizing box with a mixture of 5% isoflurane in oxygen. Anesthesia was maintained by inhalation of a mixture of 3% isoflurane in oxygen using a mask. The fur at the left knee joint was clipped and the area disinfected with chlorhexidine solution (Hibiscrub, Sumitomo Dainippon Pharma Co., Ltd., Osaka, Japan) and 70% alcohol. Approaching from the lateral portion of the knee joint, an incision was made vertically on the skin from the central part of the femur toward the tibial tuberosity. The articular capsule was incised, and the patella of the stifle joint was exposed completely by artificially dislocating the patella toward the medial side. Three holes measuring 2 mm in diameter and 4 mm in depth were made using a hand drill (Micro-engine BL-F, Osada Medical Co., Ltd., Tokyo, Japan) at the articular cartilage of the medial trochlea (one hole) and the trochlear sulcus (two holes) of the distal femur. The wound was rinsed afterwards with saline solution, and the articular capsule was sutured and closed with a synthetic absorbent thread (3-0 PDSII, Johnson & Johnson, Tokyo, Japan). The subcutaneous tissues and skin were sutured with nylon (USP 3-0 suture, Suprylon, Vomel, Germany). During the 1-week period after the operation, the wound surface was disinfected by povidone-iodine (Isodine, Meiji confectionery,

Tokyo, Japan) once a day, and 10 mg/kg of oxytetracycline (“Terramycin,” Pfizer, Tokyo, Japan) was subcutaneously administered twice a day to prevent infection.

2.4. ADE and EADE administration

The experimental subjects were divided into four groups as follows: the control group, the ADE group, the low dosage of EADE (Low EADE) group, and the high dosage of EADE (High EADE) group. ADE contained 0.04% 20-hydroxyecdysone and EADE contained 1.38% 20-hydroxyecdysone. 500 mg ADE/kg/day (0.2 mg 20-hydroxyecdysone/kg/day) was administered to the ADE group; 500 mg EADE/kg/day (0.69 mg 20-hydroxyecdysone/kg/day) was administered to the Low EADE group; and 500 mg EADE/kg/day (6.9mg 20-hydroxyecdysone/kg/day) was administered to the High EADE group. ADE and EADE were dissolved in tap water and each dosage was orally administered from the bottle every day for 3 weeks. The control group had free access to tap water. At 3 weeks post-operation, the rabbits were euthanized by overdose (80 mg/kg) of pentobarbital (“Nembutal,” Sumitomo Dainippon Pharm Co., Ltd., Osaka, Japan) through intravenous injection. The stifle joints were opened and were macro- or microscopically observed at the site of operation for assessment of the injured cartilage.

2.5. Assessment of macroscopic changes

For the macroscopic analysis, the extent of restoration within the defective holes was scored as previously reported (Gao *et al.*, 2008). The restoration score was as follows: 0 points: less than 50%; 1 point: 50–60%; 2 points: 60–80%; 3 points: 80–100%. The degree of restoration was scored separately for the trochlear sulcus

and the medial trochlear ridge for each subject to give a group mean, and the average score of these two areas combined was also calculated.

2.6. Assessment of histological changes

Histological assessment was performed using the femurs of the rabbits within each group. The recovered left femur was fixed using a 10% neutral buffered formaldehyde solution. After fixation, the stifle joint that had been operated on was trimmed to a thickness of 5 mm and decalcified by shaking in 5% formic acid solution for one day. After decalcification, the tissue was soaked in a 5% sodium sulfate solution for one day to allow neutralization, and was then washed for approximately 10 h under running water. After application of the standard paraffin embedding method, the tissue was sliced into 5- μm slices using a microtome. Staining was performed using the hematoxylin/eosin (HE), Safranin O, and Alcian blue methods. The images of restored areas, articular cartilage, and the growth zone were captured by the OpticLab H850 (Plustek Japan, Tokyo, Japan) and manipulated by ImageJ (Wayne Rasband, MD, USA). The depth of restoration in the cartilaginous matrix and the subchondral bone matrix were measured at each area via HE staining. With Safranin O staining, the red-colored pixels that indicated the presence of PGs were counted so that non-specific colored pixels were not included. With Alcian blue staining, the indigo-colored pixels that indicated the presence of glycosaminoglycans (GAGs) were also counted. We recorded the difference between restored substances at the injured sites in all groups using a microscope (BX51-FL, Olympus, Tokyo, Japan). The proportion of the pixels counted for the desired hue from a total of 120,000 pixels (random sampling of 20,000 pixels at six locations at each

cartilaginous matrix) was then calculated using an image processing technique. The number of osteoclasts at the surface of the subchondral bone, and the number of osteoblasts at the subchondral bone were recorded at 10 random areas under the microscope. The average number of osteoclasts per 100 counts of osteoblast was calculated.

2.7. Culture and cytochemical staining of hMSCs

Human MSCs (hMSCs) derived from umbilical cord matrix were obtained from PromoCell GmbH (Heidelberg, Germany). The cells were seeded at 1×10^5 cells/well in a 96-well plate and cultured for 3 weeks in growing 3D spheroids in each medium. Negative control cells were cultured in 0.2 ml of growth medium (PromoCell GmbH, Heidelberg, Germany); positive control cells were cultured in a complete chondrogenic differentiation medium (PromoCell GmbH); and EADE cells were cultured in the desired concentration of EADE resolved in chondrogenic differentiation medium. The media were changed twice-weekly. After the culture period, histochemical quantitation of chondrogenic differentiation was assessed as PG accumulation, as measured by staining of cell clusters with Alcian blue. Cells were first rinsed with PBS three times and then fixed with 100% methanol for 10 min at room temperature. Staining was accomplished by applying a solution of 0.1% Alcian blue pH 2.5 (Nacalai Tesque, Kyoto, Japan) to the cells for 18 h at 4°C. To quantify the intensity of the staining, the stained culture plates were rinsed with 0.1N HCl twice, and each well was extracted with 6 M guanidine-HCl overnight at room temperature. The optical density of spheroid and extracted dye was measured at 630 nm.

2.8. Chondrocyte culture and measurement of prostaglandin E2

Human chondrocytes derived from normal human femoral cartilage were obtained from Cell Applications (San Diego, CA, USA). The cells were maintained in growth medium (Cell Applications, San Diego, CA, USA) at 37°C in a humidified atmosphere consisting of 95% air and 5% CO₂. For treatment with interleukin (IL)-1 β (PeproTech, Rocky Hill, NJ, USA), the cells were seeded at 1 × 10⁴ cells/well in a 24-well plate. After overnight incubation, the growth medium was changed and cells were stimulated with 1 ng/mL IL-1 β in the presence of 10 or 100 µg/ml of EADE prior to incubation for 24h. After incubation, the supernatants were collected to measure the levels of prostaglandin E₂ (PGE₂) using a PGE₂ Parameter Assay Kit (R&D systems, Minneapolis, MN, USA) in accordance with the manufacturer's instructions.

2.9. Statistical Analysis

Data are expressed as the mean ± standard deviation of the mean. Experimental data were assessed by Dunnett's test. We considered p < 0.05 to indicate a statistically significant difference between groups. Microsoft Excel was used for statistical analysis using the t-test, and IBM SPSS Statistics 19 was used for other tests.

3. Results

3.1. Effect of EADE in vivo

3.1.1 Macroscopic effect of EADE on cartilage regeneration

Figure 1 shows the average degree of restoration score for each group. In the control group, the degree of restoration score were lightly increased at trochlear sulcus (A) and the medial trochlear ridge (B). In the ADE group, the Low and High EADE group, the degree of restoration score was dose-dependently increased by 20-hydroxyecdysone and was significantly increased in the High EADE group compared to the control group in the trochlear sulcus ($p < 0.01$). Conversely, while the degree of restoration score was increased in the ADE, Low EADE, and High EADE groups in the medial trochlear ridge, there were no significant differences compared to the control group. Importantly, the average degree of restoration score, taken from pooling the data from the trochlear sulcus and medial trochlear ridge, was significantly increased in the High EADE group compared to the control group ($p < 0.01$), which was similar to that seen with the trochlear sulcus alone.

3.1.2 Effect of EADE on cartilage regeneration as assessed by histological HE staining

Figure 2 shows representative HE staining images of the trochlear sulcus 3 weeks after surgery. Tissue defects were still clearly visible in the control group (a) at the trochlear sulcus. In contrast, tissue regeneration was apparent in the cartilage matrix and subchondral matrix in the ADE (b), Low EADE (c), and High EADE (d) groups, with defective areas filled with proliferating fibroblasts, cartilaginous cells, and subchondral bone matrix. The surface of the wound area was covered with regenerated connective tissue; at a deeper zone, a lack of bone trabeculae, migration of mononuclear cells and neutrophils was observed. These tissues were surrounded by proliferating cells (fibroblasts and cartilaginous cells), giving the tissue an

appearance that was almost identical to the formation of mature cartilaginous substrates. In the deeper zone, subchondral bone matrix was massively regenerated in ADE (b), Low EADE (c), and High EADE (d) groups. The cancellous bone structure was also somewhat regenerated in these groups, particularly in the High EADE group (d). Figure 3 shows the proportion of restoration depth in the cartilage matrix (A, B, C) and the subchondral bone matrix (D, E, F). The proportion of regenerated cartilage matrix was greater in the ADE, Low EADE, and High EADE groups compared to the control group in the trochlear sulcus. At the medial trochlear ridge, the restoration area in depth was more developed in the High EADE group, compared to the control group, and the average depth of the two areas combined was significantly higher in the High EADE group ($p < 0.05$). In contrast, the proportion of regenerated subchondral bone matrix in the trochlear sulcus and medial trochlear ridge, and the average combined depth, was greater in ADE, Low EADE, and High EADE groups compared to the control group.

3.1.3 Effect of EADE on cartilage regeneration as assessed by histological Safranin O and Alcian blue staining

To evaluate cartilaginous matrix regeneration more clearly, histological staining using Safranin O and Alcian blue was carried out. Figure 4 shows Safranin O staining of the trochlear sulcus (A) and Alcian blue staining of trochlear sulcus (B) with PGs in the cartilage matrix stained dark red. There was very little staining of the cartilage matrix in the control group in the trochlear sulcus (A-a). Conversely, the cartilage matrix was stained more strongly in the ADE (A-b), Low EADE (A-c), and High EADE (A-d) groups compared to the control group. In Alcian blue staining, the GAGs in the

cartilage matrix were stained blue. The stained areas and color strength were similar to what was observed with Safranin O staining (A). Strong staining was seen in the cartilage matrix in the trochlear sulcus in the ADE (B-b), Low EADE (B -c), and High EADE (B -d) groups compared to the control group (B -a). Figure 5 shows the results of image analysis of the cartilage matrix following Safranin O and Alcian blue staining. The regeneration areas were significantly increased in the Low and High EADE groups as seen with Safranin O (A) and Alcian blue (D) staining in the trochlear sulcus ($p < 0.05$), with the two staining methods resulting in almost identical output. This demonstrates that EADE increased the amount of GAGs and PGs in the cartilage matrix in the trochlear sulcus in a dose-dependent manner. In the medial trochlear ridge, the greatest level of staining with either method was observed in the High EADE group (B, E) and the average of the total stained area (that is, staining within the trochlear sulcus and the medial trochlear ridge combined) was significantly higher in the Low and High EADE groups compared to the control group ($p < 0.05$).

3.1.4 Effect of EADE on osteogenesis as assessed by histological HE staining

To estimate the effect of EADE on the balance of osteogenesis, osteoclasts and osteoblasts were counted in the subchondral bone following histological HE staining. Figure 6 shows the osteoclasts and osteoblasts in subchondral bone in HE staining. In control group, the osteoclasts were increased and little osteoblasts were observed. In the ADE group, the osteoblasts were observed and little osteoclasts were observed. In the Low EADE and the High EADE group, the osteoblasts were massively observed. Figure 7 shows the average number of osteoclasts (A, B, C) and we calculated the average number of osteoclasts per 100 counts of osteoblast

in the trochlear sulcus (D) and the medial trochlear ridge (E). The number of osteoclasts was significantly reduced in the High EADE group at the trochlear sulcus (A). However, the average number of osteoclasts was slightly increased in the ADE, Low EADE, and High EADE groups in the medial trochlear ridge (B). Interestingly, the average number of osteoclasts per 100 counts of osteoblast in the medial trochlear ridge (E) was slightly decreased in the ADE, Low EADE, and High EADE groups. These data show that osteogenesis was more highly activated at the medial trochlear ridge as a result of EADE treatment. Moreover, the average total number of osteoclasts per 100 counts of osteoblast for the trochlear sulcus and the medial trochlear ridge combined (F) was slightly decreased in the ADE, Low EADE, and High EADE groups. This suggests that EADE activates osteogenesis in subchondral bone.

3.2. Effect of EADE *in vitro*

3.2.1. Effect of EADE on chondrogenic differentiation in hMSCs

To determine whether chondrogenic differentiation was associated with cartilage matrix regeneration, we estimated MSC differentiation to chondrocytes following EADE treatment. Figure 8 shows the results of the cytochemical analysis carried out using Alcian blue staining after 3 weeks of hMSC culture. The hMSCs were cultured for 3 weeks with a normal growth medium or a chondrogenic differentiation medium containing the desired concentration of EADE. While undifferentiated MSCs have little extracellular matrix, chondrogenic differentiation results in the formation of cartilage with a typical extracellular matrix, composed of the PG aggrecan and other glycosaminoglycans. Aggrecan and other

glycosaminoglycans is used as an indicator of cartilage formation and can be detected via Alcian blue staining. Analysis showed that EADE dose-dependently increased aggrecan and other glycosaminoglycans -associated staining.

