

Sialyllactose Prevents Cartilage Damages via M0 Macrophage Maintenance in Yucatan Mini-Pig Osteoarthritis Model

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Abstract

Sialyllactose, known to be abundant in human breast milk, has anti-inflammatory properties, but its preventive effect on osteoarthritis remain unclear. Here, we demonstrated the efficacy of 3' sialyllactose (3' SL) and 6' sialyllactose (6' SL) in preventing osteoarthritis in Yucatan mini-pigs. Twelve female Yucatan mini-pigs were administered 0, 200, 400 mg 3' SL or a combination of 200 mg 3' SL + 200 mg 6' SL for 12 weeks (4 weeks before and 8 weeks after surgery); then, osteoarthritis was induced in the left knee by anterior cruciate ligament transection surgery. Kinematic variables were used to quantify gait analysis on the treadmill, and the degree of osteoarthritis was analyzed in the femur and tibia cartilage. It was confirmed that lameness of the left hind limb was reduced in all treated groups compared to the control group. Cartilage disruption was alleviated through macroscopic and microscopic observation of the knee joint. In addition, the expression of pro-inflammatory cytokines (IL-1 β , TNF- α) and anti-inflammatory cytokines (IL-10, TGF- β) was decreased in human macrophages (THP-1) by 3' SL. This reduction in cytokine expression was due to the maintenance of M0 macrophages, which did not differentiate into M1 or M2 macrophages. Thus, we suggest that 3' SL and 6' SL have the potential to act as natural therapeutic agents for the prevention of osteoarthritis.

Keywords: Natural oligosaccharides, Osteoarthritis, Pro-inflammatory cytokines, Macrophages

Introduction

Osteoarthritis is the most common form of arthritis and is mainly caused by age-related cartilage damage. In general, joint cartilage cannot grow on its own, and the occurrence of arthritis leads to pain and sequelae. Painkillers and non-steroidal anti-inflammatory drugs (NSAIDs) that are used to treat osteoarthritis, mainly relieve pain and inflammation. Therefore, it is important to develop treatments for degenerative arthritis that prevent or slow the disease progression. Previous reports have shown that osteoarthritis is associated with excessive production of interleukin (IL)-1 β . IL-1 β is known to

increase the expression of metalloproteinases (MMPs) that induce collagen degradation in the cartilage. Moreover, it is known that inflammatory cytokines such as IL-6, IL-17, and tumor necrosis factor (TNF)- α also induce osteoarthritis [1-3]. Thus, NSAIDs are mainly used in osteoarthritis treatment to reduce inflammation in the cartilage, but their continued use is limited due to various side effects.

Several recent studies have suggested the possibility that macrophages play an important role in regulating joint inflammation and osteoarthritis severity through various cytokines. Macrophages are one of the most numerous

immune cells in the synovial membrane of joints and are important for maintaining homeostasis of synovial tissue. In addition, macrophages are known to exist in tissues around joints, such as the infrapatellar fat pad, ligament, tendon, and muscle, and the mechanism of arthritis control by macrophages present in these is being actively studied [4,5]. Indeed, a previous study has reported a high frequency of macrophages at osteoarthritis sites, and suggested a direct relationship between macrophages and osteoarthritis. In particular, macrophages are known to regulate immune function by differentiating into inflammatory M1 macrophages and anti-inflammatory M2 macrophages. M1-polarized macrophages release IL-1 β , TNF α , and MMPs to induce osteoarthritis as well as osteophyte formation, causing pain. The treatment of osteoarthritis using the characteristics of these macrophages has been limited so far, and the control of macrophage differentiation can be used as a new treatment strategy for osteoarthritis [6-8].

Human milk contains an abundance of sialyllactose-containing oligosaccharides. Among these, 3' SL (with N-acetylneuraminic acid connected to 3' position of lactose) and 6' SL (with N-acetylneuraminic acid connected to 6' position of lactose) are the major components of sialylated oligosaccharides. The anti-inflammatory functions of these sialyllactose have been established [9]. In addition, 3' SL and 6' SL were recently shown to be safe even when ingested for a long period of time [10,11]. Thus, oral 3' SL and/or 6' SL have the potential to be used as alternatives to NSAIDs that cause side effects during long-term treatment for osteoarthritis. In particular, 3' SL was found to have therapeutic and protective effects in osteoarthritis and rheumatoid arthritis in mice via inhibition of NF- κ B signaling [12,13]. However, it is still not clear whether these effects identified in mice will also be effective in human patients.

To overcome species differences in mice and humans, we investigated the protective effects of sialyllactose in Yucatan mini-pig osteoarthritis model. Further, we investigated their effects on pro-inflammatory and anti-inflammatory cytokine expression in vitro using a human macrophage cell line.

Material and Methods

Ethics statement

All experimental protocols were approved by the Animal Care and Use Committee of the Korea Institute of Toxicology (KIT) and complied with the Association for Assessment and Accreditation of Laboratory Animal Care International Animal Care Policies (Approval No. 1908-0276, 1912-0403).

