Protective Effect of Deer Bone Oil on Cartilage Destruction in Rats with Monosodium Iodoacetate (MIA)-Induced Osteoarthritis

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The anti-osteoarthritic activity of the methanol fraction of deer bone oil extract (DBO-M) was evaluated in interleukin (IL)-1β-infamed primary rabbit chondrocytes and in rats with monosodium iodoacetate (MIA)-induced osteoarthritis. The active component in DBO-M was analyzed using a direct infusion liquid chromatography quadrupole (LCQ) ion-trap electrospray ionization (ESI)-mass spectrometer (MS). DBO-M significantly suppressed the IL-1β-induced sulfated-glycosaminoglycan (s-GAG) release from chondrocyte, and lowered mRNA levels of the collagen-degrading enzymes matrix metalloproteinase (MMP)-1 and MMP-3 in a dose-dependent manner. Upon treatment with high doses of DBO-M, the levels of IL-1β, tumor necrosis factor (TNF)-α, and IL-6 decreased by around 40%, 70%, and 50%, respectively, compared to the control in the serum of rats with MIA-induced osteoarthritis. Bone volume fraction (BV/TV) and trabecular thickness (Tb.Th) increased by over 40% in rats treated with DBO-M compared to the values reported for the MIA-treated control group, while trabecular separation (Tb.Sp) showed a significant decrease (ca. 38%), as confirmed through micro-computed tomography (CT) analysis of MIA-induced destruction of articular bones. Furthermore, direct infusion ESI-MS analysis showed that DBO-M contains gangliosides, which are glycosphingolipids with monosialic acid (GM3), as a major compound. Our results suggest that DBO-M effectively improves MIA-induced osteoarthritis by suppressing inflammatory responses, and that gangliosides could be one of the DBO-derived anti-inflammatory components.

Key words osteoarthritis (OA); deer bone oil; chondrocyte; monosodium iodoacetate (MIA); micro-computed tomography (Micro-CT); ganglioside

Osteoarthritis (OA) is a degenerative joint articular disorder, which is characterized by disruption of cartilage homeostasis, which is the balance between destruction and regeneration of the tissue. Such degeneration around connective tissue is known to be affected by various factors, including mechanical stress and biochemical changes. Articular cartilage serves as a cushion covering the ends of the femur and tibia, absorbing physical impact on the joint region. It wears out upon exposure to mechanical and chemical stress, resulting in serious deteriorative functional impairment. The pathophysiology of OA includes local inflammation induced mainly by pro-inflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α, which in turn leads to up-regulation of matrix metalloproteinases (MMPs) in the articular region.

OA-mediated clinical manifestations limit daily activities, such as walking and performing household chores. These may in turn lead to other disorders including depression, sleep disorder, disability, and deterioration in the quality of life (QOL). Treatment of OA has been focused on the partial relief of the symptoms and pain caused by OA-induced inflammation, without any certain remedy for this disease. Non-steroidal anti-inflammatory drugs (NSAIDs) have been recognized as the most common and important treatment option for the symptoms of OA. Although their analgesic and anti-inflammatory effects are efficacious in relieving pain and symptoms of OA, NSAIDs have various adverse effects, including gastroododenal ulceration, renal insufficiency, and prolonged bleeding.

Deer bone oil has been traditionally used to treat various illnesses in East Asia, including Korea, where it is called nok-gol. Recent studies have reported the biological activities of nok-gol extract, such as anti-aging, anti-inflammatory, anti-oxidant, and anti-amnesic activity. Deer bone extracts are known to contain constituents involved in cartilage protection, such as glucosamine, chondroitin sulfate, and collagen hydrolsate. However, active components for suppression of arthritis have not been identified in deer bone extract. Several previous studies reported the inhibitory effect of water extract of deer bone on cartilage damage and inflammatory cytokines in osteoarthritic rats. However, the beneficial effects of oil derived from deer bone have not been widely studied, although it is produced abundantly during the preparation of deer bone water extract.

Various types of oils from different sources have been proven to have therapeutic effects on osteoarthritic symptoms and on the inflammatory response. In particular, emu oil has been traditionally used as an anti-inflammatory agent by Australian aboriginals. Several studies have shown the therapeutic effect of emu oil in osteoarthritis. In recent years, we previously reported anti-inflammatory effect of deer bone oil extract (DBOE). However, anti-osteoarthritis of deer bone oil extract has not been confirmed, and its active component has not been analyzed. The current study describes the effect of DBOE on osteoarthritis in an animal model with analysis of the active component. To analyze the anti-osteoarthritis effect and active components of deer bone

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oil, methanol fractions of DBOE (DBO-M) were prepared via liquid–liquid fractionation. The effect of DBO-M on arthritis in monosodium iodoacetate (MIA)-induced OA rats was evaluated via topical application, and the active component was identified via electrospray ionization (ESI)-MS analysis. Our study suggests the potential use of deer bone oil as an anti-osteoarthritic agent.