3.2.2 Effect of EADE on PGE₂ production in chondrocytes

To evaluate its influence on osteogenesis, we measured the repressive effect of EADE on PGE₂ production. Human chondrocytes were cultured in the absence or presence of 1 ng/mL IL-1 β with the desired concentration of EADE, and PGE₂ production was determined using the ELISA method (Figure 9). In the absence of IL-1 β (control group), a minimal amount of PGE₂ was apparent in cultured cells. In contrast, those cells cultured in the presence of IL-1 β produced PGE₂ in markedly higher amounts than control cells. The effect of EADE on the IL-1 β -induced production of PGE₂ was also examined. Culture of cells with 10 or 100 μ g/mL EADE appeared to significantly block the stimulation of PGE₂ production by IL-1 β in a concentration-dependent manner ($p < 0.05$).

4. Discussion

In the present study, we evaluated whether concentration of the effective fraction of ADE (EADE), including 20-hydroxyecdysone, could be more effective in the treatment of OA and cartilage regeneration. In a cartilage injury model, the degree of restoration within the defective holes was dose-dependently increased by EADE administration (Figure 1). In particular, the cartilage matrix was strongly regenerated in the and High EADE groups ($p < 0.05$) (Figure 2, 3). In addition, the regeneration

area was significantly enhanced in a dose-dependent manner in the Low and High EADE groups as seen following Safranin O and Alcian blue staining ($p < 0.05$) (Figure 4, 5). The data obtained from Safranin O and Alcian blue staining were consistent with each other, and the extent of staining corresponded to the concentration of EADE, showing that the compound increases the amount of GAGs and PGs in cartilage matrix. Moreover, in our previous study, the number of osteoclasts was significantly decreased following administration of ADE or 20-hydroxyecdysone (Sawada *et al.*, 2014), but the influence on bone metabolism was unclear. Bone metabolism is regulated by the balance of osteoclast and osteoblast cells. ADE has been shown to inhibit the differentiation of osteoclasts (Ono *et al.*, 2008), while 20-hydroxyecdysone also has beneficial effects with respect to epiphyseal cartilage and trabecular bone in ovariectomized rats (Kapur *et al.*, 2010). In the present study, the average number of osteoclasts per 100 counts of osteoblast in subchondral bone was decreased in the High ADE group compared to the control group (Figure 7). It is possible that EADE could influence not only the number of osteoclasts, but also, bone metabolism, and the regeneration of subchondral bone. As it has been reported that 20-hydroxyecdysone stimulates MSC osteogenic differentiation (Gao *et al.*, 2008), its presence as an active component in EADE could stimulate MSCs and enhance osteogenesis in subchondral bone.

When cartilage is injured, natural healing tends to be slow and usually results in the formation of nonfunctional fibro-cartilage, while regeneration of hyaline cartilage seldom occurs (Tamai *et al.*, 2002). However, the administration of GlcN does regenerate hyaline cartilage (Tamai *et al.*, 2002). Experimental data have shown that the mechanism underlying this action is an association with chondroblast

activation (Hashida *et al.*, 2003). Glucosamine promotes a chondrogenic phenotype in MSCs (Derfoul *et al.*, 2007) and inhibits matrix metalloproteinase (MMP)-13 expression and matrix degradation (Derfoul *et al.*, 2007). MMPs and IL-1 β have been shown to play a central role in the degradation of articular cartilage (Farahat *et al.*, 1993, Campbell *et al.*, 1990, Tetlow *et al.*, 2001). IL-1 β is produced by mononuclear cells in the arthritic synovium and chondrocytes (Nakamura *et al.*, 2004), and enhances the production of various chemical mediators such as PGE₂ (Campbell *et al.*, 1990), nitric oxide (NO), and MMPs (Tetlow *et al.*, 2001) from chondrocytes. To more clearly delineate the mechanisms of the regeneration process following cartilage injury, the ability of EADE to induce differentiation of MSCs, and its anti-inflammatory effects in chondrocytes, were assessed. EADE stimulated PG production and induced *in vitro* chondrogenic differentiation of MSCs (Figure 8). Moreover, EADE significantly attenuated the stimulation of PGE₂ production by IL-1 β in chondrocytes (Figure 10). It has been reported that 20-hydroxyecdysone suppresses IL-1 β -induced catabolic gene expression in cartilage (Sheu *et al.*, 2015), and that ADE inhibits expression of iNOS and NO production from macrophages (Ono *et al.*, 2008). It has also been shown that PGE₂ induces receptor activator of nuclear factor kappa-B ligand (RANKL) expression by osteoblasts and directly enhances RANKL-induced osteoclastogenesis from precursors (Kotake *et al.*, 2010). In addition, 20-hydroxyecdysone has been reported to stimulate MSC osteogenic differentiation (Gao *et al.*, 2008). Therefore, 20-hydroxyecdysone, the active component in EADE, could suppress IL-1 β -induced inflammation and PGE₂-induced RANKL expression. By this action, EADE could have an anti-inflammatory effect and suppress osteoclastogenesis.

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Figures

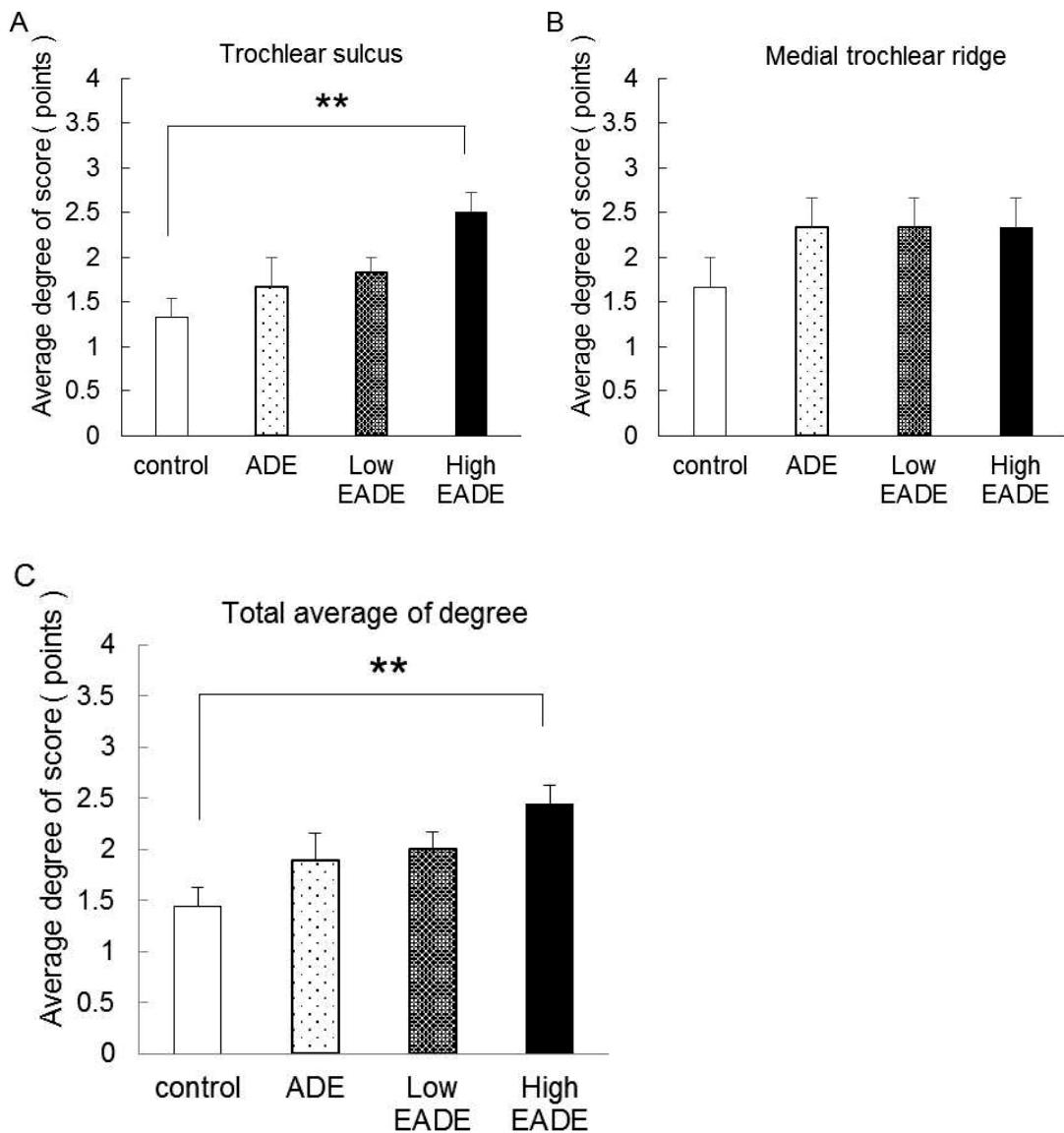


Figure 1. The degree of restoration in the defective holes.

The degree of restoration in the defective holes was quantified using the following scoring system: 0 points: Less than 50 %; 1 point: 50–60 %; 2 points: 60–80 %; 3 points: 80–100 %. Scores for the degree of restoration of two holes in the trochlear sulcus (A), one hole in the medial trochlear ridge (B), and the average of the combined data for all holes (C). The degree of restoration in the defective holes was

calculated as the average of the scores obtained from subjects within each group.

(A) The degree of restoration in the trochlear sulcus was dose-dependently increased by 20-hydroxyecdysone, with the High EADE group score being significantly higher than that of the control group. (B) The degree of restoration in the medial trochlear ridge was increased to a similar level within the ADE, Low EADE, and High EADE groups. (C) The average degree of restoration seen when the data from the trochlear sulcus and the medial trochlear ridge were pooled was also dose-dependently increased, and the score from the High EADE group was significantly higher compared to the control group. The data here were similar to the scores seen for the trochlear sulcus. **: p < 0.01 versus Control using Dunnett's test.

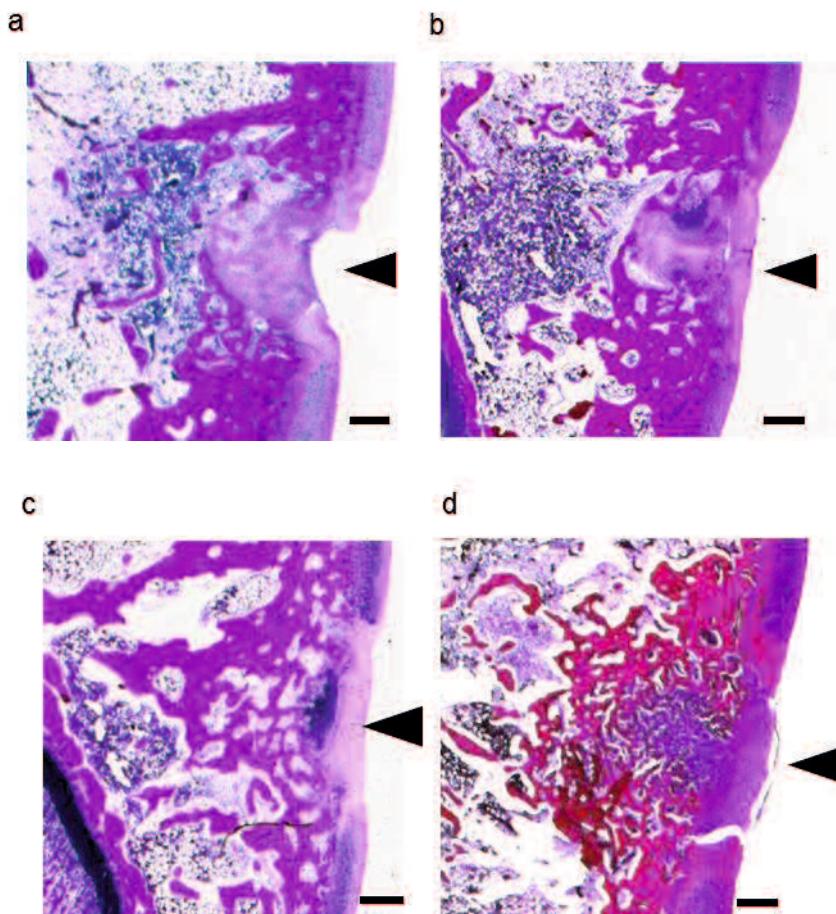


Figure 2. Histological HE staining 3 weeks after surgery.

Histological HE staining in the trochlear sulcus. Tissue defects (black arrowheads) were still clearly visible in the control group (a) at the trochlear sulcus. The holes were filled with proliferating fibroblasts, cartilaginous cells, and subchondral bone matrix in the ADE (b) and Low EADE (c) groups. It also appears that all holes were fully filled with cartilaginous matrix and subchondral matrix in the High EADE group (d). Bars = 500 μ m.

