MASTER'S THESIS

Comprehensive *in vitro* and *in vivo* investigations of the therapeutic potential of Jeju lava seawater salt in osteoarthritis

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Interdisciplinary Graduate Program of Advanced Convergence Technology & Science

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Comprehensive *in vitro* and *in vivo* investigations of the therapeutic potential of Jeju lava seawater salt in osteoarthritis

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국문 초록

소금은 체액의 양을 조절하는 등 인간의 건강을 유지하는데 중요한 역할을 한다. 하지만 일반적인 해수를 이용한 소금은 미세플라스틱의 오염에 노출되어 있고 건강상의 문제를 야기할 가능성이 있다. 제주 용암해수(Jeju Lava Seawater, JLS)는 제주에만 존재하는 자원으로 미세플라스틱이 존재하지 않고 마그네슘, 칼슘 등 필수적인 미네랄이 풍부하다. 하지만 용암해수의 섭취가 퇴행성 관절염에 미치는 영향에 대해서 연구된 바가 없어 해당 연구를 진행하였다.

연골세포에 제주 용암해수유래 소금과 대조군인 천일염, 심층해수유래 소금을 처리한 세포독성 실험 결과 0.5%의 농도에서 모든 소금은 독성이 없음을 확인하였다. 연골세포에 농도별 (0.1, 0.2, 0.5%)로 소금들을 처리하여 확인한 결과 제주 용암해수유래 소금에 의한 anabolic factor (Aggrecan, Sox9, Col2a1)의 발현 증가와 catabolic factor (Mmp3, Mmp13, Adamts4, Adamts5)의 발현 감소를 확인하였다. 전염증성 사이토카인과 동시에 소금들을 처리한 결과 제주 용암해수유래 소금에 의한 anabolic factor 의 발현 증가와 catabolic factor 의 발현 감소를 확인하였다.

8 주령 수컷 C57BL/6 생쥐에게 일반 식수 (TAP)와 1% JLS 를 4 주 동안 급여 후 내측반월판절제술 (DMM)을 시행, 8 주 간 급여를 더 진행했다. 그 결과 연골의 퇴행이 미세하게 회복되었음을 확인하였다.

이 결과로 JLS 의 섭취가 관절염의 증상 완화에 도움을 줄 수 있다는 것을 알 수 있다.

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ABSTRACT

Salts play a crucial role in maintaining human health by regulating fluid levels and supporting various physiological processes. However, conventional seawater-derived salts are associated with microplastic pollution and pose potential health risks. Jeju lava seawater (JLS), sourced exclusively from Jeju Island, has emerged as a unique alternative, devoid of microplastics and enriched with essential minerals such as magnesium, calcium, zinc, and iron. In this study, we investigated the effects of JLS on osteoarthritis (OA) pathogenesis, with emphasis on chondrocyte metabolism and OA development. Surgical destabilization of the medial meniscus was performed to establish a murine model of OA. Additionally, we examined the expression of catabolic and anabolic factors in JLS-treated chondrocytes. Cell viability assay revealed that JLS treatment was not cytotoxic to chondrocytes at concentrations ≤ 0.5%. Additionally, JLS treatment caused a concentration-dependent increase in the expression of anabolic factors such as Aggrecan, SOX9, and COL2A1, but decreased the expression of catabolic factors such as Mmp3, Mmp13, Adamts4, and Adamts5 in chondrocytes stimulated with pro-inflammatory cytokines. Although not statistically significant compared with the control group, JLS intake slightly attenuated OARSI score, osteophyte score, synovitis score, subchondral bone thickness, and osteophyte size in a mouse model of OA. Conclusively, these results suggest that JLS ameliorates OA by positively influencing chondrocyte metabolism, making it a therapeutic candidate for the OA management.

Keywords: Jeju Lava Seawater Salt, osteoarthritis, chondrocytes

I. INTRODUCTION

Arthritis is associated with severe joint pain, and there is currently no effective treatment strategy. Although pharmaceutical interventions exist, natural remedies such as salt therapy are equally effective for arthritis and joint pain (Garver et al., 2015). Salt therapy focuses on addressing inflammation, which is the primary cause of pain, by introducing natural salt into the joints and supplying calcium for bone strength. Notably, the natural and organic salts utilized in this therapy not only relieve pain but also contribute to overall mental and physical well-being, and they do not possess side effects (Shah 2019).

Salt therapy involves deep penetration of the cells of the body, allowing negative ions and properties to be inhaled or absorbed through the skin. These elements target the inflamed joints and dehydrating cells responsible for inflammation at the molecular level. Unlike prescription medications, which simply mask pain, salt therapy directly addresses the underlying inflammation, leading to consistent improvements with regular sessions (Wasik and Tuuminen 2021). In addition to relieving arthritic pain, salt therapy promotes bone health by increasing calcium deposition (Gregory et al., 2015). This dual benefit makes salt therapy the preferred alternative to prescription medications. The simplicity and flexibility of salt therapy sessions make them accessible and offer safer options than prescription drugs, which have potential side effects. Additionally, salt therapy provides various health benefits, including skin cleansing, stress reduction, improved sports performance, and improved immune system. Moreover, the positive effects of salt therapy extends to conditions such as

osteoarthritis (OA), rheumatoid arthritis, asthma, and chronic obstructive pulmonary disease, leveraging its natural properties for effective pain and inflammation relief (Wasik and Tuuminen 2021).