MATERIALS AND METHODS

Materials

MIA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), collagenase from Clostridium histolyticum (crude), recombinant human IL-1β, dimethyl methylene blue (DMMB), guanidine hydroxychloride (GuHCl), chondroitin sulfate, protease K, sodium formate, formic acid, sodium acetate, acetic acid, dipotassium hydrogen phosphate, and eucalyptol were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Phenol was purchased from Junsei (Tokyo, Japan), and sulfuric acid was obtained from Showa (Tokyo, Japan). Solvents including hexane, methanol, acetone, chloroform, and ether were purchased from Daejung Chemical & Materials (Gyonggi-do, Korea). Fetal bovine serum (FBS), antibiotic antimycotic solution, penicillin streptomycin, and Dulbecco’s modified Eagle’s medium (DMEM) with high glucose were purchased from Welgene (Fresh Media, Daegu, Korea). Enzyme-linked immunosorbent assay (ELISA) kits for IL-1β, IL-6, and TNF-α were purchased from R&D System, Inc. (Minneapolis, MN, U.S.A.). Gangioside authentic GM3 (from canine blood) and GD3 (as ammonium salt from bovine milk) mixtures were obtained from Sigma-Aldrich. HPLC-grade water, methanol, and acetonitrile were obtained from J. T. Baker, and ammonium acetate and acetic acid used as additives for mobile phase preparation were purchased from Sigma-Aldrich. All other reagents were of the highest commercial grade available.

Preparation of DBOE and Solvent Fractionation by Liquid–Liquid Extraction

Deer bone (Cervus elaphus) from adult male New Zealand elks (aged 2 to 4 years) was obtained from Nongshim Co. (Seoul, Korea). The extraction of deer bone was executed as described by Lee et al.38 Oil separated from the deer bone extract was dehydrated by sodium sulfate and filtered with filter paper. DBO was fractionated using two immiscible solvents, hexane and methanol, with a ratio of 2:1 (v/v). DBO was first dissolved in hexane to 10% (w/v), and half the volume of methanol was added to the solution and vigorously shaken for 1 min in a separating funnel. The funnel was stillled until the two separate layers appeared. The DBO-M and hexane fraction of DBO (DBO-H) of each layer were separately collected, and solvents were eliminated with a high vacuum rotary evaporator. DBO-M fraction was used in this study.

Isolation and Culture of Chondrocytes

Chondrocytes were released from slices of articular cartilage that were taken from 8-week-old male white New Zealand rabbits by enzymatic digestion (collagenases).17 The pieces of cartilage were collected in Dulbecco’s modified Eagle’s medium with high glucose (DMEM; Welgene, Fresh Media, Daegu, Korea). The cartilage was sliced into pieces of 2 to 3 mm2 and placed in 40 mL culture medium with DMEM high glucose, 2% FBS, 1% antibiotic antimycotic solution, 1% penicillin streptomycin, and 0.2% collagenase in a 50 mL conical tube. The tube containing cartilage fragments was shaken and incubated in a shaker incubator at 50 rpm and 37°C for 3 to 4 h with the lid of the tube secured with parafilm. The suspension of digests was filtered through a 70-μm nylon cell strainer (BD Falcon, U.S.A.) in order to eliminate any aggregates or non-digested residues. Then, the suspension was centrifuged at 168×g for 10 min. The supernatant was removed and the pellet was gently resuspended with 40 mL of culture medium. Cells were centrifuged and washed twice in the conditions mentioned above. Then, the suspension was placed into cell culture flasks (75 cm2) at a concentration of 2.5×105 cells/mL.

Cell Culture and Treatment in Primary Chondrocytes

The isolated chondrocytes were cultured in DMEM containing 10% FBS, 1% antibiotic antimycotic solution, and 1% penicillin streptomycin. The cells were maintained in a humidified 95% air and 5% CO2 atmosphere at 37°C. The culture medium was changed 4 d after primary culture, while for secondary and subsequent cultures, it was changed every 3 d. Cells were trypsinized and split at a ratio of 1:4 when they reached 70–80% confluence.

Cell Viability Assay

The chondrocytes were seeded onto 24-well plates at a concentration of 2.5×104 cells/mL for MTT assay. The cells were pre-incubated for 48 h and then treated with DBO-M at various concentrations in the presence or absence of IL-1β (25 ng/mL). Control groups were treated with dimethyl sulfoxide (DMSO). After 3 d of treatment, media were removed and the cells were treated with 1 mg/mL MTT reagent for 4 h. Then, 20% sodium dodecyl sulfate (SDS) in acidic isopropl alcohol was added. After overnight incubation, the absorbance at 540 nm was detected.