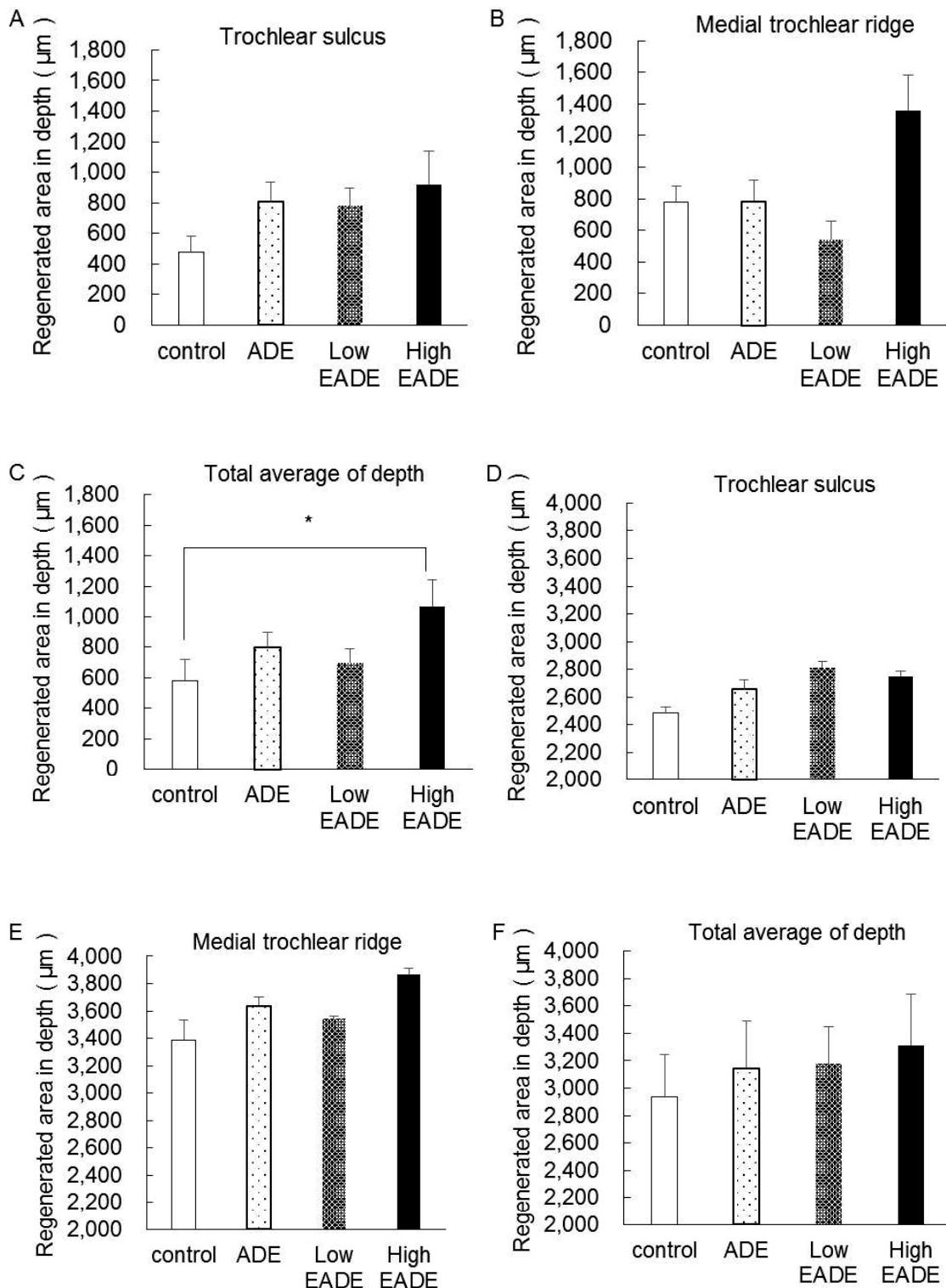


Figure 3. Restoration depth in the cartilage matrix and subchondral matrix (measured using HE staining).

The proportion of the regenerated area in depth at each part of the cartilage matrix (A), (B), (C) and the subchondral bone matrix (D), (E), (F). The cartilage matrix was regenerated to a greater extent in the ADE, Low EADE, and High EADE groups compared to the control at the trochlear sulcus (A); it showed enhanced regeneration in the High EADE group at the medial trochlear ridge (B); and it was significantly regenerated in the High EADE group when the total average depth was calculated (C). The subchondral bone matrix showed regeneration in the ADE, Low EADE, and High EADE groups in both areas and when the data were averaged (D), (E), (F), compared to the control group. *: p < 0.05 versus Control using Dunnett's test.

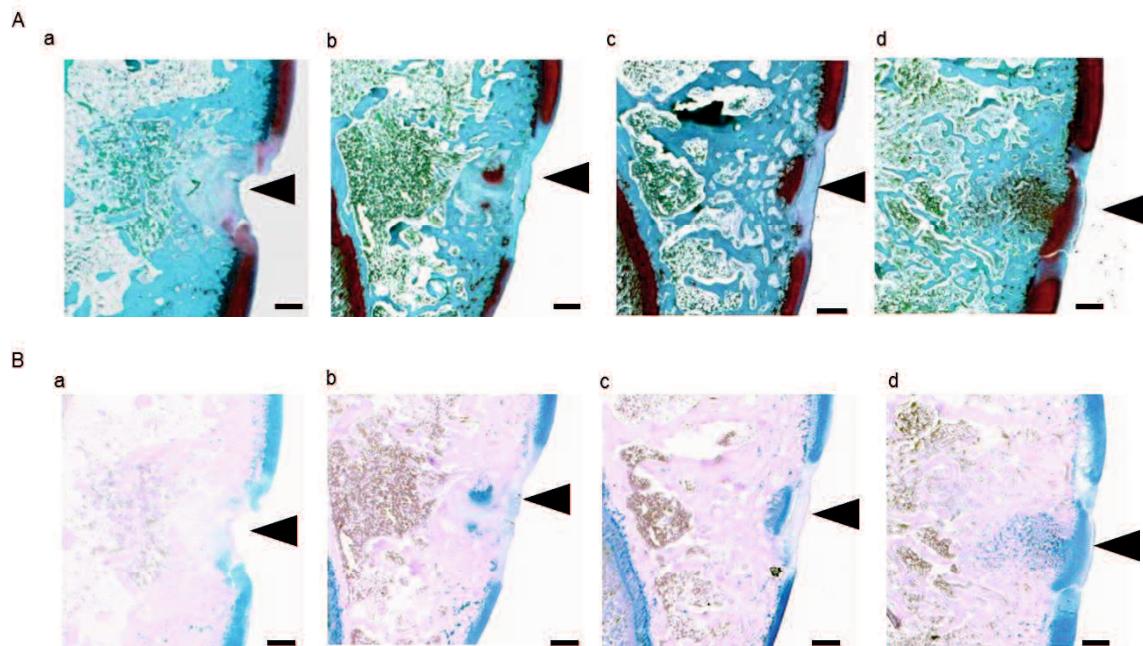


Figure 4. Histological Safranin O staining and Alcian blue staining 3 weeks after surgery.

Histological Safranin O staining in the trochlear sulcus (A) and Alcian blue staining in the trochlear sulcus (B). The proteoglycans in the cartilage matrix were darkly stained. Black arrowheads indicate the tissue-defective areas. At the trochlear sulcus (A), green and dark red staining was still weak in the control group, but the stained area appears to spread dose-dependently in the ADE (A-b), Low EADE (A-c), and High EADE (A-d) groups, compared to the control group (A-a). The defects were filled with collagen fibers in the control (A-a), ADE (A-b), Low EADE (A-c), and High EADE (A-d) groups and it seems that the cartilage matrix was strongly stained dark red in the High EADE group (A-d) only. The GAGs in the cartilage matrix were stained blue (B). The stained areas and intensity of color were similar to what was observed with Safranin O staining (A). The defects were filled with proteoglycan in the ADE (B-b), Low EADE (B-c), and High EADE (B-d) groups and it seems that the cartilage matrix was strongly stained blue in the High EADE group (B-d). Bars = 500 μ m.

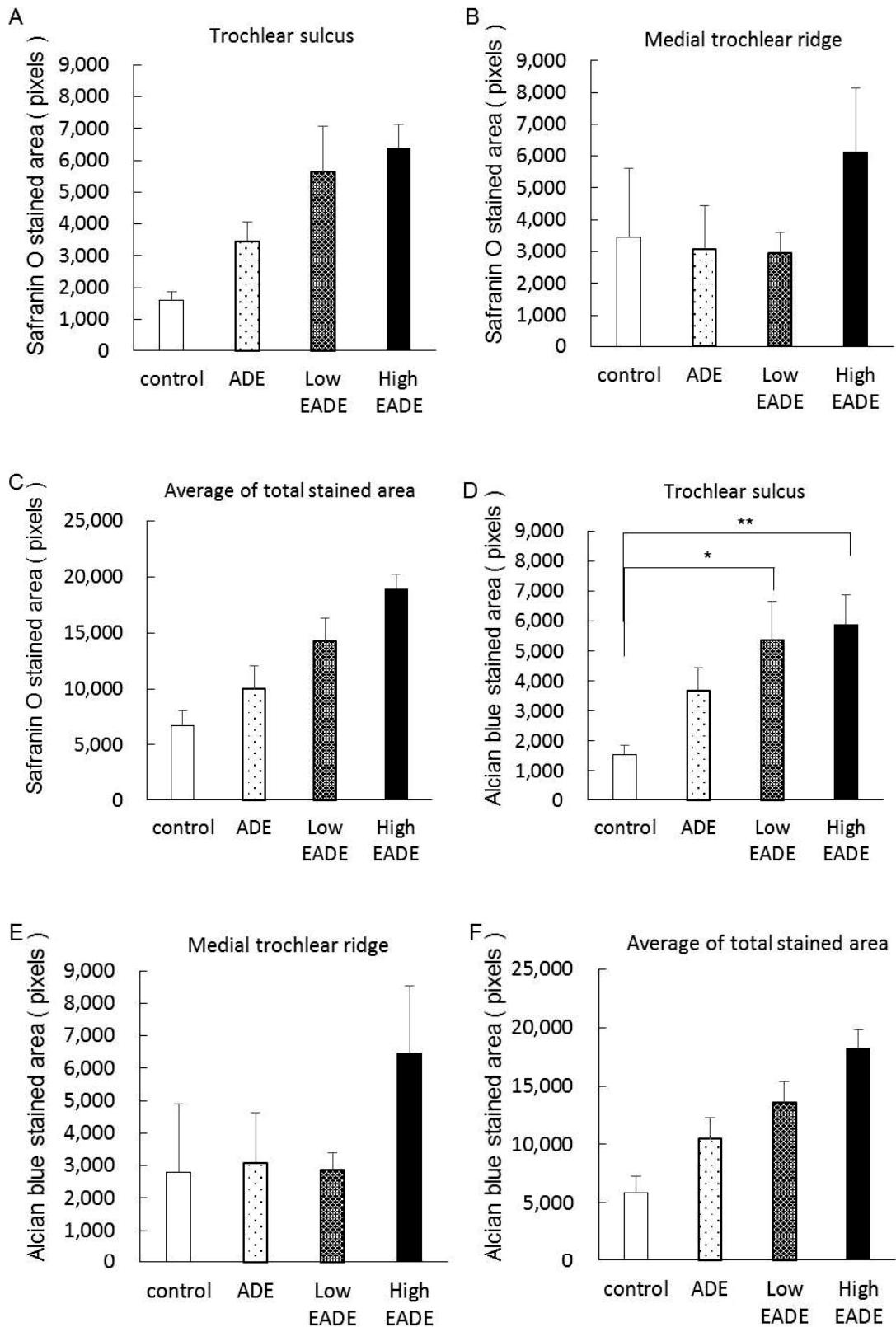


Figure 5. Image analysis of the cartilage matrix following Safranin O and Alcian blue staining.

The proportion of pixels counted for the desired hue in Safranin O (A), (B), (C) and in Alcian blue (D), (E), (F) staining at each cartilage area (six areas in each group) was calculated. The increase in the stained area seen using either method, which represents enhanced PG and GAG content in the cartilage matrix, indicated significant dose-dependent regeneration in the Low ADE and High ADE groups compared to the control group at the trochlear sulcus (A), (D) and when the data were averaged for both areas (C), (F). It also appears that the stained area was greater in the High EADE group at the medial trochlear ridge (B), (E). *: $p < 0.05$, **: $p < 0.01$ versus Control using Dunnett's test.

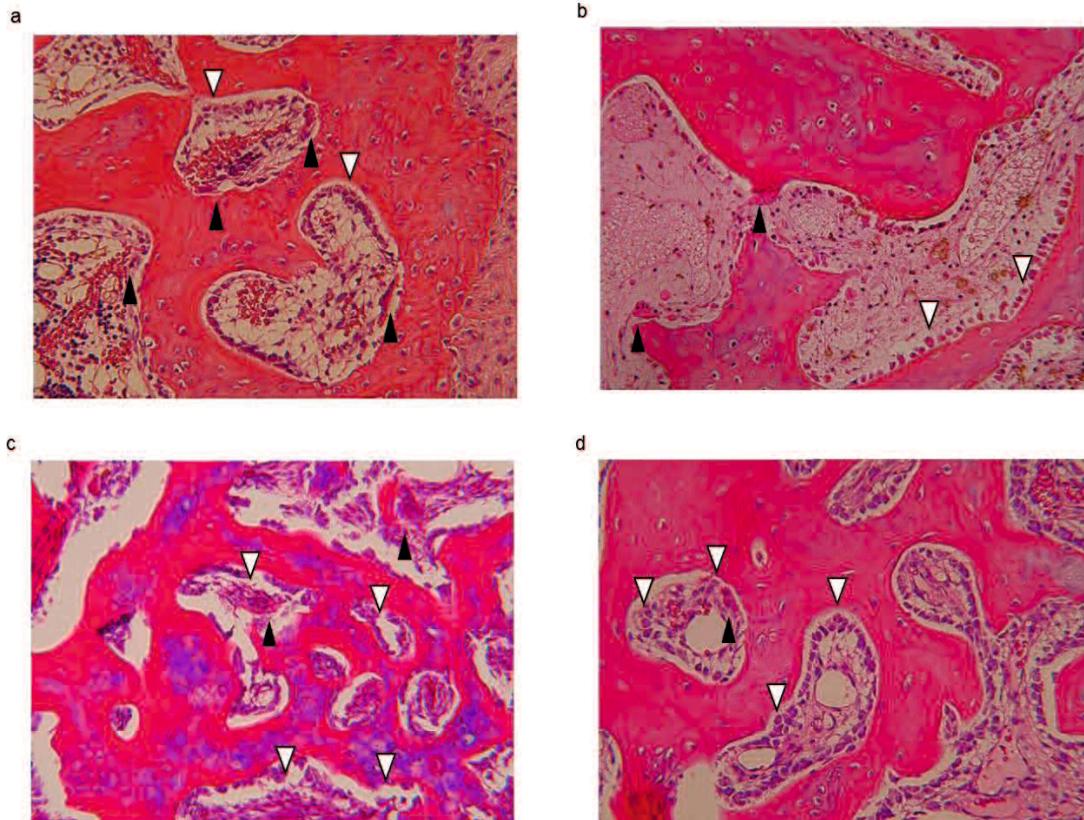


Figure 6. Histological HE staining in subchondral bone 3 weeks after surgery.