Traditionally, salt is derived from common seawater; however, salt derived from seawater may be susceptible to contaminants such as microplastics, which are minute particles smaller than 5 mm in size. Recent studies have highlighted the presence of microplastics in dietary salts, raising concerns regarding human exposure to these particles (Rubio-Armendáriz et al., 2022). Once absorbed into the human body, microplastics can exert various health effects, including oxidative stress, cellular damage, inflammation, immune reactions, and neurotoxic effects (Yong et al., 2020). Therefore, it is importance to address and mitigate potential health implications associated with the presence of microplastics in salt sources.

Jeju lava seawater (JLS), a unique resource found exclusively on Jeju Island, undergoes specialized filtration as it traverses volcanic rock masses (Lee and Sim 2013). This distinctive filtration method distinguishes it from conventional seawater salts, ensuring the absence of microplastics. Moreover, seawater undergoes meticulous sterilization and contains essential functional minerals such as magnesium and calcium, and less common minerals such as zinc, iron, and vanadium (Kim et al., 2012). Notably, JLS possess anti-inflammatory properties (Lee et al., 2019), making it a promising material for treating inflammations. Moreover, the application of JLS in balneotherapy has been explored clinically, particularly in the context of OA (Kim et al., 2020). This underscores the distinctive qualities and potential therapeutic benefits of JLS.

Although the dietary intake of JLS has been associated with mineral-related

benefits, including anti-inflammatory and antioxidant effects, studies are yet to explore the specific effects of JLS on OA. Therefore, this study aimed to investigate the anti-inflammatory effects and mechanisms of JLS in OA using *in vitro* and *in vivo* experiments. Particularly, we compared the therapeutic effects of JLS in a mouse model of OA induced via destabilization of medial meniscus (DMM).

II. MATERIALS AND METHODS

2.1. Ethical statement and preparation of Salt samples

The JLS, supplied by Jeju Mineral Salt (Jeju-si, South Korea), was obtained from lava seawater containing 3.5% salt. The original concentration of JLS was increased to 17% via evaporation using spray drying. Natural Korean sea salt (KS) and deep ocean seawater salt (DS) were purchased from reputable suppliers. All animal experiments were conducted in accordance with the guidelines and regulations approved by the Jeju National University Institutional Animal Care and Use Committee (approval numbers: 2020-0002, 2022-0059).

2.2. Inductively coupled plasma-mass spectrometry (ICP-MS)

Salts were prepared using a high-performance microwave digestion system (ETHOS UP; Milestone Srl, Italy). The procedure involved melting the samples at a temperature of 80 ℃ for 48 h. Thereafter, the mineral contents of the samples were analyzed using the NexION® 2000 ICP-MS (PerkinElmer, USA). This approach ensured accurate and comprehensive determination of the mineral contents of the salts.

2.3. Chondrocyte culture

To obtain chondrocytes for *in vitro* culture, ICR mice (4–5 days old) were euthanized and whole knee joints were removed and stored in phosphate-buffered saline (PBS) containing antibiotics. After thoroughly washing the knee joints with

antibiotic-containing PBS (2–3 times), the joints were treated with trypsin-ethylenediaminetetraacetic acid solution containing type 2 collagenase, followed by incubation at 37 ℃ for 3 h in a 5% CO_2 atmosphere under intermittent vortexing. Thereafter, the cartilage tissue was extracted from the knee joints, washed three times with PBS, and incubated in DMEM containing type 2 collagenase for 1.5 h. After incubation, the isolated chondrocytes were centrifuged at 1,000 rpm for 3 min, washed twice with PBS, and seeded in either 6-well plates (at a density of 3×10^5 cells) or 96-well plates (at a density of 3×10^4 cells) for subsequent experimental procedures.

2.4. MTT assay and real-time polymerase chain reaction (RT-PCR)

Chondrocytes were seeded in 96-well plates (3×10^4 cells/well) and incubated at 37 ℃ for 12–16 h under a 5% CO_2 atmosphere. The culture medium was replaced with solutions containing 0.1, 0.2, and 0.5% salts, followed by incubation for an additional 24 h. After removing the culture medium, the MTT solution was added and incubated for 4 h. Finally, 50 μl of isopropanol was added to the wells, and the optical density was measured at 450 nm using a SpectraMaxi3X spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at the Bio-Health Materials Core-Facility, Jeju National University.