Sulfated-Glycosaminoglycan (s-GAG) Assay

The chondrocytes were seeded and treated with substances as mentioned above in MTT assay. s-GAG assay was performed by a method of Barbosa et al.38 Cells and media were collected separately, and the cells were further enzymatically digested in a solution of 50 μg/mL protease K in 100 mM K2HPO4, pH 8.0 at 56°C overnight. Enzymatic reaction was inactivated by heating the preparation for 10 min at 90°C. After centrifugation (10000×g, 10 min, 4°C), the supernatant was collected as sample. One milliliter of DMMB solution and 100 μL of sample were mixed and vortexed vigorously for 30 min. The mixture was then centrifuged at 17000 rpm for 10 min at 4°C. After discarding the supernatant, the pellet was dissolved with 1 mL of DMMB decomplexation solution and sonicated for 30 min. Absorbance was measured at 656 nm. Calibration curves using chondroitin sulfate as standard were included into each assay to quantify s-GAG contents in the sample. s-GAG released into the culture media by degradation of proteoglycan was estimated and presented as percent ratio to the total amount of s-GAG from both cell lysates and culture media.

Values are presented as percent ratio of s-GAG released into media over total amount of s-GAG.

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\text{(% s-GAG Release)} = \frac{(\text{s-GAG in Media})}{(\text{s-GAG in Media} + \text{s-GAG from Cell Lysate})} \times 100
\]

ESI-MS/MS Analyses

Analyses of active substances in DBO-M were performed using a direct infusion LCQ ion-trap ESI-mass spectrometer (ThermoFinnigan, San Jose, CA, U.S.A.).
CA, U.S.A.). Stock solutions containing authentic GM3 and GD3 mixtures (each 100 µg/mL) were separately prepared in methanol, and were stored in an amber vial in a refrigerator at −20°C before being diluted for direct infusion ESI-MS/MS analyses. Two stock solutions of GD3 and GM3 were serially diluted with a mobile phase (acetonitrile–methanol–20 mM ammonium acetate–acetic acid, 45:45:0.9:0.1 [v/v/v/v]). Each working solution (5 µg/mL) was used for MS instrument tuning. Infusion into the mass spectrometer operated in the negative ion mode was done as follows: the standard solution from the built-in syringe pump at a flow rate of 5 µL/min was mixed with the previously mentioned phases (100 µL/min) through a T-piece. The optimized instrument settings were as follows: capillary temperature of 295°C, spray voltage of 4.2 kV, capillary temperature of 24°C with 60% atmospheric humidity and a 12 h light/12 h dark cycle. Before the experiment, the animals had ad libitum access to water and a commercial diet (Samyang Co., Seoul, Korea). The protocol for this experiment was reviewed and approved by the Korea University Animal Care Committee (KUIACUC-2014-49).

**Experimental Animals** White New Zealand male rabbits (aged 8 weeks) and seven-week-old male Wistar male rats were obtained from Daehan Biolink, Daejeon, Korea. The rabbits and rats were individually housed in stainless steel cages and plastic cages, respectively. The colony room was maintained at 24±1°C with 60% atmospheric humidity and a 12 h light/12 h dark cycle. Before the experiment, the animals had ad libitum access to water and a commercial diet (Samyang Co., Seoul, Korea). The protocol for this experiment was reviewed and approved by the Korea University Animal Care Committee (KUIACUC-2014-49).

**Quantitative Real-Time Polymerase Chain Reaction (qPCR)** After the full treatment procedure of chondrocytes, which were seeded on a 60-mm cell culture dish as mentioned above, RNA from the cells was extracted using RNaseasy® Plus Mini Kit (QIAGEN®), following the manufacturer’s instructions. cDNA was synthesized from 1 µg of isolated RNA using oligo-dT and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer’s protocol. One-hundred nanograms of the synthesized cDNA was subjected to qPCR using TaqMan® Gene Expression Assay primer/probe and TaqMan® Gene Expression Master Mix (Applied Biosystem, Life Technologies®), following the instructions provided. The TaqMan® primers used in this assay are listed in Table S1.

Using StepOnePlus™ Real-Time PCR System (Applied Biosystem, Life Technologies™), the relative amount of an individual gene of interest compared to that of the endogenous control gene, eukaryotic 18S ribosomal RNA (rRNA), was calculated to determine the mRNA expression level of each gene. The values were expressed as RQ values (relative quantification), which was computed by comparative methods by the StepOne software ver. 2.3.

**OA Induction in the Rat** Rats were anesthetized with 3–5% isoflurane/N₂O/O₂ mixture (Hana Pharm Co., Ltd., Seoul, Korea) vapors using Fluovac System (Harvard Apparatus Inc., Holliston, MA, U.S.A.) and were given an intra-articular injection of MIA twice in both knees during the experiment. The first injection was performed on day 0 (3 mg/50 µL in saline) and the second injection was done on day 22 (1.5 mg/50 µL in saline) through the infra-patellar ligament of the knee joint of the hind limb, using a 31 gauge BD Ultra-fine™ II insulin syringe (BD, NJ, U.S.A.).