Histological HE staining in the trochlear sulcus and the enlarged photo in the subchondral bone. Black arrowheads indicate the osteoclasts and white arrowheads indicate the osteoblast. In control group (a), the osteoclasts were increased and little osteoblasts were observed. In the ADE group (b), the osteoblasts were observed and little osteoclasts were observed. In the Low EADE (c) and the High EADE (d) group, the osteoblasts were massively observed. At 200x magnification.

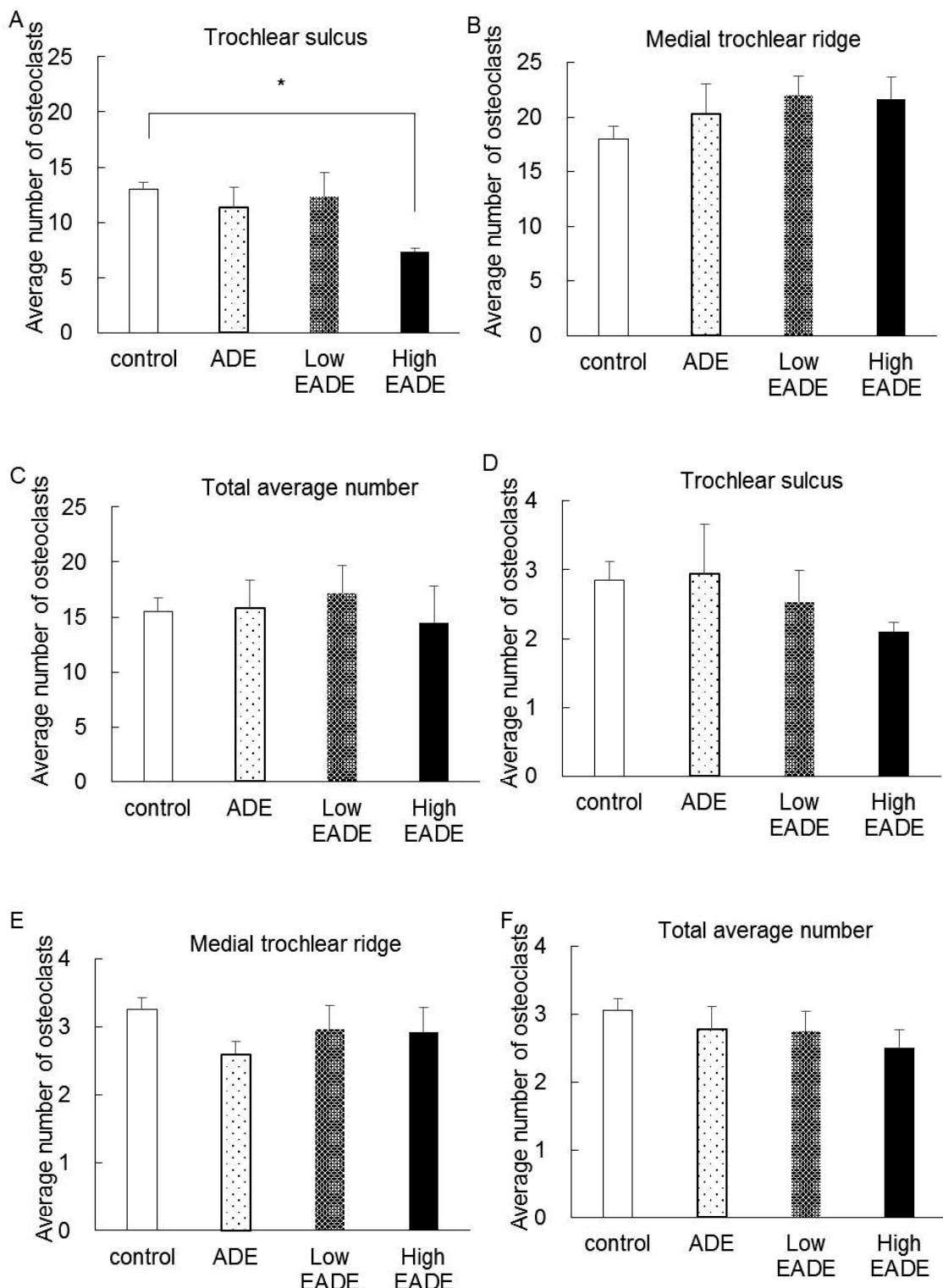


Figure 7. The average number of osteoclasts and the average number of osteoclasts per 100 counts of osteoblast.

The number of osteoclasts at the surface of the subchondral bone was recorded at the trochlear sulcus (A), and the medial trochlear ridge (B) at 10 random areas under the microscope, and the average total number was calculated (C). In addition, the number of osteoblasts at the subchondral bone was recorded and the average number of osteoclasts per 100 counts of osteoblast was calculated for each area (D), (E), (F). The number of osteoclasts was significantly reduced in the High EADE group in the trochlear sulcus (A). While the number of osteoclasts was increased in the ADE, Low EADE, and High EADE groups, compared to the control group, at the medial trochlear ridge (B), the balance of osteoblasts and osteoclasts was inclined towards osteogenesis in these groups, compared to the control (E). The average total number of osteoclasts per 100 counts of osteoblast was inclined towards osteogenesis in the ADE, Low EADE, and High EADE groups, compared to the control group (F). *: p < 0.05 versus Control using Dunnett's test.

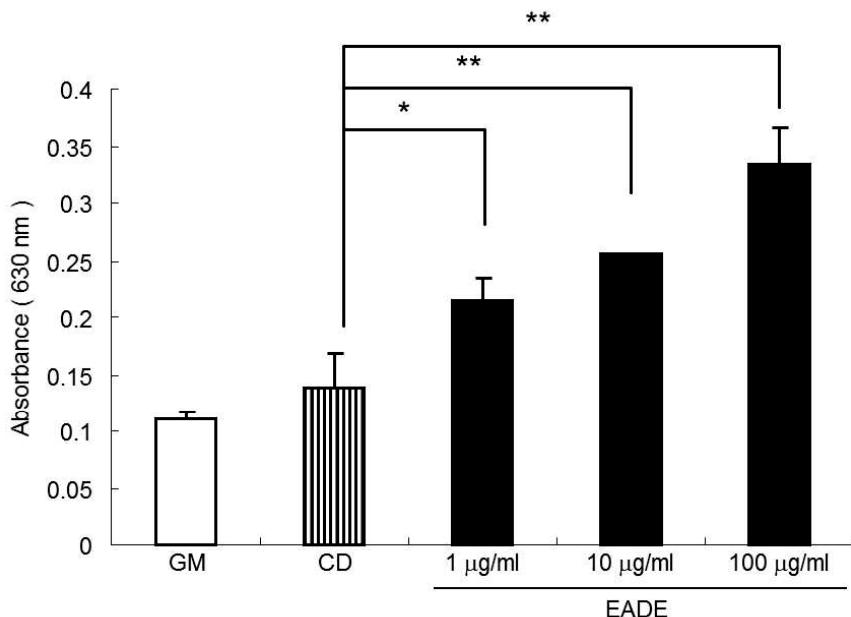


Figure 8. Chondrocyte differentiation from MSCs.

The hMSCs were cultured for 3 weeks with normal growth medium (GM) or chondrogenic differentiation medium (CD) with the desired concentration of EADE. CD results in the formation of cartilage with a typical extracellular matrix consisting of the proteoglycan aggrecan. This is used as an indicator of cartilage formation and can be detected using Alcian blue staining at an absorbance of 630 nm. Absorbance was significantly increased by EADE in a concentration-dependent manner. *: $p < 0.05$ versus CD using Dunnett's test.

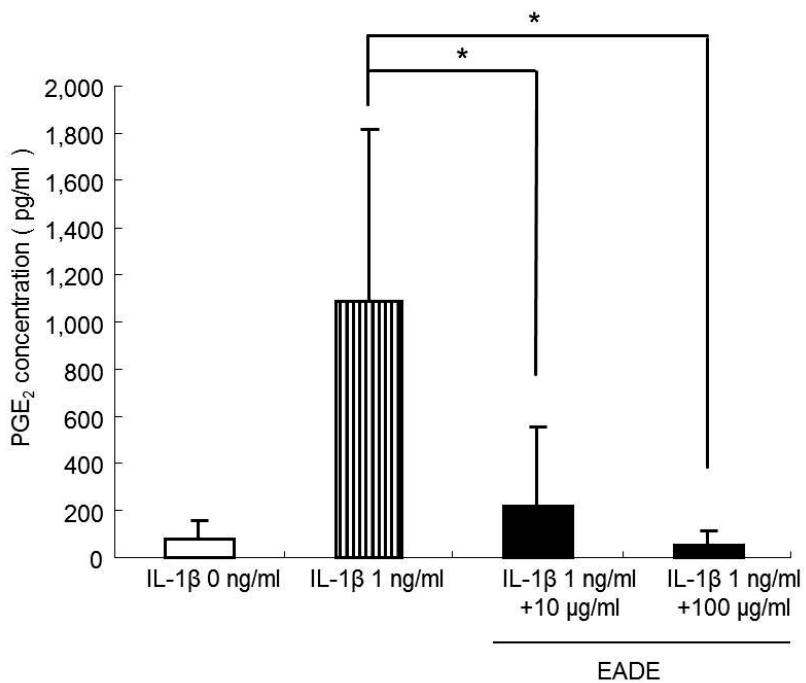


Figure 9. PGE₂ levels in cytokine-stimulated chondrocytes.

Human chondrocytes were stimulated with IL-1 β (1 ng/ml) and treated with EADE (10 and 100 μ g/ml). The concentration of PGE₂ was increased by IL-1 β stimulation, and EADE dose-dependently suppressed this increase. *: p < 0.05 versus IL-1 β 1 ng/ml using Dunnett's test.

Chapter II.

Evaluation of the efficacy of *Ajuga decumbens* extract supplement in individuals with knee discomfort associated with physical activity: a randomized, double-blind, placebo-controlled study

Abstract

The purpose of this study was to assess the efficacy and safety of the oral administration of *Ajuga decumbens* extract (EADE) supplement to individuals with knee discomfort associated with physical activity. A randomized, double-blind, placebo-controlled study was conducted using 48 subjects. The subjects were randomly allocated to an EADE diet group (oral administration of EADE-containing diet, n = 24) and a placebo group (n = 24), and the intervention was continued for 12 weeks. Finally, 22 subjects in the placebo group and 22 subjects in the EADE diet group assessed to be eligible for assessment of the efficacy of supplement. The knee function was assessed by changes in the scores of the Japanese Knee Osteoarthritis Measure (JKOM) questionnaire and the scores of the Japan Orthopedic Association (JOA) criteria as well as by analyzing the levels of type II collagen synthesis and degradation biomarkers (Procollagen II C-Terminal propeptide: PIICP, Degradation products of C-terminal telopeptides of type II collagen: CTX-II, C-terminus of the piece of type II collagen cleavage product: C2C, and matrix metalloproteinase-13: MMP13). Outcomes were measured at the baseline and 4, 8, and 12 weeks from the start of administration. The subscale II (Joint flexion/stiffness) of the JOA criteria was substantially improved in the EADE diet group compared with the placebo group at 8 and 12 weeks during the intervention. Moreover, in the subgroup analyses using subjects with less knee discomfort, the subscale II (Pain/stiffness) and IV (General

activities) scores of JKOM were significantly improved and total score was substantially improved in the EADE diet group compared with the placebo group. No adverse effects were identified for the administration of EADE. In conclusion, these observations suggested that the administration of EADE-containing diet was safe and improved the joint function (flexion and stiffness) and general activities in the subjects with mild knee discomfort. Thus, EADE could be a promising candidate as a functional food, which would be useful for the joint health.

1. Introduction

Knee osteoarthritis (OA) is characterized by the progressive destruction of articular cartilage and is the leading cause of pain and physical disability in the elderly (Yoshimura *et al.*, 2009). Early-stage of OA limits the movement and causes the knee discomfort, thereby impairing the activities of daily life (ADL) and quality of life (QOL) (Yoshimura *et al.*, 2009). The prevalence of radiographic knee OA in Japan is increasing and estimated to affect 30 million people in Japanese senior people over 50 years old (Yoshimura *et al.*, 2009). According to the treatment guidelines for OA of the Knee Osteoarthritis Research Society International (OARSI), non-steroidal anti-inflammatory drugs, selective cyclooxygenase-2 inhibitors, and acetaminophen are listed as high level of evidence drugs for treatment of OA (McAlindon *et al.*, 2014). However, these drugs enhance cartilage destruction, and promote OA (Xu *et al.*, 2006, Ou 1989). Therefore, novel substances with a chondroprotective action have been sought. In our research, we focused on the extract of *Ajuga decumbens* (AD) as a candidate that exhibits a protective action on OA. AD is a natural herb and has long been used as a medicine for pain relief in Japan (Rashad *et al.*, 1989, Herman

et al., 1984). Importantly, AD has been shown to have anti-inflammatory action on a rat arthritis model (Ono *et al.*, 2008). Moreover, AD promotes the osteoblastic differentiation of cultured osteoblasts (Ono *et al.*, 2008) and the repair of articular cartilage and subchondral bone in a cartilage damaged model (Sawada *et al.*, 2014). However, these beneficial effects on the joint have not been studied in humans. In this study, we investigated the effect of orally administered AD extract (EADE) on the subjects, who experienced the knee discomfort associated with physical activity but clinically did not need the treatment. Dietary supplement containing EADE was administered to the subjects, and the effect on the knee joint was evaluated using OA scores (the Japanese Knee Osteoarthritis Measure, JKOM and the Japan Orthopedic Association criteria, JOA). In addition, type II collagen degradation (Degradation products of C-terminal telopeptides of type II collagen: CTX-II and C-terminus of the piece of type II collagen cleavage product: C2C) and synthesis (Procollagen II C-Terminal propeptide: PIICP) markers and matrix metalloproteinase (MMP)-13 were analyzed to evaluate the effects of EADE on cartilage metabolism. Moreover, to make the effect of EADE more clear, subgroup analyses of the subjects with less knee discomfort (Kellgren–Lawrence grades of 0–I and >75 points of JOA score) were performed.