For RT-PCR, chondrocytes were harvested after a 24-h incubation period, and total RNA was extracted from the cells using TRIzol reagent. Total RNA was reverse-transcribed to generate cDNA using PrimeScript RT kit. PCR amplification of anabolic and catabolic genes was performed on a the Veriti Thermal Cycler (Thermo Fisher Scientific, Applied Biosciences) PCR system or BIO-RAD Real-Time PCR system

(CFX96™ Real-Time System, Bio-Health Materials Core-Facility, Jeju National University) using SYBR premixed Extaq reagent (Takara Bio, Mountain View, CA, USA) and specific primers. The primers used in this study are listed in **Table 1**.

Table 1. List of primers used for RT-PCR

Primer	Sequence (5'->3')	Tm value (℃)	Length (bp)
Mo Gapdh forward	TCACTGCCACCCAGAAGAC	58	450
Mo Gapdh reverse	TGTAGGCCATGAGGTCCAC		
Mo Sox9 forward	ATGCTATCTTCAAGGCGCTG	60	272
Mo Sox9 reverse	GACGTCGAAGGTCTCAATGT		
Mo Col2a1 forward	CACACTGGTAAGTGGGGCAAGACCG	58	173
Mo Col2a1 reverse	GGATTGTGTTGTTTCAGGGTTCGGG		
Mo Aggrecan forward	GAAGACGACATCACCATCCAG	60	581
Mo Aggrecan reverse	CTGTCTTTGTCACCCACACAT		
Mo Mmp3 forward	AGGGATGATGATGCTGGTATGG	58	434
Mo Mmp3 reverse	CCATGTTCTCCAACTGCAAAGG		
Mo Mmp13 forward	TGATGGACCTTCTGGTCTTCTGG	58	474
Mo Mmp13 reverse	CATCCACATGGTTGGGAAGTTCT		
Mo Adamts4 forward	CATCCGAAACCCTGTCAACTTG	58	287
Mo Adamts4 reverse	GCCCATCATCTTCCACAATAGC		

Mo forward	Adamts5	GCCATTGTAATAACCCTGCACC	58	292
Mo reverse	Adamts5	TCAGTCCCATCCGTAACCTTTG		
Mo forward	Mmp9	TGCACTGGGCTTAGATCATTCC	58	450
Mo reverse	Mmp9	CCGTCCTTGAAGAAATGCAGAG		
Mo forward	Col10a1	GCCAGGTCTCAATGGTCCTA	58	484
Mo reverse	Col10a1	AAAAGCAGACACGGGCATAC		
Mo forward	Runx2	GCCACCTTTACCTACACCCC	60	117
Mo reverse	Runx2	ACTCTGGCTTTGGGAAGAGC		

2.5. Destabilization of the medial meniscus (DMM)

C57BL/6 mice (4-week-old) were randomly divided into two groups (n = 20 mice/group: tap water (TAP) and JLS groups. Mice in the TAP and JLS groups received tap water and 1% JLS, respectively, for 4 weeks. After this period, surgical destabilization of the medial meniscus (DMM) was to establish a murine model of OA, as previously described (Son et al., 2017, Son et al., 2019). Thereafter, the mice continued their respective drinking regimens for an additional 8 weeks. After 8 weeks post-surgery, the knee joints of the mice were collected, fixed with 4% paraformaldehyde, and decalcified with 0.5 M of ethylenediaminetetraacetic acid (EDTA) for 2 weeks. This experimental design aimed to assess the effects of JLS on DMM-induced OA in mice.

2.6. Histological analysis

Briefly, knee samples were dehydrated, paraffin-embedded, cut into 5-μm thick sections, and stained with safranin-O to assess the extent of cartilage degeneration. Four experienced researchers individually assessed and scored the samples to evaluate parameters, such as osteophyte formation and synovitis, using the Osteoarthritis Research Society International (OARSI) scoring system. Subchondral bone thickness and osteophyte size were quantified using Adobe Photoshop 2021 (Adobe Systems, Inc., CA, USA). This analysis aimed to provide a comprehensive evaluation of knee joint condition, including cartilage health, bone morphology, and pathological changes.

III. RESULTS

3.1. Differences in mineral ratio between JLS and others salts

The mineral compositions and concentrations of JLS, KS, and DS were analyzed inductively coupled plasma mass spectrometry (ICP-MS), and the concentrations of the minerals were expressed as parts per million (ppm). Notably, the concentrations barium (Ba) and rubidium in JLS, KS, and DS were 0.650, 0.228, and 0.168 ppm, respectively, and 1.161, 0.752, and 2.614 ppm, respectively. Additionally, the lithium (Li) and molybdenum (Mo) concentrations of JLS, KS, and DS were 1.745, 1.519, and 4.196 ppm, respectively, and 3.717, 1.104, and 11.825 ppm, respectively. Moreover, the concentrations of vanadium (V) and boron (B) in JLS, KS, and DS were 2.625, 2.526, and 2.849 ppm, respectively, and 37.581, 36.827, and 94.031 ppm, respectively. However, selenium (Se) and uranium (U) were not detected in JLS, KS, and DS. The concentrations of strontium (Sr) in JLS, KS, and DS were 231.017, 55.232, and 190.351 ppm, respectively. Additionally, the concentrations of selected trace elements (Mn, Co, Ni, Cu, Cd, Pb, Zn, and Fe) and major elements (Na, Ca, K, and Mg) were provided in PPB and PPM, respectively.