**Sample Treatment** DBO-M was topically applied to the rats’ dorsal skin after shaving about 7×5 [(width)×(length)] cm² of their back. Topical application was performed 7 d after MIA injection and continued until day 56. The rats were randomly divided into five groups (eight rats per group). Samples were prepared with 15% (v/v) eucalyptol (Sigma) as a permeation-enhancer, and 2 mL/kg of sample was dermally applied to the shaved dorsumner day. Olive oil (OO) was used as a diluent for the sample and emu oil (EO) (Proemu, AEA certified, Birmingham, AL, U.S.A.) was used as the positive control.

**Biochemical Analysis of Blood** The rats were fasted for 24 h before being sacrificed. Blood samples were collected in a tube coated with ethylenediaminetetraacetic acid (EDTA), and then analyzed to determine the red blood cell (RBC) count, the white blood cell (WBC) count, hematocrit (HCT), the hemoglobin (Hb) concentration, the mean corpuscular volume (MCV), the mean corpuscular hemoglobin (MCH), and the mean corpuscular hemoglobin concentration (MCHC).

Total triglyceride (TG), total cholesterol (TCO), and high-density lipoprotein cholesterol (HDL-C) contents in the serum obtained from the blood sample were analyzed by FUJI DRI CHEM 3500 (Fuji Photo Film Co., Osaka, Japan).

**Serum Levels of IL-1β, IL-6, and TNF-α** The collected blood samples were stored at 25°C for 30 min, and then centrifuged at 3000 rpm for 10 min at 4°C. Supernatants were collected and divided into separate tubes in several aliquots and stored at −80°C until analysis. The levels of IL-1β, TNF-α, and IL-6 in the serum were determined using an ELISA kit (R&D System, Minneapolis, MN, U.S.A.) following the manufacturer’s guide.

**Micro-Computed Tomography (CT) Evaluation** The micro-CT evaluation was performed by SkyScan 1176 (Bruker MicroCT) software ver. 1.1. The specimens were stored in 4% buffered formalin for fixation until analysis. The samples were scanned by a camera with a pixel size of 12.44 µm, and frames were achieved using a 0.5 mm aluminum filter with a rotation degree of 0.5° and angle of 180° with frame averaging number of 3. The X-ray operation was performed at a voltage of 50 kV, a current of 500 µA, and an exposure time of 210 ms. A total of 395 scan slices per sample with an image pixel size of 18.02 µm were taken. Their serial reconstructed images were obtained from the raw images using the NRecon software (SkyScan NV. Belgium), and their serial transaxial images were obtained by DataViewer (SkyScan, Belgium) and analyzed by CTan software (Bruker microCT®) for 2D and 3D analyses. The proximal metaphysis of the tibia was chosen as the region of interest (ROI), covering from 2.12 mm above the end of the growth-plate tissue to a height of 3.89 mm. The area of trabecular bone in the designated ROI was manually selected by 3D data analysis software (CTAn, Bruker microCT®). Bone volume fraction (BV/TV, %) representing the ratio of trabecular bone volume (BV) to tissue volume (TV), trabecular thickness (Tb.Th, mm), trabecular number (Tb.N, mm⁻¹), trabecular separation (Tb.Sp, mm), and SMI (structure
model index) indicating whether the structures of trabeculae are rod-like or plate-like structure were analyzed.

**Statistical Analysis** The results are presented as the mean±standard deviation (S.D.) or standard error (S.E.). The significance of the differences of the groups was analyzed using one-way ANOVA with Tukey's multiple range tests. Statistic values of *p*<0.05 were considered statistically significant. All of the statistical analyses were done with the Statistical Package for Social Sciences (SPSS, IBM® SPSS® Statistics 21).

**RESULTS**

**Effects of DBO-M on Cell Viability and s-GAG Release in IL-1β-Treated Chondrocytes** DBO-M itself did not show any adverse effects on viability of rabbit cartilage-derived chondrocytes at concentrations of 0–400 µg/mL (Fig. 1A). IL-1β treatment caused a slight cytotoxic effect on the cells by decreasing viability (80%), but DBO-M treatment was shown to prevent the IL-1β-induced cell cytotoxicity (100–400 µg/mL) (Fig. 1A). This result showed that DBO-M was not cytotoxic to the cells within the range of 0–400 µg/mL, but rather protected the cells from IL-1β-induced cytotoxicity. In addition, DBO-M treatment caused a significant reduction in s-GAG release from chondrocytes (Fig. 1B), being parallel to the result of cell viability. Since the increase of s-GAG from the cell or tissue means the degradation of cell surface component, this result indicates that DBO-M protects chondrocyte from IL-1β-induced cell degradation.