2. Materials and Methods

2.1. Study Design

A prospective, randomized, double-blind, placebo-controlled and parallel-group comparative study was designed to assess the efficacy and safety of a diet supplemented with EADE. The study was conducted from November 2014 to June

2015 and involved one medical clinical service organization center under the control of one medical investigator in Japan. The protocol was submitted to and approved by the institutional ethics committee in October 2014 (20141030-2), and the study was conducted in accordance with the Declaration of Helsinki and Ethical Guidelines for Epidemiological Research (recognized by the Japanese Government in 2008). The objective of the study was explained to all the subjects and written informed consent was provided prior to enrollment in the study.

2.2. Subjects

Male and female Japanese subjects aged 40–80 years with knee discomfort associated with physical activity but without radiographic evidence of knee osteoarthritis (Kellgren and Lawrence (K/L) grades 0–II, mainly 0–I) were enrolled; K/L classification of 0 means ‘definite absence of X-ray changes’ and I means ‘doubtful absence of X-ray changes’ (Kellgren *et al.*, 1957). The major exclusion criteria were the presence of gout or rheumatoid arthritis that may cause joint pain; suffering from any other injuries that may have required the use of anti-inflammatory or other medications or physiotherapy treatment by an orthopedist; previous surgical treatment of knee joints; routine use of dietary supplements or medicines containing EADE, hyaluronic acid, glucosamine, chondroitin sulfate, collagen peptides, or rich in calcium; need to undergo pharmacological treatments during the study period; performing hard exercise; history of osseous or articular diseases within the past year; diagnosis as having malignant tumor, hypertension, heart disease, kidney disease, thyroid disorder or other serious illness that may have required other physiotherapy treatment; pregnancy; nursing mothers or women of childbearing

potential; participation in another clinical study; and presence of any medical condition judged by the medical investigator to preclude the subject's inclusion in the study.

2.3. Study Interventions

The study diet was manufactured in the form of a 1.5 g powder. The ingredients were EADE (>1% 20-hydroxyecdysone), dextrin, hydroxypropyl methylcellulose, cellulose, calcium stearate, and palatinose. EADE was purchased from Matsuura Yakugyo Company. The amount of EADE was 10 mg in the 1.5 g EADE diet. The placebo diet comprised crystalline cellulose, dextrin and caramel pigment instead of EADE. The study supplement was wrapped in a single pack (1.5 g) and provided with a wafer, and the subject took the supplement after breakfast once a day with two cups of water for 12 weeks.

2.4. Randomization and Blinding

Because the symptoms, especially pain, vary according to sex in arthritis (Muraki *et al.*, 2009), research coordinators created an allocation table for males and females, randomly assigned eligible subjects and granted allocation numbers to test diet. The allocation table was sealed until the end of the study. All of the research staff and subjects were blinded to the treatment allocation during the test period. Only the statistics experts and data monitoring committee were unblinded, but they had no contact with the subjects. After the study, the subjects' data were fixed, and the allocation table was made available for reviewing the study information.

2.5. Evaluation of Efficacy

The outcomes for the evaluation of efficacy were based on the changes in the subscale scores of the Japan Orthopedic Association criteria for osteoarthritic knees (JOA) (Koshino *et al.*, 1988); the changes in the subscale scores of the Japanese Knee Osteoarthritis Measure (JKOM) (Akai *et al.*, 2005); the levels of urinary CTX-II and serum C2C as type II collagen degradation biomarkers (Reijman *et al.*, 2004, Poole *et al.*, 2004); the levels of serum PIICP as a type II collagen synthesis biomarker (Nelson *et al.*, 1998), the levels of serum MMP-13 (Wang *et al.*, 2014), the ratio of CTX-II/PIICP, and the ratio of C2C/PIICP. These parameters were measured at the baseline and 4, 8, and 12 weeks. Serum and urine samples were stored at -80°C until measurement. After the end of study period, CTX-II, C2C, PIICP, and MMP-13 were measured on the same day using the respective ELISA assay kits; CTX-II, Urine CartiLaps® EIA, Immunodiagnostic Systems Ltd, Tyne&Wear, United Kingdom.; C2C, Collagen Type II Cleavage ELISA, IBEX Pharmaceuticals Inc, Quebec, Canada; PIICP, Enzyme-linked Immunosorbent Assay Kit For Procollagen II C-Terminal Propeptide, Uscn Life Science Inc, Houston, USA.; MMP-13, and Enzyme-linked Immunosorbent Assay Kit For Matrix Metalloproteinase 13, Uscn Life Science Inc, Houston, USA. Serum C2C and urinary CTX-II were used as markers for type II collagen degradation, and serum PIICP was used as a marker for type II collagen synthesis. The ratios of C2C/PIICP and CTX-II/PIICP were also analyzed to assess the changes in the balance of type II collagen degradation and synthesis (cartilage metabolism). The concentrations of CTX-II were corrected for urinary creatinine (Cre) and expressed as ng/mmol/Cre. Serum MMP-13 was also used as a marker for cartilage degradation, because MMP-13 is a collagenase that

contributes to cartilage degradation by cleaving type II collagen triple helix (Poole *et al.*, 2003).

The JOA is the criteria established by the Japanese Orthopedic Association (Akai *et al.*, 2005), which is used by physicians for subjectively evaluating the treatment, based on the following subcategories; I) Pain/walking function; II) Pain/step-up and -down function; III) Joint flexion/stiffness; IV) Swelling; and V) Aggregated total symptoms. The first four subscales are rated from 0 to 30, from 0 to 25, from 0 to 35, and from 0 to 10, respectively. The maximum value indicates no symptoms or functional disability, and a score of 0 indicates a condition resulting in extreme difficulty to perform daily living tasks. The sum of the scores of these four subscales represents the score of the fifth subscale (Total symptoms score).

The JKOM is a self-answered, evaluation questionnaire that includes five subcategories: I) Pain was evaluated by a visual analog scale (VAS); II) Pain and stiffness during the past few days (8 questions); III) Activities of daily living during the past few days (10 questions); IV) General activities during the past month (5 questions); and V) General health conditions during the past month (2 questions). VAS values range from 0 (no pain) to 100 (pain that cannot be tolerated). The responses to each question (II-V subcategories) are assigned 1 to 5 points, with 1 point indicating good functional status and 5 points indicating the worst functional status. The JKOM score is higher in subjects with more pain and physical disability, and this evaluation modality is reported to be sufficiently reliable and valid for studying the clinical outcomes of knee OA (Braham *et al.*, 2003). The outcome of JKOM has been shown to be closely correlated with that of other arthritis-related scales, such as the Western Ontario and McMaster Universities Arthritis Index

(WOMAC) and the Medical Outcomes Study 36-Item Short-Form Health Survey (SF-36) (Braham *et al.*, 2003).

2.6. Evaluation of Safety

Tolerability and safety were evaluated on the basis of the incidence and severity of dietary supplement-related adverse events reported throughout the study and by changes in physical parameters and laboratory test variables, including hematology, blood biochemistry, and urinalysis. Each outcome was assessed at 4, 8, and 12 weeks from the start of administration. In addition, throughout the intervention period, the change in physical condition and joint, and use of pharmaceutical products were indicated in a diary.

2.7. Sample Size and Statistical Analysis

We estimated that a total of about 50 subjects are necessary for accurately assessing the efficacy and safety, based on the previous study, in which a dietary supplement was administered to the individuals with knee discomfort but without need of medication (Nagaoka *et al.*, 2013). To obtain enough subjects for analysis, 123 subjects were screened, and 48 subjects without any of the exclusion criteria were selected, and randomized to the EADE diet group and placebo group, and evaluated. Finally, 22 subjects in the EADE group and 22 subjects in the placebo group were analyzed (Figure 1). Moreover, to further make the effect of test supplement more clearly, we focused on the subjects with mild knee discomfort. Thus, the subjects with K/L grades of II and ≤ 75 points of JOA score were excluded, and the subjects (18 subjects in the EADE group and 14 subjects in the placebo group)

with K/L grades of 0–I and >75 points of JOA score were evaluated. All data were expressed as the mean \pm standard deviation unless otherwise specified. The baseline characteristics of the entire subject population were compared between the two groups (EADE diet and placebo) by using Student's t-test for continuous variables and the Mann-Whitney U-test for categorical variables. Changes in the JOA and JKOM scores, and biomarker levels during the intervention were compared with the baseline values by using Student's t-test (for quantitative variables) and the Wilcoxon signed-rank test (for qualitative variables). Comparison between the two groups was performed by Student's t-test (for quantitative variables). p-values < 0.1 for comparisons between the two groups and p-values < 0.05 for comparisons with the baseline values were considered statistically different based on the previous study (Watanabe *et al.*, 2007). Microsoft Excel was used for statistical analysis using the t-test, and IBM SPSS statistics 21 was used for other tests.

3. Results

3.1. Study Population

A flow chart of subjects in this study is shown in Figure 1. The 123 candidates were screened based on the K/L grades and the JOA score by orthopedists. In addition, a lifestyle questionnaire was administered and body measurements (height, body weight, and calculation of the body mass index), physical examinations (blood pressure and pulse measurement), JKOM assessments, and clinical examinations were performed. Moreover, the levels of uric acid and rheumatoid factor were measured. The 48 candidates without any of the exclusion criteria were finally selected and randomized to the EADE diet group and placebo group. This section

may be divided by subheadings. It should provide a concise and precise description of the experimental results, their interpretation as well as the experimental conclusions that can be drawn.

3.2. Evaluation of the efficacy in the study population

3.2.1. Baseline Characteristics

Table 1 shows the baseline characteristics of the study population in the EADE diet ($n = 22$) and placebo ($n = 22$) groups. There were no significant differences in any of the baseline characteristics between the EADE diet and placebo groups. The baseline characteristics included demographic characteristics (age, and distribution of male and female subjects), physiological characteristics (body height, body weight, body mass index, systolic blood pressure, diastolic blood pressure, and pulse rate), distribution of K/L grades, aggregated JOA scores, VAS and total scores of JKOM, and the levels of biomarkers for type II collagen metabolism (CTX-II, C2C, PIICP, and MMP-13).

3.2.2. Assessment Based on the JOA Subscale Scores

Table 2 shows the changes in the individual subscale and aggregate scores of the JOA criteria in the EADE diet and placebo groups during the 12-week intervention. Both in the EADE diet and placebo groups, three subscale scores, i.e., I) Pain/walking function; II) Pain/step-up and -down function; and V) Aggregated total symptoms, were similarly increased (improved) relative to the baseline values during the 12-week intervention ($p < 0.05$ at 12 weeks). Notably, the subscale III) Joint flexion/stiffness was significantly increased in the EADE diet group at 8 and 12 weeks

of the intervention compared with the placebo group ($p < 0.1$).

Figure 2 shows the change of subscale III. The baseline scores (0 week) were not different between the two groups; however, the score significantly increased in the EADE diet group compared with the placebo group, suggesting that EADE containing diet improves the joint flexion and stiffness.

3.2.3. Assessment Based on the JKOM Subscale Scores

Table 3 shows the changes in the five subscale scores of the JKOM responses during the 12-week intervention. In the subscale scores (I–V) and total score, there is no significant difference between the EADE diet and placebo groups at the baseline. Of note, in the EADE diet group, I (Pain evaluated by a VAS) and V (General health conditions) subscale scores were significantly decreased at 4, 8, and 12 weeks relative to the baseline ($p < 0.05$). Moreover, in the EADE diet group, II (Pain and stiffness during the past few days), III (Activities of daily living during the past few days), IV (General activities during the past month) and total score were significantly decreased at 8 and 12 weeks compared with the baseline ($p < 0.05$). In the placebo group, the subscales (I, II, and III) and total score were similarly decreased relative to the baseline; however, subscales IV and V were not changed.

In the changes of percentage from the baseline, II and III subscales were significantly decreased in the placebo group relative to the EADE diet group at 4 weeks after the intervention; however, I, III, IV, and V subscales and total score were more decreased in the EADE diet group compared with the placebo group at 8 or 12 weeks after the intervention. Especially, the subscales of III, IV, and V were more decreased in the EADE diet group at 12 weeks than the placebo group; $-15.5 \pm$

19.8% in the EADE diet group vs. $-8.9 \pm 56.4\%$ in the placebo group for subscale III; $-6.8 \pm 20.0\%$ in the EADE diet group vs. $-0.3 \pm 31.7\%$ in the placebo group for subscale IV; $-13.6 \pm 28.9\%$ in the EADE diet group vs. $-3.9 \pm 57.5\%$ in the placebo group for subscale V.