Mini-pig osteoarthritis modeling

Twelve female Yucatan mini-pigs aged 12–18 months and weighing 40–70 kg were obtained from OPTIPHARM (OPTIPHARM Co., Ltd., Korea). Osteoarthritis was induced

in their left knee by anterior cruciate ligament transection surgery, as previously described [14–16]. Briefly, intramuscular injections of ketamine (30 mg/kg) and xylazine hydrochloride (3 mg/kg) were used for general anesthesia, and inhalation of isoflurane (2~3% v/v) was used to sustain the anesthesia during anterior cruciate ligament transection surgery. During the surgery, body temperature and respiratory rate of the animals were monitored continuously.

Oral administration of 3' SL and 6' SL

Placebo tablets, 200 mg 3' SL tablets, and 200 mg 6' SL tablets were provided by GeneChem Inc (GeneChem Inc., Daejeon, Korea). Placebo tablets contained 84.6% microcrystalline cellulose. The Three Yucatan mini-pigs per group were orally administered 0, 200, or 400 mg/head/day 3' SL tablets, or 200 mg/head/day 3' SL tablets plus 200 mg/head/day 6' SL tablets with feed daily for 4 weeks before osteoarthritis modeling and 8 weeks after the modeling.

Gait analysis

Videos of all experimental mini-pigs walking on the treadmill were recorded using a method previously described [17–19]. Mini-pigs were acclimated before walking on the treadmill for 5–10 min and walked at 2 km/h on the treadmill. During walking on the treadmill, the left side of the mini-pig was recorded using a Sony video camera (HDR-CX240, Sony). For each animal, a 20 s video (more than 10 steps) was analyzed for knee angle, stride time (time between two consecutive first contacts of the left hind limb), swing time (time between first contact and toe-off), and stance time (stance time divided by stride time) using Kinovea program (Kinovea 0.9.1, Charmant, Joan).

Hematology and serum biochemistry

Twelve females Yucatan mini-pigs were starved for more than 17 hours prior to blood collection. For hematology, about 0.5 mL of blood was harvested in tubes containing an anticoagulant (EDTA-2K). Total leukocyte count, mean corpuscular hemoglobin, total red blood cell count, mean corpuscular hemoglobin concentration, hemoglobin, platelet count, hematocrit, reticulocyte count, mean corpuscular volume, and differential WBC count were measured by using an ADVIA2120i hematology analyzer (Siemens, USA). For serum biochemistry analysis, about 1.5 mL of blood was harvested into tubes without the anticoagulant and was incubated for at least 90 minutes at room temperature and centrifuged to separate the serum. Glucose, alanine aminotransferase, blood urea nitrogen, total bilirubin, creatinine, alkaline phosphatase, total protein, gamma glutamyl transpeptidase, albumin, creatine phosphokinase, albumin/globulin ratio, calcium, total cholesterol, inorganic phosphorus, triglyceride, sodium, phospholipid, potassium, aspartate aminotransferase, and chloride levels were measured using a Toshiba 120 FR chemistry analyzer (Toshiba Co., Japan).

Scoring of cartilage destruction and histochemistry

Macroscopic scoring of cartilage and synovium was performed as previously described [20,21]. Briefly, articular damages were scored as following: normal, 0; surface roughening, 1; fibrillation and fissures, 2; small erosions down to subchondral bone (<5 mm diameter), 3; larger erosions down to subchondral bone(>5 mm diameter), 4. Synovium pathology was scored as normal to severe as following: normal, 0; slight (focal involvement, slight discoloration, visible fibrillation/thickening, notable increase in vascularity), 1; mild (diffuse involvement, slight discoloration, visible fibrillation/thickening, notable increase in vascularity), 2; moderate (diffuse involvement, severe discoloration, consistent notable fibrillation/thickening, moderate vascularity), 3; marked (diffuse involvement, severe discoloration, consistent and marked fibrillation/thickening, marked synovial proliferation with diffuse hypervascularity), 4; severe (diffuse involvement, severe discoloration, consistent and severe fibrillation, thickening to the point of fibrosis, severe proliferation and hypervascularity), 5. Mini-pig knee joints were fixed in 10% neutral-buffered formalin and decalcified with 10% formic acid for 4 weeks. After decalcification, knee joint cartilages were embedded in paraffin and sectioned at a thickness of 4 μ m. H&E staining and Safranin-O staining were performed on tibia and femur cartilage sections and scored using Osteoarthritis Research Society International (OARSI) guidelines [20,21]. Briefly, cartilage damages were scored as following: normal, 0; slight surface irregularities 1 to 3; fissures (from transitional zone to calcified zone), 4 to 6; erosion or severe fibrillation (from mid zone to subchondral bone), 7 to 10.