**Effect of DBO-M on Gene Expression of MMP-1 and MMP-3 in Chondrocytes** IL-1β markedly increased mRNA levels of MMP-1 and MMP-3 in chondrocytes (Fig. 2). However, co-treatment with DBO-M effectively suppressed the IL-1β-induced elevation of MMP-1 and MMP-3 mRNA expression, although 100 µg/mL DBO-M caused a slight increase in mRNA levels compared to the control group. At a concentration of 400 µg/mL, DBO-M decreased IL-1β-induced expression of MMP-1 by over 80% compared to the control (Fig. 2A), and 400 µg/mL DBO-M decreased MMP-3 mRNA levels by over 70% (Fig. 2B). This result indicated that DBO-M...
effectively suppressed the IL-1β-induced expression of MMPs, which are responsible for the remodeling and pathogenesis of tissues.

**Analysis of Active Components of DBO-M** The main product ion found in the tandem MS spectra of both gangliosides was m/z 290.1, representing the terminal sugar moiety 5-N-acetyl-neuramic acid. Direct infusion ESI-tandem MS analyses of the authentic GD3 mixtures showed mass transitions (m/z precursor [M–2H]2–>m/z product ion) specific to a number of components in the GD3 mixtures, as shown in Supplementary Fig. 1S: 755.9>290.0 for GD3 (d39:1), 762.9>290.0 for GD3 (d40:1), 769.9>290.0 for GD3 (d41:1), and 776.9>290.0 for GD3 (d42:1). With respect to the authentic GM3 mixtures, the mass transition from the precursor ion [M–H]− to product ion distinct to some components in the GM3 mixtures were also detected as follows: 1235.7>290.0 for GM3 (d40:1), 1263.7>290.0 for GM3 (d42:1), and 1277.7>290.0 for GM3 (d43:1). Our MS analysis of the gangliosides obtained from DBO-M is depicted in Fig. 3. Tracing the mass transitions match to the GD3 components into the DBO-M did not give any relevant mass transitions specific to GD3. On the other hand, we detected the mass transition (1263.7>290.0) specific to GM3 (d42:1) (Fig. 3A). Additionally, one more mass transition from m/z 1261.7 to m/z 290.0 was detected (Fig. 3B). With respect to not only the two Dalton differences compared with the molecular ion (m/z 1263.7) of GM3 (d42:1), but also to the existence of the dominant product ion (m/z 290.0) corresponding to 5-N-acetyl-neuramic acid, this mass transition seems to represent the structural derivative of GM3 (d42:1). Indeed, the molecule could be considered a GM3 analog such as GM3 (d42:2), in which an additional double bond is present in the ceramide scaffold of a ganglioside. Interestingly, when we explored this distinctive mass transition in the authentic GM3 mixtures (Fig. 3C), we were also able to detect the same, indicating that this ganglioside was one of the components found in GM3 originating from canine blood.

**Average Weight Gain and Daily Food Intake in MIA-Injected OA Rats** We determined the average weight gain and food intake of rats during the experimental period (56 d, 8 weeks) (Fig. 4). Intra-articular MIA injection caused a significant decrease in weight gain (ca. 40%) and food intake (ca. 20%) of rats, compared to the control. However, topical

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**Fig. 3. Mass Spectra Obtained from Both DBO-M and GM3 Authentic Mixtures**

Analyses of DBO-M-derived samples and authentic GM3 and GD3 mixtures (each 100µg/mL) were performed using a direct infusion LCQ ion-trap ESI-MS. GM3 (d42:1) present in DBO-M (A); GM3 analog found in DBO-M (B); GM3 analog present in GM3 authentic mixtures (C).
Effects of DBO-M on Serum Biochemical Parameters in MIA-Injected OA Rats
Levels of triglycerides (TG) and low-density lipoprotein cholesterol (LDL-C) in serum were significantly lower in the DBO-M groups compared to the MIA and control groups. TG levels were lowest in the high-dose DBO-M group, while LDL-C was lowest in the low-dose DBO-M group. These results indicate anti-inflammatory and anti-oxidative effects of DBO-M.