Table 2. ICP-MS analysis of Jeju lava seawater (JLS), Korean natural sea salt (KS), and deep ocean sea water salt (DS).

	JLS	KS	DS
Ba	0.650	0.228	0.168
Ga	N.D	N.D	N.D
Ge	N.D	N.D	N.D
Rb	1.161	0.752	2.614
Li	1.745	1.519	4.196
Mo	3.717	1.104	11.825
V	2.625	2.526	2.849
B	37.581	36.827	94.031
Cs	N.D	N.D	N.D
Se	N.D	N.D	N.D
Sr	231.017	55.232	190.351
U	N.D	N.D	N.D
As	0.024	0.009	0.048
Mn	0.259	0.331	0.221
Co	0.099	0.021	0.021
Ni	1.404	1.959	0.904
Cu	0.441	0.789	0.574
Cd	0.013	0.006	0.008
Pb	0.613	0.414	0.534

Zn	1.953	1.773	2.273
Fe	N.D	0.315	0.908
Na	286,828	245,257	223,075
Ca	7,692	517	8,662
K	7,725	7,140	12,939
Mg	14,256	13,539	38,159

N.D: Not Detected; Unit: ppm

3.2. Effects of salts on the viability of primary cultured chondrocytes

Treatment with 0.1–0.5% of JLS, KS, and DS exhibited no cytotoxic effects on the chondrocytes (Figure. 1). Notably, treatment with 0.5% of JLS significantly increased chondrocyte viability (Figure. 1a). However, treatment with the salts at concentrations > 0.5% showed evidence of cytotoxicity (not reported). Overall, these results suggest that salt treatment at concentrations of 0.1–0.5% did not adversely affect chondrocyte viability, whereas higher salt concentrations may by cytotoxic to chondrocytes. Based on these results, we decided to use 0.5% as the maximum salt concentration in subsequent experiments.

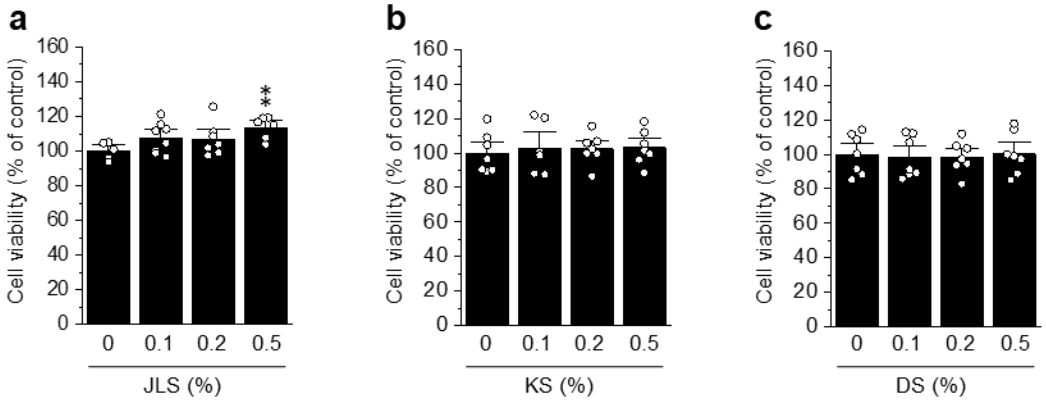

Figure. 1. The effects of salts on the viability of chondrocytes were examined using MTT assay. Chondrocytes were treated with Jeju lava seawater salt (JLS), Korean sea salt (KS), and deep ocean seawater salt (DS) at concentrations of 0.1, 0.2, and 0.5% for 24 h, followed by MTT assay. $^{**}p < 0.01$.

3.3. Effects of salts on anabolic and catabolic factors of chondrocytes

To elucidate the mechanism of JLS on chondrocytes, we examined the effects of the salts on the expression of anabolic and catabolic factors in cultured primary chondrocytes. Salt treatments did not significantly affect SOX9 and COL2A1 expression in chondrocytes, suggesting that the investigated salts did not affect the crucial anabolic factors associated with chondrocyte function (Figure. 2a–c). In contrast, JLS treatment caused a concentration-dependent increase in Aggrecan expression but decreased Mmp3, Mmp13, and Adamts5 expression in chondrocytes (Figure. 2d–f). Collectively, these results indicate that JLS may suppress the degradation of extracellular matrix (ECM) components by inhibiting the activities of Mmp3, Mmp13, and Adamts. Additionally, the decrease in the expression of catabolic factors suggests that JLS may prevent cartilage degradation by maintaining ECM integrity.