Cell culture

The human monocyte cell line THP-1 (American Type Culture Collection, VA, USA) was cultured in RPMI 1640 (SH30027.01, Hyclone, Logan, UT, USA) containing 10% heat-inactivated fetal bovine serum (SH30919.03, Hyclone) and 1% penicillin/streptomycin (P/S, 15140, Thermo Fisher Scientific, MA, USA) at 37 °C in a 5% CO₂ incubator. MEM non-essential amino acid solution (1%; 11140, Thermo Fisher Scientific, MA, USA) was added to encourage the growth and prolong cell survival in the culture. To differentiate THP-1 cells into macrophages, 10 ng/mL phorbol-12-myristate-13-acetate (P8139, Sigma-Aldrich, MO, USA) was used to stimulate THP-1 cells in complete culture media for 24 h. The medium was changed the next day, followed by further incubation for 24 h. Differentiated THP-1 cells were treated with 0 (control), 10, 100, 500, or 1,000 μ g of 3' SL for 24 h, followed by 0.1 ng/mL lipopolysaccharide (LPS) (L2637, Sigma-Aldrich, MO, USA) treatment for 4 h.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and ELISA

Total RNA was isolated from differentiated THP-1 cells using TRIzol (15596018, Thermo Fisher Scientific, MA, USA), and

was cDNA was synthesized with 1 μ g RNA using a Quantitect Reverse Transcription Kit (205313, Qiagen, Hilden, Germany) according to the manufacturer's instructions. qRT-PCR was performed using Power SYBRTM Green PCR Master Mix (4368702, Applied Biosystems, CA, USA) and human primers for GAPDH, IL-1 β , IL-10, TGF β , TNF α , CCR7, and CD163. qRT-PCR conditions were as follows: 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min on a PCR machine (A28134, Applied Biosystems, CA, USA). mRNA levels of target genes were normalized to that of GAPDH ($\Delta C_t = C_t$ gene of interest – C_t GAPDH) and described as relative mRNA expression ($2^{\Delta C_t}$ sample- ΔC_t control) or the fold-change. IL-1 β and TNF- α protein levels was measured using the Quantikine ELISA kit (DLB50, DTA00D, R&D Systems, Inc., MN, USA) by manufacturing protocol.

Statistical analysis

The dataset was statistically analyzed using the Prism8 (GraphPad Software, CA, USA). Gait behavior was analyzed by One-way analysis of variance. Synovial pathology and microscopic observations cartilage were analyzed by Kruskal-Wallis one-way analysis of variance. Quantification of cytokines by qRT-PCR and ELISA was analyzed by One-way analysis of variance. All results are presented as means $\pm$ SD. P values <0.05 were considered to be significant.

Results

Changes in mini-pig lameness gaits by 3' SL and 6' SL treatment

To investigate their protective effect against osteoarthritis, Yucatan mini-pigs were orally administered 0 (control), 200 (200 mg 3' SL), or 400 mg/head/day 3' SL (400 mg 3' SL), or a combination of 200 mg/head/day 3' SL + 200 mg/head/day 6' SL (200 mg 3' SL + 200 mg 6' SL) for 12 weeks. After 4 weeks of administration, anterior cruciate ligament transection surgery was performed on the left hind limb in all groups (**Figure 1A**). Before necropsy, we investigated gait behavior 8 weeks after anterior cruciate ligament transection surgery to verify the effectiveness of 3' SL and 6' SL in preventing osteoarthritis. Yucatan mini-pigs were walked on the treadmill, and mobility of the left hind limb was analyzed (**Figure 1B**). For accurate analysis, kinematic variables were used to quantify gait characteristics [17,18]. First, we analyzed the range of motion of the left knee during one cycle (**Figure 1C and 1D**). The average range of motion in the left knee was only 13.10% lower in the 200 mg 3' SL treatment group, while the other groups did not show any difference compared with the control group. However, the knee angle at each time point was different in all groups compared to the control group. Especially stride time was reduced in all groups compared to the control group (**Figure 1E**). But the swing time and stance time did not alter (**Figure 1F and 1G**). These data indicate that lameness of the left hind limb was changed by oral administration of 3' SL and 6' SL.

Figure 1.