Table 1. TG, HDL-C, LDL-C, and TCHO Levels in Serum of the Experimental OA Rats

| Group                  | TG (mg/dL) | HDL-C (mg/dL) | LDL-C (mg/dL) | TCHO (mg/dL) |
|------------------------|------------|---------------|---------------|--------------|
| CON                    | 96.8±17.1  | 52.4±2.2     | 19.4±4.2      | 86.1±3.1     |
| NC                     | 116.4±20.4 | 48.9±3.6     | 25.3±5.2      | 76.5±5.0     |
| EC                     | 126.0±9.7  | 56.0±2.8     | 23.5±3.7      | 87.9±4.4     |
| LDBO                   | 102.3±4.4  | 58.6±3.2     | 22.5±4.1      | 97.3±5.5     |
| HDBO                   | 103.1±15.2 | 57.5±6.8     | 21.2±3.9      | 91.3±6.5     |

Values are expressed as the mean ± S.E. NS; not significant. Means with different superscript letters indicate significant differences with p<0.05 by ANOVA with Tukey’s multiple range tests. CON; control (without MIA injection), NC; negative control (MIA injection + olive oil (OO, diluent)), EC; emu oil control (MIA injection + emu oil, positive control), LDBO; low-dose-DBO-M (MIA injection + OO/DBO-M (2 : 1, v/v), and HDBO; high-dose-DBO-M (MIA injection + OO/DBO-M (1 : 1, v/v)).

Table 2. The Hematologic Parameters in the Experimental OA Rats

| Group                  | RBC (10^6/µL) | WBC (10^3/µL) | HCT (%) | Hb (g/dL) | MCV (fL) | MCH (pg) | MCHC (g/dL) | Platelet (10^3/µL) |
|------------------------|---------------|--------------|---------|-----------|---------|---------|------------|------------------|
| CON                    | 8.1±0.4       | 7.8±0.9      | 49.0±2.2| 15.0±0.3  | 60.6±0.4| 18.8±1.0| 31.0±1.5    | 1115.9±73.8      |
| NC                     | 8.7±0.2       | 4.4±0.7      | 52.6±0.7| 16.0±0.1  | 60.9±0.6| 18.5±0.3| 30.4±0.5    | 1102.9±26.9      |
| EC                     | 8.4±0.1       | 5.5±0.8      | 50.3±0.8| 14.7±0.2  | 59.9±0.4| 17.5±0.2| 29.2±0.1    | 1249.3±61.7      |
| LDBO                   | 8.8±0.2       | 6.0±0.8      | 52.4±1.0| 15.3±0.3  | 59.6±0.4| 17.4±0.1| 29.2±0.2    | 1235.4±82.9      |
| HDBO                   | 8.7±0.2       | 7.2±0.7      | 50.7±1.1| 15.0±0.3  | 58.6±0.4| 17.4±0.2| 29.6±0.2    | 1253.1±127.6     |

Values are expressed as the mean ± S.E. NS; not significant. Means with different superscript letters indicate significant differences with p<0.05 by ANOVA with Tukey’s multiple range tests. CON; control (without MIA injection), NC; negative control (MIA injection + olive oil (OO, diluent)), EC; emu oil control (MIA injection + emu oil, positive control), LDBO; low-dose-DBO-M (MIA injection + OO/DBO-M (2 : 1, v/v), and HDBO; high-dose-DBO-M (MIA injection + OO/DBO-M (1 : 1, v/v)).

Application of emu oil significantly ameliorated the MIA-induced weight loss and reduction of food intake (Figs. 4A, B). DBO-M at both low and high concentrations also improved body weight (b.w.) and food intake, but those effects were not significant (Fig. 4). This result indicated that MIA-induced destruction of the knee joint contributed to the reduction of b.w. and food intake, and that DBO-M and emu oil suppressed the MIA-induced decrease of b.w. and food intake. However, there were no significant differences in weights of the liver, heart, lung, kidney, or spleen among the experimental groups (Table S2).

Effect of DBO-M on Serum Biochemical Parameters in MIA-Injected OA Rats
Levels of triglycerides (TG) and low-density lipoprotein cholesterol (LDL-C) in serum were...
shown to decrease with DBO-M treatment, while high-density lipoprotein cholesterol (HDL-C) was slightly increased by DBO-M. However, these changes were not significant among the groups. On the other hand, total cholesterol (TCHO) significantly increased in low-dose DBO-M-treated rats (LDBO) (Table 1). White blood cell (WBC) count was significantly lowered by MIA injection (NC), but its level was slightly recovered in the EC, LDBO, and HDBO groups (Table 2). The Hb level was slightly higher in the NC group than in the CON group, but its level decreased in the EC and HDBO groups to that of the CON group. Values of mean corpuscular volume (MCV) in the CON and NC groups were similar, but the MCV levels in the EC and LDBO groups slightly decreased compared to that in the NC and CON groups (yet not significant), while MCV in the HDBO group significantly decreased. Other values including red blood cell (RBC) count, hematocrit (HCT), mean corpuscular hemoglobin (MCH), mean cell hemoglobin concentration (MCHT), and platelet count showed no significant variation among the groups (Table 2).

The analysis of biochemical and hematologic parameters suggested that DBO-M does not affect the lipid profile and serum value of rats when compared to the CON group, except for the levels of total cholesterol, WBC, and MCV, which showed only slight changes.