3.2.4. Assessment Based on Type II Collagen Metabolism

To evaluate the effect of the EADE diet on type II collagen metabolism, urine and serum samples were used for measuring cartilage metabolism markers (CTX-II, C2C, and PIICP) and a collagenase (MMP-13), as shown in Table 4. CTX-II (a type II collagen degradation marker) was significantly increased relative to the baseline in both the EADE and placebo groups at 8 weeks, whereas C2C (another type II collagen degradation marker) was not significantly changed during the intervention period. PIICP (a type II collagen synthesis marker) was significantly increased in both the groups at 4, 8, and 12 weeks. Notably, PIICP was substantially increased at 8 weeks in the EADE diet group compared with the placebo group ($p < 0.1$) (Figure. 3A), suggesting that EADE containing diet may enhance the type II collagen synthesis. Moreover, MMP-13 was significantly reduced at 8 and 12 weeks in both the groups relative to the baseline (Figure. 3B).

To further determination of the balance of type II collagen metabolism (degradation and synthesis), we evaluated the ratio of CTX-II or C2C to PIICP. In the EADE diet group, the CTX-II/PIICP ratio was significantly reduced at 4 and 8 weeks, and C2C/PIICP ratio was significantly reduced at 4 week relative to the baseline ($p < 0.05$). In contrast, the C2C/PIICP ratio was not significantly changed in the placebo group, although the CTX-II/PIICP ratio was significantly reduced at 4, 8, and 12

weeks relative to the baseline in this group.

3.3. Evaluation of the efficacy by the subgroup analysis

3.3.1. Baseline Characteristics

To determine the EADE effect on the subjects with less knee discomfort, the subjects with lower K/L grades (0–I) and higher JOA score (>75) were selected, and the subgroup analysis was performed. The baseline data were shown in Table 5. The EADE diet and placebo groups contained 18 and 14 subjects, respectively. There were no significant differences in any of the baseline characteristics between the two groups.

3.3.2. Assessment Based on the JOA Subscale Score

The JOA subscales are shown in Table 6. In both the EADE diet and placebo groups, the subscales I and V were significantly increased at 4, 8, and 12 weeks, and the subscale II was significantly increased at 12 weeks relative to the baseline; however, there was no significant difference in these subscales between the two groups. Interestingly, the subscale III was slightly increased and reached to the maximum score (35 points) at 8 and 12 weeks in the EADE diet group, although the subscale was not essentially changed in the placebo group.

3.3.3. Assessment Based on the JKOM Subscale Scores

Table 7 shows the changes in the JKOM subscale scores during the 12-week intervention. In the EADE diet group, all subscale scores (I–V) and total score decreased relative to the baseline at 8 or 12 weeks ($p < 0.05$). Furthermore, the

subscales II and IV significantly decreased in the EADE diet group compared with the placebo group at 8 weeks ($p < 0.05$) (Fig. 4A and B). Similarly, total score substantially decreased in the EADE diet group compared with the placebo group at 8 weeks ($p < 0.1$) (Figure. 4C).

Moreover, in the changes of percentage from the baseline, the subscale scores (I, II, IV, and V) and total score more decreased in the EADE diet group than the placebo group at 8 weeks (Table 7). Importantly, the subscale IV was substantially decreased in the EADE diet group compared with the placebo group ($p < 0.1$).

3.5. Safety Assessment.

We evaluated the safety of 48 subjects allocated to the EADE diet and placebo groups. Eleven (45.8%) subjects in the placebo group ($n = 24$) and thirteen (54.2%) subjects in the EADE diet group reported adverse events. Major adverse events reported were respiratory symptoms (sore throat, cough, rhinorrhea, and/or fever) and joint pain (knee, hip, shoulder, or elbow). All adverse events were of mild intensity and judged by the medical investigator to be unrelated to the intervention.

Furthermore, among the physical measurement parameters (body weight and body mass index), physiological examinations (systolic and diastolic blood pressures, and pulse rate) and laboratory tests (urinalysis, hematology, and blood chemistry), body mass index and some parameters in hematology and blood chemistry indicated the significant changes during the intervention in a few subjects; however, these changes were within the reference values.

Based on these findings, we concluded that the EADE-containing food does not induce adverse events.

4. Discussion

In this randomized, double-blind, placebo-controlled clinical trial, we evaluated the effects of oral administration of EADE in subjects with knee discomfort associated with physical activity. The effectiveness of EADE was assessed mainly on the basis of the JOA and JKOM scores. In EADE diet group, the scores of JOA criteria were mostly improved from the baseline values in both groups (Table 2). Notably, the subscale III (Joint flexion/stiffness) was substantially improved in the EADE diet group compared with the placebo group at weeks 8 and 12 after the intervention ($p < 0.1$) (Figure 2). Interestingly, it has been revealed that EADE extract promotes the repair of articular cartilage in a rabbit cartilage damaged model (Sawada *et al.*, 2014). Moreover, the cartilage defect was reported to correlate with the disturbance of knee extension during the progression of OA (Watanabe *et al.*, 2010, Nozaki *et al.*, 1994). Thus, it was considered that the improvement of joint flexion/stiffness may be due to the potential of EADE to repair the injured cartilage of knee joint. In contrast, the subscales ‘Pain/walking function’ and ‘Pain/step-up and down function’ of the JOA criteria increased (improved) in not only the EADE group but also the placebo group during the intervention, and there was no significant difference between the two groups. The unexpectedly high scores in the placebo group may be due to the characteristics of these pain-associated subscales, which depend on the self-report and subject-based measurements. The subscale ‘Swelling’ did not change during the intervention in both the EADE and placebo groups. This can be explained by the fact that none of subjects participating in this study had swelling in any knee joints, as evidenced by the high scores (full score of 10 points) at the baseline and during the

intervention.

In the assessment with the JKOM score, the percentages of change from the baseline were more (1.5 - 20-fold) decreased (improved) in the EADE diet group compared with the placebo group at 12 weeks in the subscales III (Condition in daily life), IV (General activities), and V (Health conditions). The questioner of III is regarding the ability to perform dairy routines during the last few days; such as 'How difficult is ascending or descending stairs?', 'How long can you walk on a flat surface without taking a rest?'. The questioner of IV is regarding the general activities during the last one month; such as 'Have you gone to an event or to department store during the last one month?', 'Were things that you usually do (some kind of lesson, meeting friends, etc.) difficult because of knee pain during the last one month?'. The questioner of V is regarding the health during the last month; such as 'Do you think your health during the last one month is average?', 'Do you think that knee pain has been affecting your health badly during the last one month?'. Thus, our results suggested that EADE would improve the ability to perform dairy routines, general activities and health conditions, which are associated with dairy or general life (including QOL).

Furthermore, to make the effect of EADE more clearly, the subjects with minimal cartilage damage (K/L 0–I and > 75 points of JOA criteria) were selected and analyzed. Importantly, the magnetic resonance imaging classification demonstrated that the irregularity and thinning but not defect were observed on the articular surface of the knee joint in subjects with >75 points of JOA criteria (Garnero *et al.*, 2003). The results indicated that the subscales II (Pain and stiffness in knees) and IV (General activities) of the JKOM were significantly improved in the EADE diet

group at 8 weeks compared with the placebo group ($p < 0.05$) (Table 7). In addition, the total score of JKOM was substantially improved at 8 weeks in the EADE diet group compared with the placebo group ($p < 0.1$) and continued to decrease until 12 weeks in the EADE diet group (Figure 4). Since EADE improved both the subscale II (Pain and stiffness in knees) of JKOM (Table 7) and the subscale II (Flexion and stiffness) of JOA (Table 2), the EADE diet is expected to alleviate the stiffness of the subjects with physical activity-associated knee discomfort. In addition, the subgroup analysis confirmed that the EADE diet significantly improves the general activities (the subscale IV of JKOM).

There is accumulating evidence that biomarkers for cartilage metabolism, particularly CII metabolism, can be used for not only evaluating the pathogenesis of joint destruction but also monitoring the structure-modifying agents or therapies (Christgau *et al.*, 2004). CII degradation biomarkers such as CTX-II, C1, 2C and C2C, were used to estimate the efficacy of chondroprotective agents, i.e., glucosamine (Cibere *et al.*, 2005, Mazieres *et al.*, 2007) and chondroitin sulfate (Yoshimura *et al.*, 2009). Moreover, CII synthesis biomarkers such as CPII (PIICP) have been used alone or in combination with CII degradation biomarkers (e.g., CTX-II and C2C) for the assessment of chondroprotective agents, i.e., glucosamine (Momomura *et al.*, 2013, Nagaoka 2014). In this study, we utilized the cartilage metabolism markers (CTX-II and C2C as type II collagen degradation markers, PIICP as a type II collagen synthesis marker, and MMP-13 as a major collagen-degrading enzyme). In addition, the ratios of the synthesis and degradation of type II collagen (C2C/PIICP and CTX-II/PIICP) were analyzed. In the EADE diet group, the levels of PIICP increased substantially at week 8 relative to the placebo group ($p < 0.1$). EADE has been

reported to promote the repair of damaged cartilage (Sawada *et al.*, 2014) and enhances the synthesis of collagen in false aged model rats (Matsuura *et al.*, 2003). Based on these findings, it was likely that EADE would enhance the synthesis of collagen, especially type II collagen, in the subjects involved in this study, as evidenced by the increase of PIICP (a type II collagen synthesis marker). Furthermore, the levels of MMP-13 were significantly reduced in both groups, but the levels of MMP-13 were more reduced at 4, 8, and 12 weeks in the EADE diet group compared with the placebo group. It has been reported that MMP-13 can be induced by inflammatory cytokines (Braham *et al.*, 2003), whereas EADE exhibits an anti-inflammatory action on an arthritis model (Ono *et al.*, 2008). Thus, the anti-inflammatory action of EADE might be involved in the suppression of MMP-13 level. In conclusion, these observations suggested that the chondroprotective and anti-inflammatory actions of EADE might contribute to improve the knee joint function (as evidenced by the subscale III of JOA, subscale II of JKOM), and general activities and QOL (as evidenced by subscale IV and total score of JKOM) in the subjects with mild knee discomfort.

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Figure and Tables

Table 1. The baseline characteristics of the study population in the *Ajuga decumbens* extract diet and placebo groups.

Variables	EADE diet (n = 22)	Placebo (n = 22)	p
Age (years)	51.8 ± 7.3	53.5 ± 8.6	0.488
Male : Female (no. of participants)	9 : 13	10 : 12	1.000
Height (cm)	165.0 ± 9.2	161.0 ± 8.4	0.135
Body weight (kg)	62.6 ± 9.1	60.3 ± 10.1	0.426
Body mass index (kg/m ²)	23.0 ± 2.6	23.2 ± 3.2	0.747
Body fat mass (%)	26.4 ± 9.2	27.0 ± 7.25	0.787
Systolic blood pressure (mmHg)	113.6 ± 11.3	116.9 ± 18.1	0.475
Diastolic blood pressure (mmHg)	72.8 ± 7.6	75.5 ± 11.7	0.371
Pulse rate (beats/min)	68.0 ± 7.2	71.5 ± 8.5	0.148
Kellgren–Lawrence grades 0 : I : II (no. of participants)	13 : 6 : 3	10 : 6 : 6	0.528
Aggregate scores of the JOA criteria	84.8 ± 8.5	81.8 ± 9.1	0.235
JKOM (I) VAS score (mm)	56.4±16.8	54.1 ± 14.8	0.636
JKOM (II)–(V) total score (points)	47.8 ± 16.7	46.5 ± 11.4	0.776
CTX-II (ng/mmol Cre)	170.4 ± 106.2	192.8 ± 100.4	0.488
C2C (ng/mL)	292.9 ± 42.9	290.9 ± 34.0	0.235
PIICP (ng/mL)	9.3 ± 2.8	9.7 ± 3.5	0.636
MMP-13 (ng/mL)	9.6 ± 4.0	8.7 ± 4.3	0.424

All values are expressed as mean ± SD except for Male : Female and Kellgren-Lawrence grades. There were no statistically significant differences between the EADE diet and placebo groups. EADE: *Ajuga decumbens* extract, JKOM: Japanese Knee Osteoarthritis Measure, VAS: Visual Analogue Scale, JOA: Japan Orthopedic

Association criteria, CTX-II: Degradation products of C-terminal telopeptides of type II collagen, C2C: C-terminus of the piece of type II collagen cleavage product, PIICP: Procollagen II C-Terminal propeptide, MMP-13: Matrix metalloproteinase-13

Table 2. Changes in the individual scores and the aggregate scores of Japan Orthopedic Association criteria during the intervention period in the *Ajuga decumbens* extract diet and placebo groups.

Variables	Group	Baseline	4 weeks	8 weeks	12 weeks
I. Pain/walking function	EADE diet	21.4 ± 5.2	25.2 ± 4.5 ^b	25.5 ± 4.3 ^b	26.4 ± 3.8 ^b
	Placebo	20.5 ± 5.3	25.7 ± 4.4 ^b	25.9 ± 2.9 ^b	28.0 ± 3.0 ^b
II. Pain/step-up and –down function	EADE diet	20.0 ± 3.5	20.5 ± 3.4	20.9 ± 3.7	22.5 ± 3.0 ^b
	Placebo	19.1 ± 2.9	21.1 ± 2.1 ^a	21.1 ± 2.1 ^a	21.8 ± 2.9 ^a
III. Joint flexion/stiffness	EADE diet	33.4 ± 3.2	33.6 ± 3.2	34.5 ± 2.1 [†]	34.5 ± 2.1 [†]
	Placebo	32.7 ± 3.4	33.4 ± 2.4	33.6 ± 2.3	33.6 ± 2.3
IV. Swelling	EADE diet	10.0 ± 0.0	10.0 ± 0.0	10.0 ± 0.0	10.0 ± 0.0
	Placebo	9.5 ± 1.5	10.0 ± 0.0	10.0 ± 0.0	9.8 ± 1.1
V. Aggregate total symptoms (point)	EADE diet	84.8 ± 8.5	89.3 ± 8.2 ^b	90.9 ± 8.5 ^b	93.4 ± 7.3 ^b
	Placebo	81.8 ± 9.1	90.2 ± 6.3 ^b	90.7 ± 5.8 ^b	93.2 ± 6.5 ^b

All values are expressed as mean ± SD. Changes in the scores during the intervention were compared with the baseline by the Wilcoxon's signed rank test. a: p < 0.05, b: p < 0.01 The Mann-Whitney U-test was used to evaluate the group difference. †: p < 0.1. EADE: *Ajuga decumbens* extract

Table 3. Changes in the individual scores and total scores of Japanese Knee Osteoarthritis Measure response during the intervention period in the *Ajuga decumbens* extract diet and placebo groups.