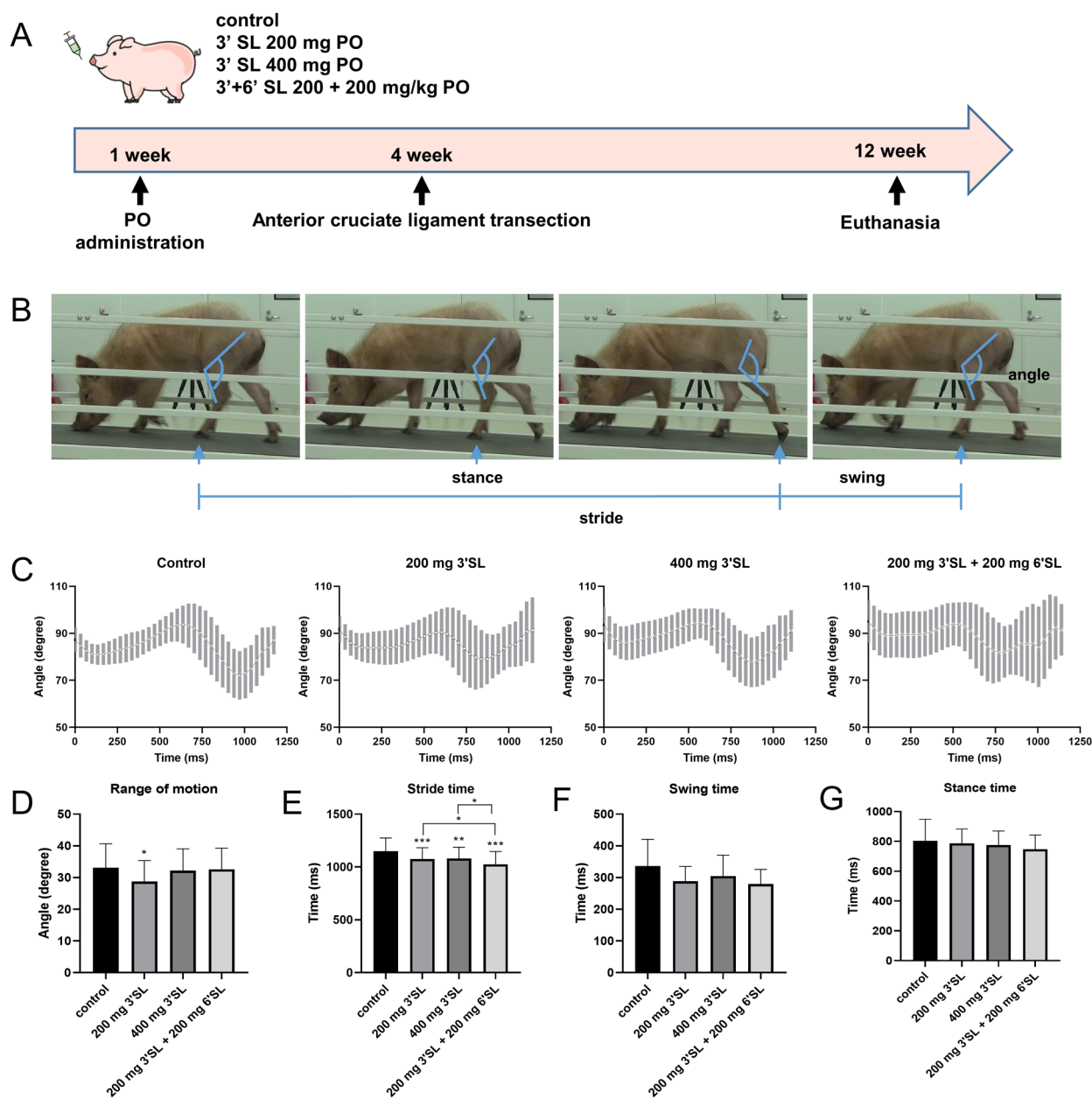


Figure 1. Effect of 3' SL and 6' SL on the gait behavior of Yucatan mini-pig osteoarthritis model. (A) Experimental procedure for evaluating the protective effect of 3' SL and 6' SL in mini-pig osteoarthritis model by anterior cruciate ligament transection. Yucatan mini-pigs were treated with oral gavage of 3' SL or 6' SL for 12 weeks. (B) Representative images of gait analysis on the treadmill. (C) Alteration of left knee angle during 1 step. Shaded areas represent standard deviations (SD) for each frame. (D-G) Quantification result of gait behavior in Yucatan mini-pigs. Error bars represent the SD; n.s., not significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, One-way analysis of variance.

Protective effect of 3' SL and 6' SL assessed by the macroscopic observation of mini-pig cartilage and synovial pathology

Eight weeks after anterior cruciate ligament transection surgery, we investigated cartilage disruption and synovial

inflammation in mini-pig knee joints after necropsy (Figure 2). Even though the lateral tibia and femur in the left and right hind limbs showed large erosions in the control group, the 400 mg 3' SL treatment group showed decreased erosion in size and number. In particular, erosion in the control group was found to extend to the subchondral bone, but in the 3'

Figure 2.