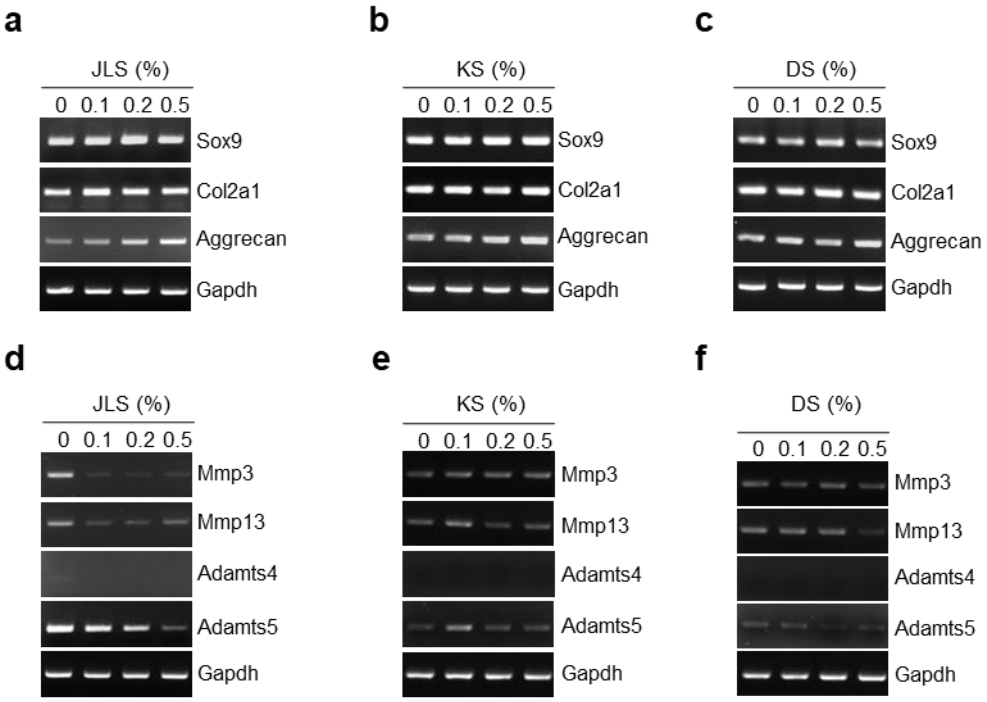

Figure. 2. The effects of salts on the expression of anabolic and catabolic factors in chondrocytes. Chondrocytes were treated with 0.1, 0.2, and 0.5% of Jeju lava seawater salt (JLS), Korean sea salt (KS), and deep ocean seawater salt (DS) for 24 h. RT-PCR was performed to analyze the mRNA expression of SOX9, COL2A1, Aggrecan, Mmp3, Mmp13, Adamts4, and Adamts5. GAPDH was used as an internal marker.

3.4. Effects of salts on hypertrophic marker genes in primary cultured chondrocytes

RT-PCR was performed to examine the mRNA expression of the hypertrophic markers Mmp9, Col10a1, and Runx2. JLS treatment did not significantly affect the expression of Mmp9, Col10a1, and Runx2 in chondrocytes (Figure. 3a), indicating that JLS did not induce hypertrophic changes in chondrocytes. Chondrocyte hypertrophic differentiation is associated with the transformation of chondrocytes into a hypertrophic phenotype, which plays a role in endochondral ossification. Notably, DS treatment (0.5%) significantly increased the expression of Col10a1 (Figure. 3c). Col10a1 is a well-established marker of chondrocyte hypertrophic differentiation, and an increase in its expression indicates that DS may promote chondrocyte hypertrophic differentiation. Hypertrophic differentiation is a critical stage in cartilage development characterized by the enlargement and mineralization of chondrocytes, and its dysregulation contributes to pathological conditions such as OA.

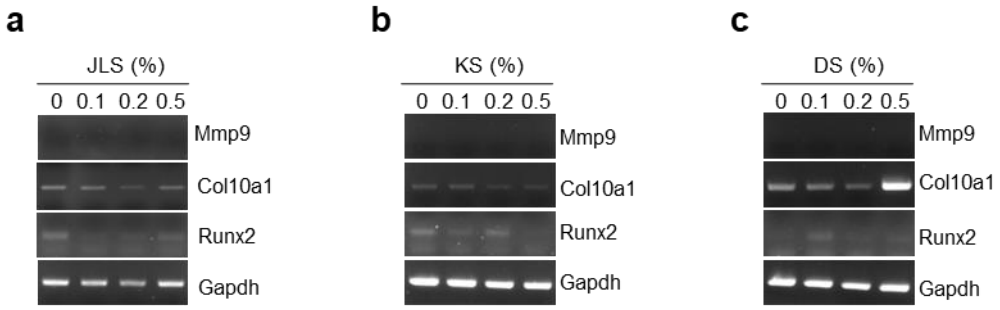

Figure. 3. The effects of Jeju lava seawater salt (JLS), Korean sea salt (KS), and deep ocean seawater salt (DS) on the expression of hypertrophic marker genes in chondrocytes. Chondrocytes were treated with JLS, KS, or DS (0.1, 0.2, and 0.5%) for 24 h. RT-PCR was performed to analyze the mRNA expression of Mmp9, Col10a1, and Runx2, with GAPDH as the internal control.