Variables	Group	Baseline	4 weeks	8 weeks	12 weeks
I : VAS	EADE diet	56.4 ± 16.8	35.5 ± 24.0 ^b (-36.9 ± 38.3)	25.5 ± 21.2 ^b (-53.5 ± 36.8)	19.5 ± 18.5 ^b (-64.9 ± 31.8 ^b)
	Placebo	54.1 ± 14.8	26.2 ± 19.2 ^b (-51.2 ± 33.1)	25.1 ± 18.1 ^b (-53.0 ± 30.8)	18.3 ± 23.0 ^b (-62.5 ± 47.6 ^b)
II : Pain and stiffness in the knees	EADE diet	17.5 ± 5.9	16.1 ± 6.8 (-8.1 ± 18.0*)	14.0 ± 6.5 ^b (-20.3 ± 18.0)	13.0 ± 6.3 ^b (-26.5 ± 16.9)
	Placebo	18.3 ± 4.6	13.6 ± 3.4 ^b (-22.8 ± 22.2)	13.7 ± 3.8 ^b (-21.6 ± 26.0)	13.1 ± 6.2 ^b (-26.9 ± 31.2)
III : Condition in daily life	EADE diet	16.4 ± 6.7	16.1 ± 7.8 (-1.5 ± 19.0**)	14.1 ± 6.2 ^a (-11.9 ± 17.2)	13.3 ± 6.0 ^b (-15.5 ± 19.8)
	Placebo	15.6 ± 4.5	12.5 ± 2.8 ^b (-15.8 ± 25.8)	12.5 ± 3.1 ^b (-17.3 ± 18.1)	13.2 ± 6.0 ^b (-8.9 ± 56.4)
IV : General activities	EADE diet	9.7 ± 3.5	9.3 ± 3.9 (-3.5 ± 21.2)	8.5 ± 3.7 ^a (-11.6 ± 22.9)	8.9 ± 3.4 (-6.8 ± 20.0)
	Placebo	8.7 ± 2.3	8.2 ± 1.8 (-1.7 ± 23.1)	8.2 ± 1.7 (-0.9 ± 26.9)	8.1 ± 1.4 (-0.3 ± 31.7)
V : Health conditions	EADE diet	4.2 ± 1.8	3.5 ± 1.3 ^a (-10.9 ± 27.0)	3.2 ± 1.1 ^b (-16.6 ± 29.1)	3.4 ± 1.4 ^b (-13.6 ± 28.9)
	Placebo	4.0 ± 1.7	3.5 ± 1.2 (0.9 ± 49.5)	3.4 ± 1.1 (-5.1 ± 37.0)	3.3 ± 1.3 (-3.9 ± 57.5)
Total Score	EADE diet	47.8 ± 16.7	45.0 ± 18.1 (-5.9 ± 14.4**)	39.8 ± 16.0 ^b (-15.8 ± 15.9)	38.6 ± 16.0 ^b (-18.2 ± 15.7)
	Placebo	46.5 ± 11.4	37.8 ± 7.5 ^b (-16.4 ± 17.6)	37.8 ± 7.6 ^b (-16.2 ± 17.7)	37.7 ± 12.8 ^b (-15.8 ± 31.4)

All values are expressed as mean ± SD. The scores in the parentheses show the percentage changes from the baseline. Changes in the scores during the intervention

were compared with the baseline by the Wilcoxon's signed rank test. a: $p < 0.05$, b: $p < 0.01$. The Mann-Whitney U-test was used to evaluate the group difference. *: $p < 0.05$, **: $p < 0.01$. VAS: Visual Analogue Scale, EADE: *Ajuga decumbens* extract

Table 4. Changes in the levels of biomarkers for Type II collagen metabolism and the ratio of Type II collagen destruction to synthesis during intervention period in the *Ajuga decumbens* extract diet and placebo groups.

Variables	Group	Baseline	4 weeks	8 weeks	12 weeks
CTX-II (ng/mmol Cre)	EADE diet	170.4 ± 106.2	156.2 ± 59.2	222.8 ± 107.1 ^a	203.8 ± 85.8
	Placebo	192.8 ± 100.4	180.6 ± 147.9	274.2 ± 172.6 ^b	236.0 ± 152.8
C2C (ng/mL)	EADE diet	292.8 ± 42.9	293.2 ± 39.1	286.9 ± 55.4	295.6 ± 38.1
	Placebo	290.9 ± 34.0	283.8 ± 30.5	283.8 ± 33.2	294.0 ± 46.4
PIICP (ng/mL)	EADE diet	9.3 ± 2.8	18.0 ± 9.5 ^b	26.0 ± 12.8 ^{†b}	11.4 ± 4.7 ^a
	Placebo	9.7 ± 3.5	15.6 ± 9.2 ^b	19.6 ± 9.1 ^b	12.5 ± 4.1 ^b
MMP-13 (ng/mL)	EADE diet	9.6 ± 4.0	9.6 ± 4.0	5.9 ± 3.6 ^b	5.6 ± 2.9 ^b
	Placebo	8.7 ± 4.3	9.2 ± 4.2	5.5 ± 3.4 ^b	5.1 ± 3.3 ^b
CTX-II/ PIICP	EADE diet	37.2 ± 26.2	20.9 ± 10.4 ^a	14.6 ± 9.5 ^b	29.3 ± 10.5
	Placebo	38.2 ± 28.5	24.4 ± 12.1 ^a	17.8 ± 8.3 ^b	26.2 ± 9.2 ^a
C2C/ PIICP	EADE diet	23.0 ± 24.1	11.4 ± 7.6 ^a	12.6 ± 15.4	19.8 ± 9.4
	Placebo	29.5 ± 37.1	17.9 ± 18.7	17.8 ± 16.1	21.1 ± 15.1

All values are expressed as mean ± SD. Changes in the values during the intervention were compared with the baseline by the paired t-test. a: p < 0.05, b: p < 0.01. The unpaired t-test was used to evaluate between the group difference. †: p < 0.1. EADE: *Ajuga decumbens* extract, CTX-II: Degradation products of C-terminal telopeptides of type II collagen, C2C: C-terminus of the piece of type II collagen cleavage product, PIICP: Procollagen II C-Terminal propeptide, MMP-13: Matrix metalloproteinase-13

Table 5. Baseline characteristics of the subgroup participants in the *Ajuga decumbens* extract diet and placebo groups.

Variables	EADE diet (n = 18)	Placebo (n = 14)	p
Age (years)	51.4 ± 7.1	54.1 ± 7.2	0.311
Male : Female (no. of participants)	7 : 11	8 : 6	0.476
Height (cm)	164.5 ± 9.4	162.3 ± 8.7	0.511
Body weight (kg)	62.1 ± 8.5	59.4 ± 9.3	0.400
Body mass index (kg/m ²)	22.9 ± 2.6	22.5 ± 2.4	0.601
Body fat mass (%)	27.4 ± 7.9	22.3 ± 8.2	0.164
Systolic blood pressure (mmHg)	111.6 ± 11.3	116.0 ± 16.7	0.389
Diastolic blood pressure (mmHg)	71.5 ± 7.3	74.43 ± 8.2	0.295
Pulse rate (beats/min)	68.4 ± 7.5	70.5 ± 8.3	0.460
Kellgren–Lawrence grades 0 : I : II (no. of participants)	12 : 6 : 0	8 : 6 : 0	0.718
Aggregate scores of the JOA criteria	86.4 ± 7.0	85.0 ± 5.5	0.363
JKOM (I) VAS score (mm)	53.1 ± 15.2	59.0 ± 13.7	0.265
JKOM (II)–(V) total score (points)	42.1 ± 9.7	43.5 ± 9.7	0.554
CTX-II (ng/mmol Cre)	166.2 ± 110.8	169.6 ± 86.2	0.924
C2C (ng/mL)	286.2 ± 43.3	286.1 ± 26.9	0.998
PIICP (ng/mL)	9.8 ± 2.2	9.0 ± 3.8	0.463
MMP-13 (ng/mL)	9.5 ± 4.1	8.4 ± 4.5	0.463

All values are expressed as mean ± SD except for Male : Female and Kellgren-Lawrence grades. There were no statistically significant differences between the EADE diet and placebo groups. EADE: *Ajuga decumbens* extract, JKOM: Japanese Knee Osteoarthritis Measure, VAS: Visual Analogue Scale, JOA: Japan Orthopedic Association criteria, CTX-II: Degradation products of C-terminal telopeptides of type

II collagen, C2C: C-terminus of the piece of type II collagen cleavage product, PIICP:
Procollagen II C-Terminal propeptide, MMP-13: Matrix metalloproteinase-13

Table 6. Changes in the individual scores and the aggregate scores of Japan Orthopedic Association criteria during the intervention period in the *Ajuga decumbens* extract diet and placebo groups.

Variables	Group	Baseline	4 weeks	8 weeks	12 weeks
I. Pain/walking function	EADE diet	22.5 ± 4.6	26.1 ± 4.0 ^b	26.7 ± 3.4 ^b	27.5 ± 3.1 ^b
	Placebo	21.8 ± 4.6	25.4 ± 5.0 ^b	26.4 ± 2.3 ^b	28.9 ± 2.1 ^b
II. Pain/step-up and-down function	EADE diet	20.3 ± 3.6	20.8 ± 3.5	21.4 ± 3.8	23.1 ± 3.0 ^b
	Placebo	19.3 ± 2.7	20.7 ± 1.8	21.4 ± 2.3	22.1 ± 2.6 ^a
III. Joint flexion/stiffness	EADE diet	33.6 ± 2.9	33.9 ± 2.7	35.0 ± 0.0	35.0 ± 0.0
	Placebo	33.9 ± 2.1	33.9 ± 2.1	34.3 ± 1.8	34.3 ± 1.8
IV. Swelling	EADE diet	10.0 ± 0.0	10.0 ± 0.0	10.0 ± 0.0	10.0 ± 0.0
	Placebo	10.0 ± 0.0	10.0 ± 0.0	10.0 ± 0.0	10.0 ± 0.0
V. Aggregate total symptoms	EADE diet	86.4 ± 7.9	90.8 ± 6.7 ^b	93.1 ± 6.4 ^b	95.6 ± 5.7 ^b
	Placebo	85.0 ± 5.5	90.0 ± 6.5 ^b	92.1 ± 4.7 ^b	95.4 ± 5.0 ^b

All values are expressed as mean ± SD. Changes in the scores during the intervention were compared with the baseline by the Wilcoxon's signed rank test. a: p < 0.05, b: p < 0.01. EADE: *Ajuga decumbens* extract

Table 7. Changes in the individual scores and total scores of Japanese Knee Osteoarthritis Measure response during the intervention in the *Ajuga decumbens* extract diet and placebo groups.