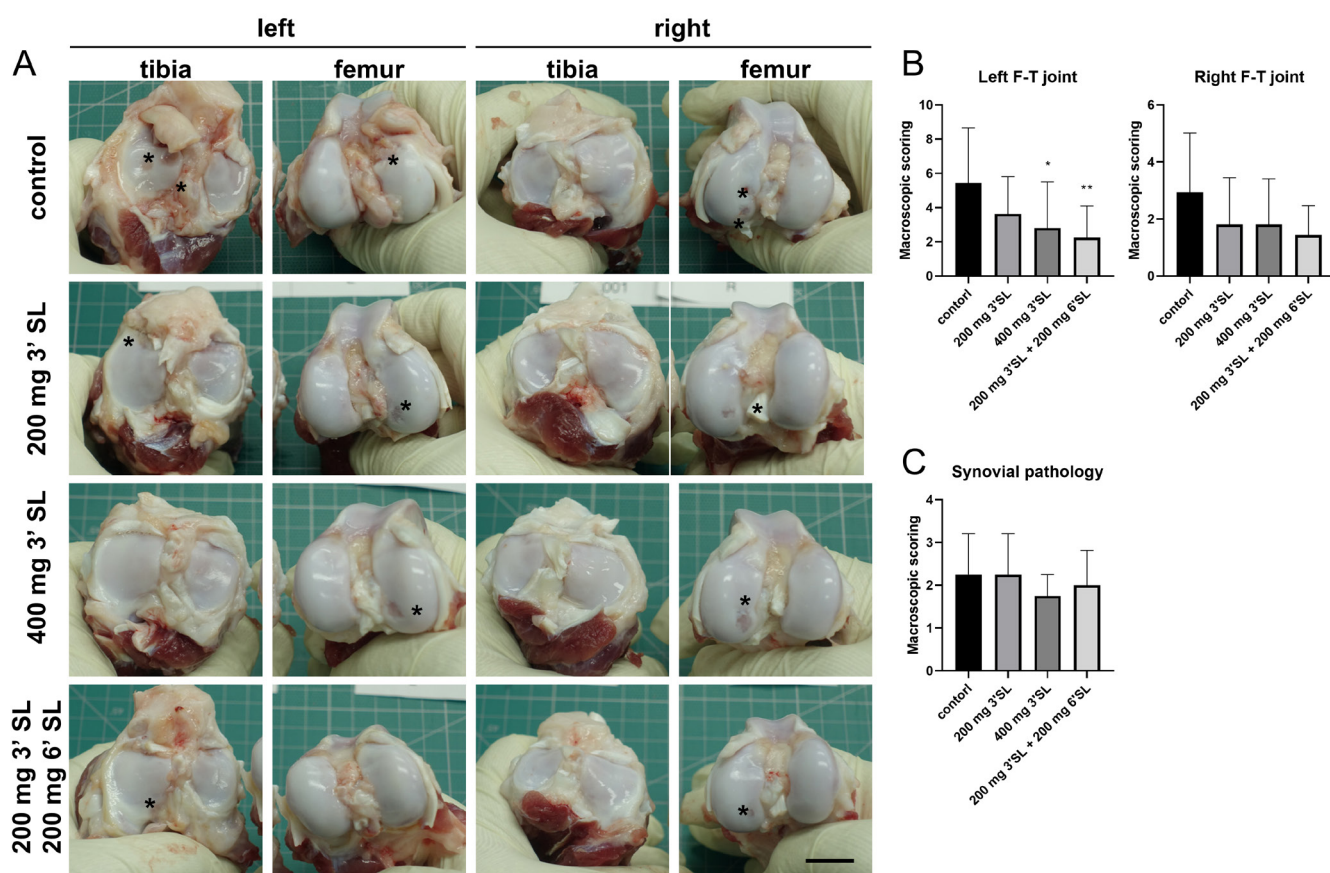


Figure 2. Effect of 3' SL and 6' SL against osteoarthritis based on macroscopic observation of Yucatan mini-pig cartilage. (A) Representative images of the mini-pig tibia and femur cartilage surface. Asterisk indicates cartilage lesion. **(B)** Quantification result of macroscopic observations of Yucatan mini-pig cartilage. Cartilage lesion of left femur–tibia joint was reduced in 400 mg 3' SL and 200 mg 3' SL + 200 mg 6' SL treated groups. **(C)** Quantification result of synovial pathology. Error bars represent the SD; *p < 0.05, **p < 0.01, ***p < 0.001, Kruskal–Wallis one-way analysis of variance. Scale bars: A, 4 cm.

SL treatment group erosion only extended to the articular cartilage. Interestingly, simultaneous administration of 3' SL and 6' SL showed surface roughening, fibrillation, and fissures rather than large erosions in the articular cartilage. We quantified cartilage disruption by macroscopic scoring of the cartilage. The 400 mg 3' SL and 200 mg 3' SL + 200 mg 6' SL treatment groups showed significantly lower macroscopic cores compared to the control group (**Figure 2B**). Synovial pathology was also examined in both hind limb knee joints. The left side of the tibial plateau and femoral condyle region showed red discoloration and an increase in vascularity in all treatment groups. We quantified synovial pathology by macroscopic scoring of the synovium and found no difference between the control and treatment groups (**Figure 2C**).