3.5. Effects of salts on proinflammatory cytokine-induced expression of anabolic and catabolic factors

Furthermore, we examined the effects of JLS on proinflammatory cytokine-induced changes in the expression of anabolic and catabolic factors in chondrocytes. JLS and KS treatment reversed IL-1β (1 ng/ml)-induced decrease in Aggrecan expression in chondrocytes (Figure. 4a and e). Similarly, treatments with the salts reversed TNF-α (10 ng/ml)-induced decrease in the expression of SOX9, COL2A1, and Aggrecan in a concentration-dependent manner (Figure. 4b, f, and j). Additionally, JLS and KS treatments significantly reversed IL-6 (100 ng/ml)- and LPS (10 ng/ml)-induced alterations in SOX9 and Aggrecan expression in primary chondrocytes (Figure. 4c, d, g, and h).

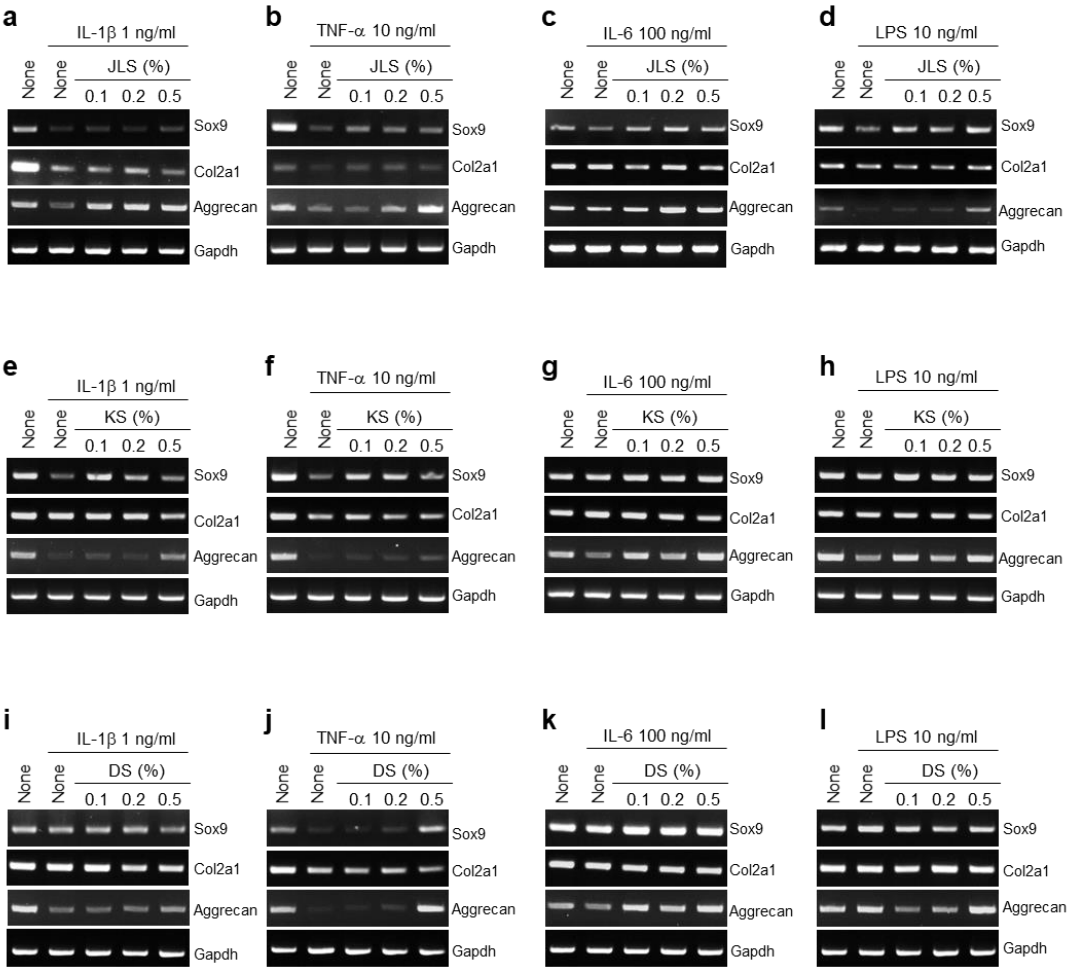

Figure. 4. The effects of Jeju lava seawater salt (JLS), Korean sea salt (KS), and deep ocean seawater salt (DS) on proinflammatory cytokine-induced expression anabolic factors in articular chondrocytes. Cells were treated with (a) 1 ng/ml of IL-1β, (b) 10 ng/ml of TNF-α, (c) 100 ng/ml of IL-6, and (d) 10 ng/ml of LPS in the absence or presence of JLS, KS, and DS (0.1, 0.2, and 0.5%) for 24 h. RT-PCR was performed to analyze the mRNA expression of SOX9, COL2A1, and Aggrecan, with GAPDH as the internal control.