**Effects of DBO-M on Cytokines in MIA-Induced OA Rats**

The effect of DBO-M on the cytokine levels in MIA-induced osteoarthritic rats was determined using ELISA. MIA injection caused a significant elevation (over 50%) in the pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 compared with the control. This result suggests that MIA-induced inflammation occurred in the joint. MIA-induced increase of cytokine levels was effectively suppressed by DBO-M treatment. IL-1β and TNF-α levels were decreased by 40–50 and 65%, respectively, in DBO-M treated groups compared to the MIA-injected group, but dose-dependent differences were not observed (Figs. 5A, B). IL-6 production was significantly decreased by DBO-M treatment in a dose-dependent manner. A high dose of DBO-M suppressed IL-6 production by 50% (Fig. 5C). Emu oil, a positive control, was shown to be effective in the suppression of IL-1β and TNF-α, but IL-6 levels were not affected. Our data showed that DBO-M treatment significantly suppressed MIA-induced increase in cytokines, which are responsible for inflammation in the body.

**Effects of DBO-M on 3D Morphometric Parameters in MIA-Induced OA Rats**

A 2D coronal plane micro-CT image displayed the damage of the articular joint region in MIA-induced osteoarthritic rats. In particular, the subchondral bone, trabecular bone, and epiphyseal region were greatly damaged in MIA-injected groups (Figs. 6A, B). DBO-M treatment was shown to significantly improve the MIA-induced destruction of the articular joint region. The area of trabecular bone in the proximal metaphysis of the tibia was assessed by 3D data analysis software (CTAn, Bruker microCT®). BV/TV, which represents the ratio of bone volume to tissue volume, significantly decreased in MIA-induced osteoarthritis. DBO-M treatment was shown to improve the BV/TV, but no statistically significant difference was observed among the groups. MIA-induced decrease of Tb.Th significantly improved with high doses of DBO-M (40%) (Fig. 6C). In contrast, Tb.Sp increased two-fold in the MIA-treated group, compared with the control. This enhancement of Tb.Sp by MIA treatment was effectively suppressed by DBO-M and emu oil. The high dose of DBO-M resulted in a 38% reduction in Tb.Sp, compared with the MIA-treated group (Fig. 6C). Emu oil also significantly improved these 3D photometric parameters, although its effects were shown to be weaker than those of DBO-M were. This result indicated that DBO-M protects against the articular cartilage destruction induced by MIA.

**DISCUSSION**

In this study, we examined the effect of the DBO-M on inflammatory responses and osteoarthritic conditions in vitro and in vivo. Our DBO-M fraction was shown to protect chondrocytes from IL-1β-induced cell cytotoxicity within the limited concentration ranges (100–400 µg/mL) (Fig. 1A). Chondrocytes, which are the only type of cells in the hyaline cartilage, are responsible for maintenance of homeostasis between catabolism and anabolism of the extracellular matrix (ECM) surroundings. Since chondrocyte cell survival is anchorage-dependent, cartilage degradation and chondrocyte apoptosis have been known to be closely connected.
particular, chondrocyte apoptosis is associated with pathogenesis of osteoarthritis, even if the cause-and-effect relationship between them is still controversial. Therefore, the chondroprotective effect of DBO-M is considered to play an important role in prevention of osteoarthritic destruction of articular cartilage, as seen in our data which showed DBO-M-mediated suppression of the s-GAG release from chondrocyte (Fig. 1B).

Inflammatory stimuli are known to promote the degradation of matrix proteins by activation of MMPs. Pro-inflammatory cytokines such as IL-1β and TNF-α were reported to up-regulate MMP-3 mRNA levels in the osteoarthritic joint. In particular, MMP-1 and MMP-13 are known to play crucial roles in degrading collagen type II, which comprises over 90% of cartilage matrix proteins. MMP-1, 3, 9, and 13 are known to be most strongly stimulated by pro-inflammatory cytokines such as IL-1β and TNF-α via an auto-crine loop. Our data showed that DBO-M effectively inhibits MMP-1 and MMP-3 mRNA expression (Fig. 2), suggesting that DBO-M mediated the suppression of ECM degradation. These in vitro results are connected to the cytokine data (Fig. 5) from the MIA-induced osteoarthritic rat. DBO-M effectively inhibited pro-inflammatory cytokine expressions (IL-1β, IL-6, and TNF-α) in the serum (Fig. 5). In particular, in prolonged inflammation, elevated levels of inflammatory cytokines are known to send signals to articular cartilage chondrocytes or fibroblasts to up-regulate MMP production, leading...
to cartilage degeneration.\textsuperscript{5,24–26} DBO-M-mediated suppression of cytokines resulted in the decrease of the articular cartilage destruction (Fig. 6). Therefore, the DBO-M-mediated anti-inflammatory response was considered to contribute, at least in part, to the ameliorating effect of DBO-M in MIA-induced osteoarthritis.