Protective effect of 3' SL and 6' SL assessed by the microscopic observation of mini-pig cartilage

We also evaluated cartilage disruption by microscopic scoring of cartilage, including structure, chondrocyte density, cell cloning on both sides of the tibia, and femur articular cartilage. Similar to macroscopic observations, cartilage disruption was observed in the control tibia and femur articular cartilage. In the control group, severe erosion and fissure to a calcified zone were observed in the articular cartilage, but these structural disruptions were decreased after SL treatment (**Figure 3A**). Thus, structure disruption scoring for the left tibia cartilage was lower in the 400 mg 3' SL and 200 mg 3' SL + 200 mg/ 6' SL treatment groups; while it was lower for the right

Figure 3.

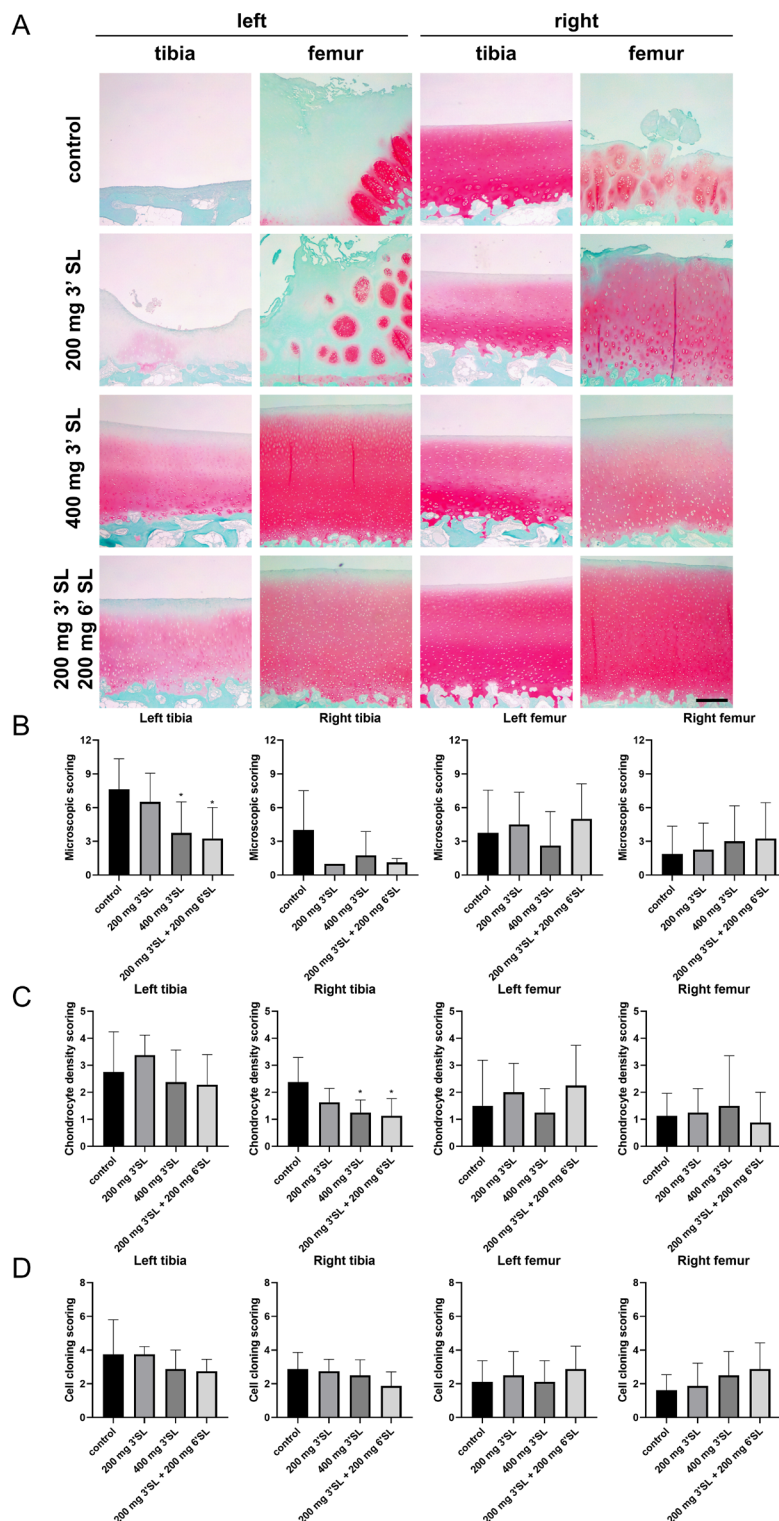


Figure 3. Effect of 3' SL and 6' SL against osteoarthritis based on microscopic observation of Yucatan mini-pig cartilage. (A) Representative cartilage destruction images obtained by Safranin-O staining in the mini-pig tibia and femur cartilage. Cartilage destruction was reduced in 400 mg 3' SL and 200 mg 3' SL + 200 mg 6' SL treated groups. **(B-D)** Quantification result of microscopic observations cartilage based on OARSI scores. Error bars represent the SD; *p < 0.05, Kruskal–Wallis one-way analysis of variance. Scale bars: A, 200 μ m.