Furthermore, JLS treatment significantly inhibited the expression of Mmp3, Mmp13, Adamts-4, and Adamts-5 induced by IL-1β (1 ng/ml), TNF-α (10 ng/ml), IL-6 (100 ng/ml), and LPS (10 ng/ml) in primary chondrocytes (Figure. 5a–d). Overall, these results underscore the anti-inflammatory role of JLS in modulating the expression of crucial factors associated with cartilage homeostasis. KS treatment significantly suppressed IL-1β (1 ng/ml)- and TNF-α (10 ng/ml)-induced expression of Adamts-4 and -5 in chondrocytes (Figure. 5e and f). Additionally, DS treatment significantly suppressed IL-1β (1 ng/ml)- and TNF-α (10 ng/ml)-induced increase in Mmp13 and Adamts5 expression (Figure. 5i and j). Moreover, DS treatment slightly decreased IL-6 (100 ng/ml)- and LPS (10 ng/ml)-induced increase in Adamts-4 and-5 expression in chondrocytes (Figure. 5k and l).

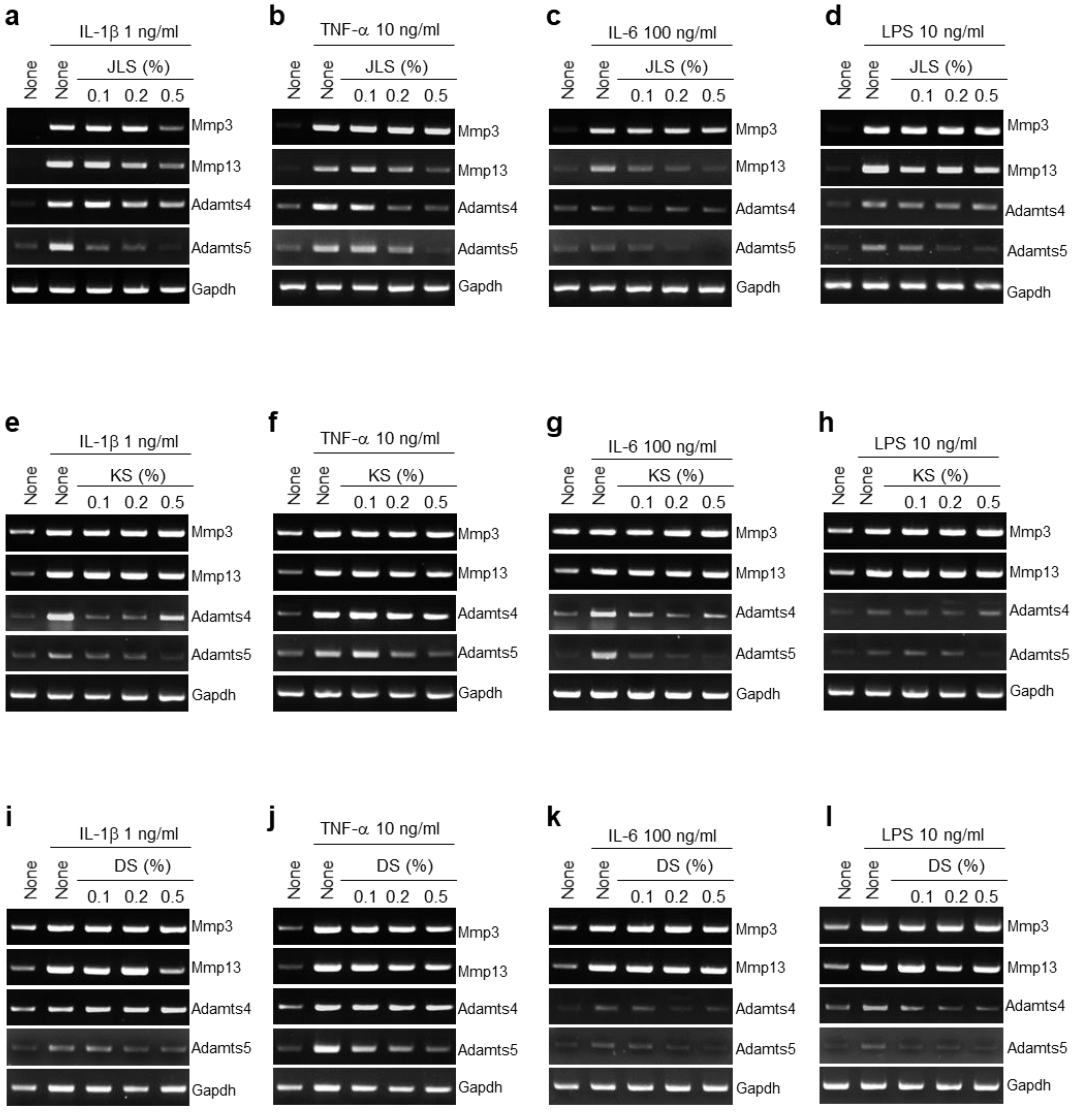

Figure. 5. The effects of Jeju lava seawater salt (JLS), Korean sea salt (KS), and deep ocean seawater salt (DS) on proinflammatory cytokine-induced expression catabolic factors in articular chondrocytes. Cells were treated