Variables	Group	Baseline	4 weeks	8 weeks	12 weeks
I : VAS	EADE diet	53.1 ± 15.2	28.3 ± 19.0 ^b (-44.9 ± 35.5 ^b)	19.3 ± 17.8 ^b (-60.0 ± 37.4 ^b)	14.1 ± 13.7 ^b (-71.9 ± 28.1 ^b)
	Placebo	60.4 ± 12.8	31.5 ± 20.5 ^b (-47.7 ± 30.1 ^b)	25.1 ± 20.9 ^b (-57.4 ± 34.9 ^b)	13.2 ± 17.0 ^b (-74.7 ± 71.9 ^b)
II : Pain and stiffness in the knees	EADE diet	15.4 ± 3.8	13.7 ± 3.8 ^a (-9.7 ± 18.0 ^a)	11.6 ± 3.6 ^{a,b} (-23.4 ± 17.2 ^b)	10.4 ± 2.4 ^b (-30.7 ± 12.3 ^b)
	Placebo	17.1 ± 4.3	13.5 ± 2.9 ^b (-17.2 ± 24.5 ^b)	13.4 ± 3.2 ^a (-16.9 ± 29.5 ^a)	11.4 ± 4.1 ^a (-31.1 ± 21.9 ^b)
III : Condition in daily life	EADE diet	14.2 ± 4.3	13.6 ± 4.5 (-2.8 ± 19.7)	12.1 ± 3.4 ^b (-12.3 ± 15.4 ^b)	11.2 ± 1.5 ^b (-16.7 ± 16.7 ^b)
	Placebo	14.1 ± 3.7	12.6 ± 2.8 (-7.1 ± 27.7)	11.6 ± 2.1 ^a (-14.3 ± 19.5 ^a)	12.8 ± 6.8 (-2.5 ± 67.8)
IV : General activities	EADE diet	8.7 ± 1.6	8.2 ± 1.4 (-2.9 ± 21.1)	7.3 ± 1.3 ^a (-12.6 ± 24.2 ^{ta})	7.8 ± 1.2 (-7.0 ± 20.9)
	Placebo	8.6 ± 2.2	8.2 ± 1.8 (-2.2 ± 20.1)	8.6 ± 1.5 (4.0 ± 28.9)	8.1 ± 1.3 (-0.3 ± 31.0)
V : Health conditions	EADE diet	3.8 ± 1.5	3.3 ± 0.8 (-8.1 ± 26.5)	3.0 ± 0.8 ^a (-13.7 ± 31.3)	3.1 ± 0.8 ^a (-12.3 ± 31.3)
	Placebo	3.5 ± 1.3	3.6 ± 1.3 (10.4 ± 55.5)	3.4 ± 1.3 (0.5 ± 40.9)	3.3 ± 1.3 (3.3 ± 66.6)
Total Score	EADE diet	42.1 ± 9.7	38.8 ± 9.4 (-6.7 ± 15.2)	34.0 ± 7.2 ^{tb} (-17.2 ± 15.8 ^b)	32.5 ± 4.0 ^b (-20.3 ± 13.1 ^b)
	Placebo	43.8 ± 9.7	37.7 ± 7.2 ^a (-10.8 ± 17.8 ^a)	36.5 ± 5.7 ^a (-12.3 ± 18.6 ^a)	35.9 ± 11.9 ^a (-15.3 ± 31.4)

All values are expressed as mean ± SD. The score in the parentheses shows the percentage changes from the baseline. Changes in the scores during the intervention

were compared with the baseline by the Wilcoxon's signed rank test. a: $p < 0.05$, b: $p < 0.01$ The Mann-Whitney U-test was used to evaluate the group difference. †: $p < 0.1$, *: $p < 0.05$. VAS: Visual Analogue Scale, EADE: *Ajuga decumbens* extract

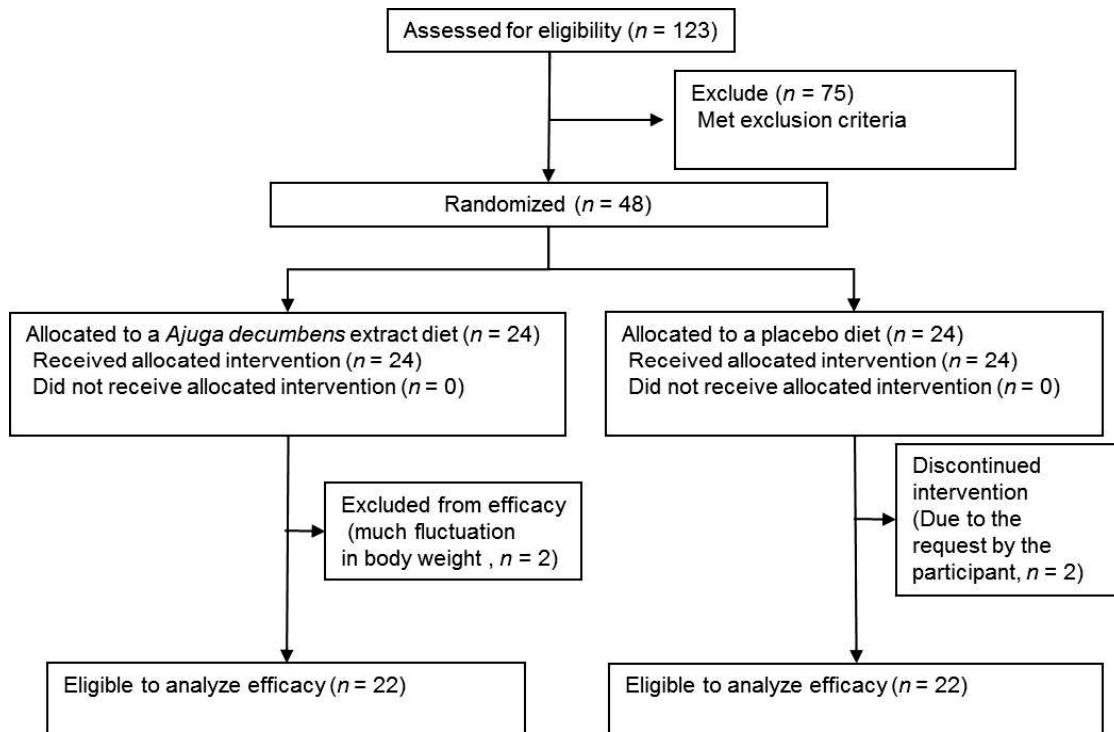


Figure 1. Flow diagram of the participants in this study.

123 subjects were screened, and 48 subjects without any of the exclusion criteria were selected, and randomized to the *Ajuga decumbens* extract (EADE) diet group and placebo group, and evaluated. 2 subjects were excluded from efficacy for much fluctuation in body weight in the EADE group and 2 subjects were discontinued intervention due to the request by the participant in the placebo group. 22 subjects in the EADE group and 22 subjects in the placebo group were analyzed to efficacy.

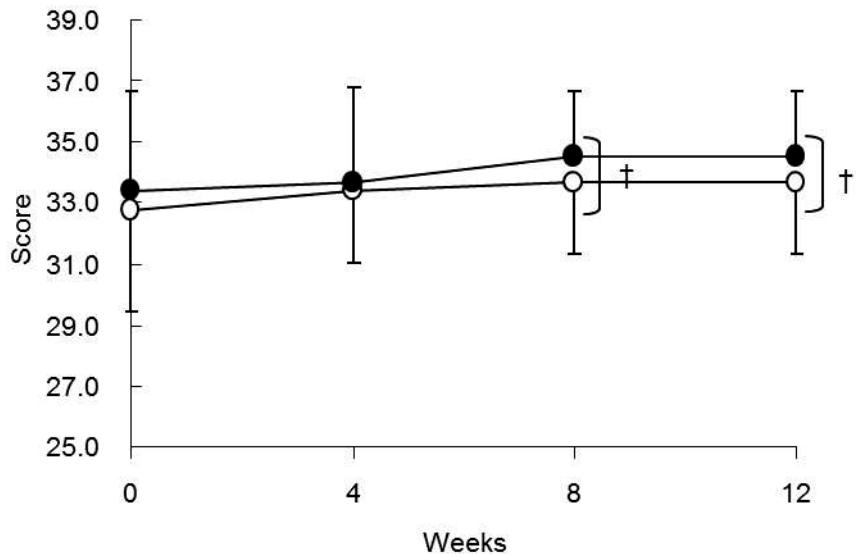


Figure 2. Changes in the subscale III of Japan Orthopedic Association criteria score.

The subscale III (Joint flexion/stiffness subscale) of the Japan Orthopedic Association criteria during the intervention in the *Ajuga decumbens* extract (EADE) (●) and placebo groups (○). Data are compared between the EADE and placebo groups. The Mann-Whitney U-test was used to evaluate the group difference. †: $p < 0.1$.

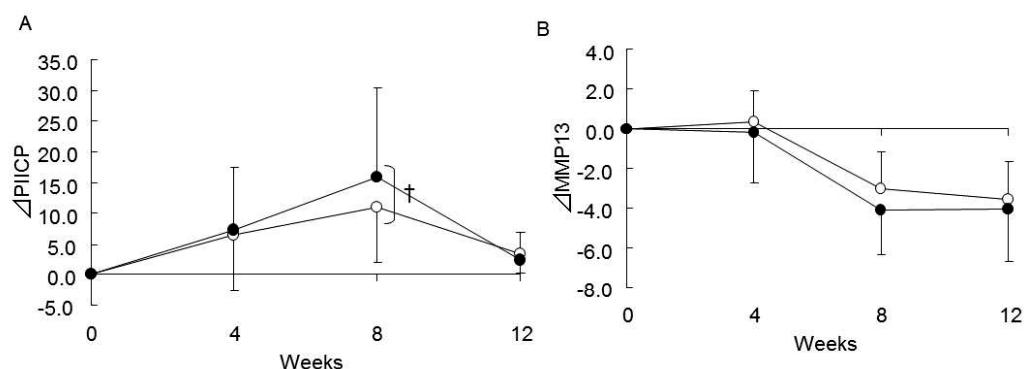


Figure 3. Changes in the levels of serum Procollagen II C-Terminal propeptide and Matrix metalloproteinase -13.

The values of Procollagen II C-Terminal propeptide (PIICP) (A) and serum Matrix metalloproteinase-13 (MMP-13) (B) from the baseline during the intervention in the *Ajuga decumbens* extract (EADE) (●) and placebo groups (○). Data are compared between the EADE and placebo groups. The unpaired t-test was used to evaluate between the group differences. †: p < 0.1.

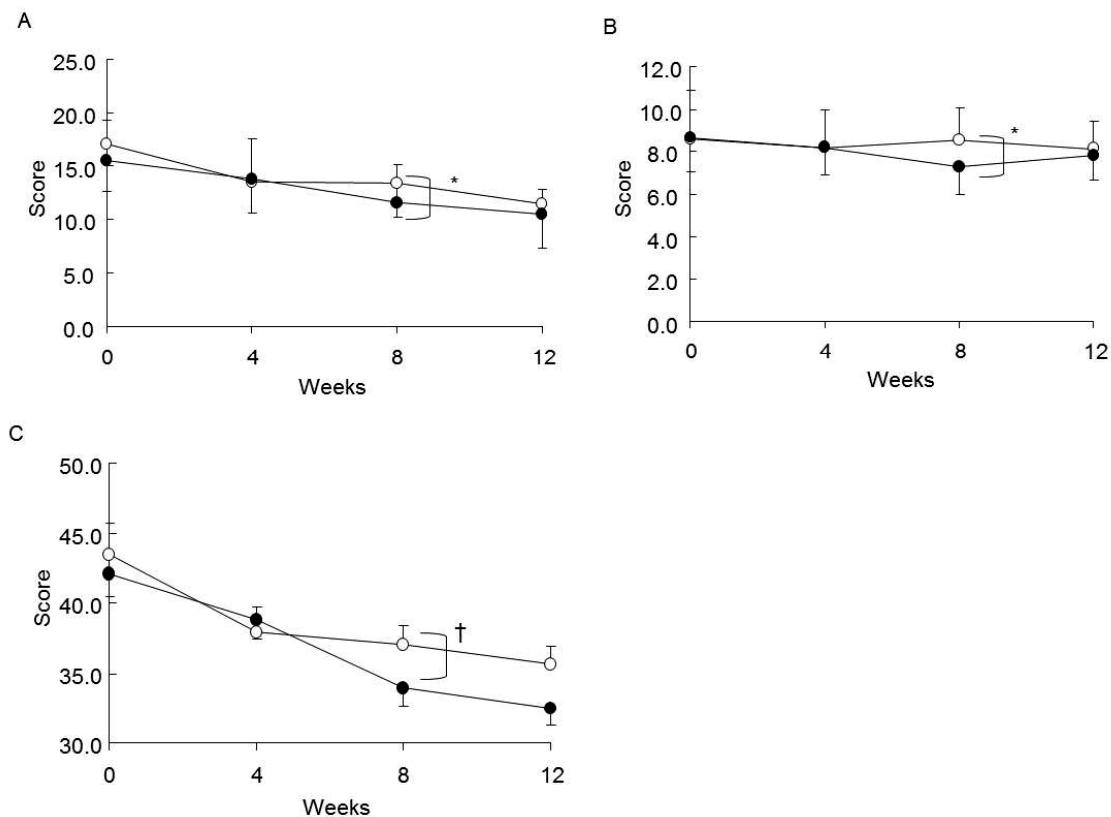


Figure 4. Changes in the subscales II and IV, and total score of Japanese Knee Osteoarthritis Measure response criteria.

The three subscale of Japanese Knee Osteoarthritis Measure response criteria II (pain and stiffness in the knees, A), IV (general activities, B) and Total score (C) during the intervention in the *Ajuga decumbens* extract (EADE) diet (●) and placebo groups (○). Data are compared between the EADE and placebo groups. The Mann-Whitney U-test was used to evaluate the group difference. †: $p < 0.1$, *: $p < 0.05$.

Conclusion

When joints are damaged by mechanical stress or age-related OA, the cartilaginous matrix decreases and bone degenerates. ADE and GlcN could improve OA associated with the degeneration of subchondral bone cartilaginous matrix and help regenerate the damaged cartilage in a synergistic fashion. BED had the similar effects on the regeneration of damaged subchondral bone and muscle atrophy to ADE. Therefore, we hypothesized that BED could be the main active component in ADE. In the present study, we evaluated the effect of EADE, which is a concentration of the effective fraction of ADE containing 20-hydroxyecdysone, on the acceleration of healing in an experimental model of cartilage injury. The cartilage matrix was strongly regenerated in the Low and High EADE groups, while the subchondral matrix was also regenerated in these groups. EADE stimulated PG production and induced *in vitro* chondrogenic differentiation of MSCs. Moreover, it significantly attenuated the stimulation of PGE₂ production by IL-1 β in chondrocytes. It was considered that EADE might not only have a chondroprotective effect but also influence bone metabolism and subchondral bone restoration.

Furthermore, we evaluated the efficacy and safety of the oral administration of EADE supplement to individuals with knee discomfort associated with physical activity. As a result, the administration of EADE-containing diet was confirmed to be safe and improved the scores concerning joint flexion and general activities in the subjects with mild knee discomfort. Notably, the management of knee discomfort was more effective at the early stage for maintaining knee function.

In conclusion, EADE could be a promising candidate as a functional food, which is useful for the joint health. EADE stimulated PG production and induced *in vitro*

Conclusion

chondrogenic differentiation of MSCs. Moreover, EADE significantly attenuated the stimulation of PGE2 production by IL-1 β in chondrocytes. The chondroprotective and anti-inflammatory actions EADE contribute to improve the knee joint function, and general activities and QOL in the subjects with mild knee discomfort.

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