tibia cartilage in the 200 mg 3' SL and 200 mg 3' SL + 200 mg 6' SL treatment groups. However, there was no difference in cartilage disruption at both sides of the femur cartilage (**Figure 3B**). Moreover, chondrocyte density mainly decreased in both sides of the tibia, but chondrocytes remained in the right tibia cartilage in the 400 mg 3' SL and 200 mg 3' SL + 200 mg 6' SL treatment groups (**Figure 3C**). However, multiple cell nests were observed in all groups, and no cell cloning scoring was statistically significant in any of the groups (**Figure 3D**). These data strongly suggest that 3' SL and 6' SL have potent roles in cartilage protection in mini-pig osteoarthritis modeling.

Anti-inflammatory effects of 3' SL in human macrophage

To understand the mechanism of action of 3' SL, we analyzed the expression of cytokines in differentiated macrophages. mRNA expression of pro-inflammatory cytokines IL-1 β and TNF- α increased after LPS treatment in differentiated macrophages. In cells treated with 3' SL 24 h before LPS treatment, the expression of IL-1 β and TNF- α was reduced (**Figure 4A and 4B**). We also confirmed protein expression by ELISA and measured the levels of pro-inflammatory cytokines in the culture supernatant; IL-1 β and TNF- α were decreased in the culture supernatant (Figure 4C and 4D). The 3' SL treatment with LPS did not reduce IL-1 β or TNF- α mRNA expression (data not shown). Interestingly, mRNA expression of anti-inflammatory cytokines IL-10 and TGF- β also decreased after 3'

SL treatment in the differentiated macrophages. These results indicated that 3' SL not only inhibited cytokine expression of IL-1 β and TNF- α in differentiated macrophages induced by LPS, but also showed a tendency to inhibit IL-10. We further investigated whether the 3' SL-mediated reduction in cytokine expression level is related to macrophage polarization by examining the expression of specific markers CCR7 and CD163 in M1 and M2 macrophages, respectively. We found that both M1 and M2 macrophage markers were decreased after 3' SL treatment. This indicates that the reduction in cytokine expression was due to the maintenance of M0 macrophages, which did not differentiate into M1 or M2 macrophages.

Discussion

Osteoarthritis is one of the major joint disorders in the elderly, and as the population ages, the number of patients with osteoarthritis is increasing rapidly. In particular, the lost articular cartilage is difficult to regenerate, and the loss of articular cartilage is accompanied by local inflammation and pain. Thus, the current treatment for osteoarthritis is focused on symptom relief, not disease-modification [22]. The present study provides viable treatment options for degenerative arthritis, aimed at preventing or slowing the disease progression.

In this study, we demonstrated the effectiveness of 3' SL and

Figure 4.