The protective effect of DBO-M on the articular cartilage destruction was identified, but MIA-induced destruction of cartilage was observed to be excessive (Fig. 6B). This is considered to be due to the overdose of MIA in twice (total 4.5 mg of MIA), which was executed to induce the definite osteoarthritids accompanying with cartilage degradation.

MIA-induced osteoarthritis in rats was shown to decrease b.w. and food intake (Fig. 4). This result, which was also observed in a previous study by Lee et al.,\textsuperscript{8} was ascribed to the destruction of cartilage. DBO-M appears to reverse the MIA-induced changes in b.w. and food intake, although this change was not statistically significant (Fig. 4).

To identify the active constituents derived from DBO-M, we performed ESI-MS/MS analysis and used two animal-derived substances (canine-GM3 and bovine-GD3) as standards. In the previous studies on the UPLC-MS/MS analysis of gangliosides obtained from bovine milk and other dairy products, the composition of both GD3 and GM3 standards was presented and verified.\textsuperscript{27} The mass transitions specific to both the GD3 and GM3 compositions described above were all in accordance with those listed in previous publications,\textsuperscript{27,28} demonstrating that ESI-MS/MS tuning performed herein appears to be efficient enough to detect and characterize each component in the GD3 and GM3 mixtures. Therefore, based on the above-established ESI-MS/MS fragmentation patterns found in authentic standards, we performed the tandem MS analyses and the mass spectra, which are supposed to be gangliosides. Our data showed that the DBO-M-derived active substance with an anti-osteoarthritic effect was considered a GM3 analog containing a ganglioside found in canine blood (Fig. 3). Further studies on the structural elucidation of the above new GM3 analog will be needed after purification followed by instrumental analyses including NMR and high resolution (HR)-MS. Our data were partially corroborated by the precedent studies. A recent study analyzed GM3 and GD3 as pharmacological agents extracted from deer antlers,\textsuperscript{29} and Lee et al. reported that deer bone water extract contains the gangliosides.\textsuperscript{30} In particular, several studies showed that GM3 plays a critical role in suppression of the pathogenesis and progression of arthritis.\textsuperscript{31,32} Supporting our data which showed the association of GM3 (or GM derivatives) on osteoarthritis. However, further study would be executed to confirm the effect of GM3 on osteoarthritis with its mechanistic action. Sasazawa et al. reported that GM3 plays an essential role in suppression of osteoarthritis by regulating metalloproteinases (MMP13 and ADAMTS-5) expressions and chondrocyte apoptosis.\textsuperscript{31} This study is also correlated to our data (Fig. 2) which showed inhibitory effect of DBO-M on metalloproteinases (MMP1 and 3). Many studies report that gangliosides have anti-inflammatory effects as regulators for local or systemic inflammation.\textsuperscript{33,34} Dietary gangliosides were shown to inhibit proinflammatory mediators such as TNF-α and IL-1β,\textsuperscript{35} while anti-inflammatory cytokine, IL-10, was enhanced by gangliosides.\textsuperscript{35} Therefore, anti-osteoarthritic effect of GM3 is recognized to be due to protection of cartilage matrix via the regulatory effect of inflammatory mediators or matrix proteinases. Additionally, many studies have been done on gangliosides and their biological activities. Exogenous administration of gangliosides was shown to be involved in the survival and protection of neurons in the central nervous system.\textsuperscript{36,37} Gangliosides have various types according to the number of sialic acid and attached saccharides. So gangliosides and its various derivatives would be widely determined on the osteoarthritic models in the next study.

Studies of the effects of deer bone on osteoarthritis have been usually performed with water extracts of deer bone.\textsuperscript{8,10,38} However, a large quantity of oil (lipids) is produced and thrown out after the water extraction of deer bone. For the purpose of using the byproducts of deer bone processing, the current study focused on the effect of deer bone oil on osteoarthritis, which had not yet been investigated. In fact, many vegetable or animal-derived oil components have been implicated in osteoarthritis. Supplements of castor oil in capsules, fish oil omega-3-fatty acids, and aromatic ginger and orange essential oil have improved the symptomatic progress of OA.\textsuperscript{31,32} In addition, topical application of emu oil has had an anti-inflammatory effect in CD-1\textsuperscript{1} mice with down-regulation of inflammatory cytokines IL-1β and TNF-α.\textsuperscript{39} In particular, Power et al. showed that emu oil would be a clinically useful agent to manage pain of OA patients when applied topically.\textsuperscript{39} Our study includes emu oil as a positive control in drug-induced OA, but DBO-M was observed to have a greater anti-osteoarthritic effect than emu oil. Accordingly, our study suggests that deer bone oil could be another potential agent for improvement of osteoarthritis.

**Conflict of Interest** The authors declare no conflict of interest.

**Supplementary Materials** The online version of this article contains supplementary materials.

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