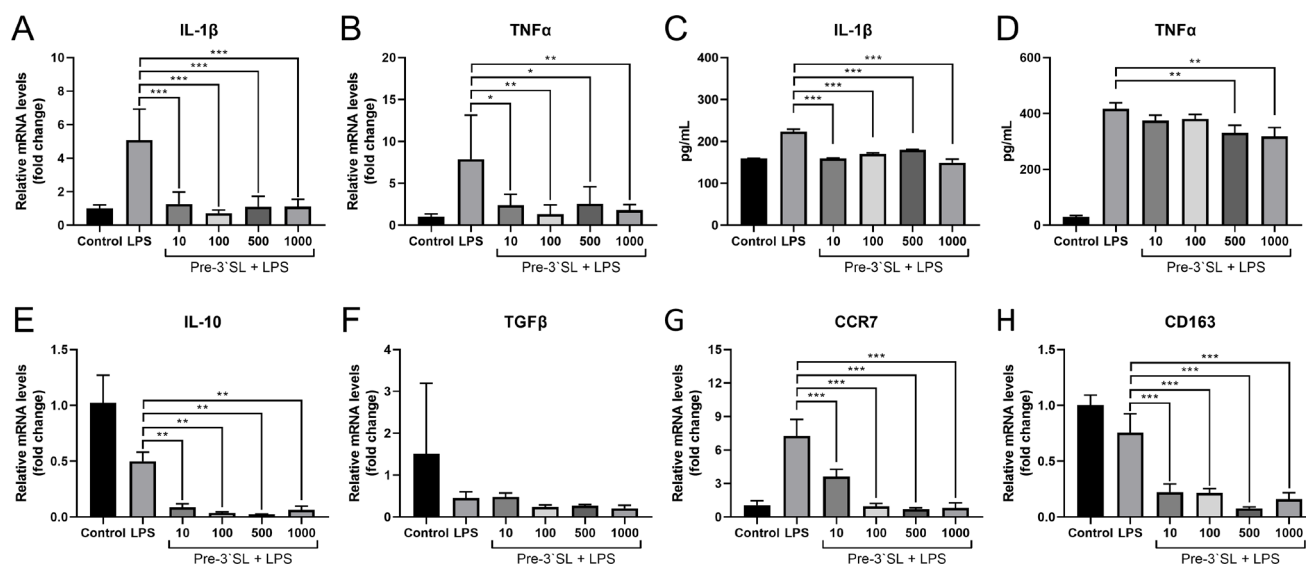


Figure 4. Inhibition of cytokine expression by 3' SL in differentiated THP-1 cells. (A–D) 3' SL inhibited LPS-induced expression of inflammatory cytokines IL-1 β and TNF- α in the differentiated THP-1 cells as determined by qRT-PCR (A, B) and ELISA (C, D). (E, F) 3' SL inhibited LPS-induced expression of anti-inflammatory cytokines IL-10 and TGF- β in the differentiated THP-1 cells as determined by qRT-PCR. (G, H) 3' SL inhibited macrophage polarization and retained M0 macrophage. Error bars represent the SD. *p<0.05, **p<0.01, ***p<0.001, One-way analysis of variance.

6' SL in preventing osteoarthritis using Yucatan mini-pigs. Osteoarthritis caused by anterior cruciate ligament incision showed cartilage destruction of the right tibial–femoral joint as well as the left tibia–femoral joint at the surgical site. However, oral administration of 3' SL and 6' SL alleviated lameness as well as cartilage disruption on both sides of the knee joint. Previously, protection effects of 3' SL against osteoarthritis were demonstrated in an osteoarthritis mouse model induced by destabilization of the medial meniscus [12]. Similar to mice results, treatment of 3' SL reduced articular cartilage damages in macroscopic and microscopic quantification. Interestingly, simultaneous administration of half-dose 3' SL and 6' SL also showed similar protective effects in the articular cartilage. Based on these mice and our mini-pigs results, 3' SL and 6' SL may have potential roles in protective effects in osteoarthritis. Moreover, continuous pain while walking is a major problem for patients with osteoarthritis that reduces the quality of life [23,24]. After oral administration of 3'-SL and 6'-SL, lameness of the left hind limb was reduced. Consequently, the improvement in gait disturbances is thought to be of great significance not only in preventing cartilage disruption but also in reducing pain.

In general, the intake of natural products for osteoarthritis prevention or treatment is meant to be for long periods of time, therefore their safety must be ensured. 3' SL and 6' SL are known to be safe substances and are used as food supplements [10,11]. Here, we conducted hematological and serum biochemistry analyses during 3' SL and 6' SL treatment periods, and any abnormalities were not detected (**Supplementary Tables 1 and 2**).

Macrophages are known to be the main inducers of osteoarthritis, releasing pro-inflammatory cytokines and MMPs. Macrophage polarization also affects the progression of osteoarthritis. The frequency of M1 macrophages, the main source of IL-1 β and TNF- α , was found to be increased in patients with osteoarthritis [25]. In addition, as M1 macrophage polarization accelerates, osteoarthritis symptoms become more severe due to an imbalance in the ratio of M1/M2 macrophages [26]. To attenuate osteoarthritis caused by macrophage-released-factors, we treated differentiated THP-1 cells with 3' SL, which was reported to protect against osteoarthritis development in mice, before inducing inflammation using LPS. We found that in addition to pro-inflammatory cytokines that were significantly decreased by 3' SL treatment, anti-inflammatory cytokines were also decreased. Moreover, there was a greater decrease in the expression of M1 and M2 specific markers in the 3' SL treatment group compared to the control group. Together, IL-1 β induce osteoarthritic pathogenesis by oxidative stress and inflammation but 3'-Sialyllactose can suppress of oxidative stress and inflammation [27]. Thus, we demonstrated that 3' SL maintains the state of resting macrophages and protects from LPS-induced inflammation.

Conclusion

In conclusion, results from this study demonstrated that mini-pig lameness gaits and cartilage disruption were alleviated 3' SL and 6' SL treatment. These protective effects on articular cartilage disruption were due to reduced pro-inflammatory (IL-1 β , TNF- α) and anti-inflammatory cytokine (IL-10, TGF- β) expression. Taken together, these are promising natural oligosaccharides that may be used as therapeutics for protective effects against osteoarthritis.

Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author Contributions Statement

Kyung-Tai Kim, Young-Kyu Kim, and Jeong Ho Hwang contributed to the conception and design of all study. Kyung-Tai Kim performed mini-pig osteoarthritis modeling, gait analysis, scoring of cartilage destruction and the statistical analysis. Mi-Jin Yang performed the hematology, serum biochemistry and histological analysis of cartilage. Min-Young Kim and Lila Kim generated 3' SL and 6' SL tablets. Young-Kyu Kim performed the THP-1 cell culture and qRT-PCR.

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