with (a) 1 ng/ml of IL-1β, (b) 10 ng/ml of TNF-α, (c) 100 ng/ml of IL-6, and (d) 10 ng/ml of LPS in the absence or presence of JLS, KS, and DS (0.1, 0.2, and 0.5%) for 24 h. RT-PCR was performed to analyze the mRNA expression of Mmp3, Mmp13, Adamts4, and Adamts5, with GAPDH as the internal control.

3.6. JLS may attenuate DMM-induced osteoarthritis

To investigate the anti-osteoarthritis effects of JLS *in vivo*, mice with DMM-induced OA were administered JLS. Compared with that in the TAP group, JLS treatment decreased cartilage erosion in mice with DMM-induced OA (Figure. 5a). Although not statistically significant, JLS treatment decreased OARSI score, osteophyte maturity, and subchondral bone plate thickness in mice with DMM-induced OA (Figure. 6b–d). Additionally, JLS treatment did not significantly affect osteophyte size or ameliorate synovitis (Figure. 6e–g).

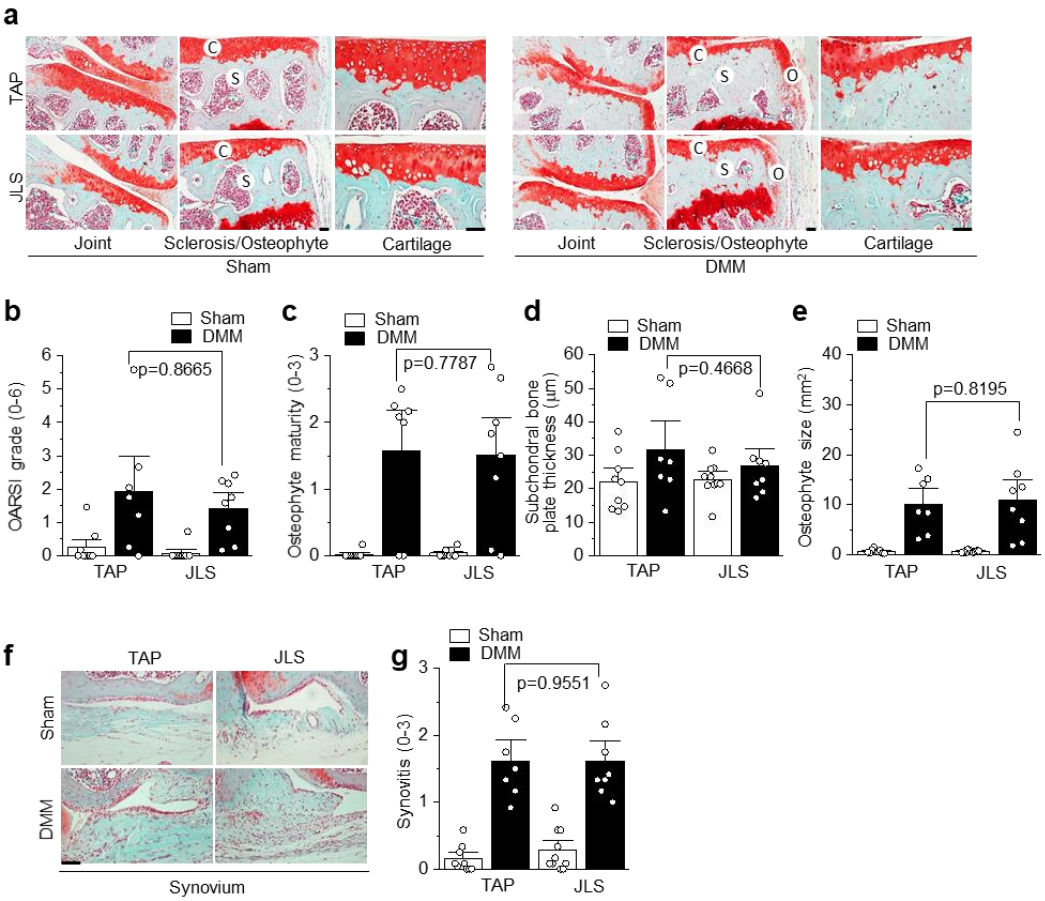

Figure. 6. The effects of Jeju mineral salt (JLS) on the pathogenesis of osteoarthritis (OA) in a murine model of DMM-induced OA. Mice in the tap water (TAP, n = 7) and JLS (n = 8) groups underwent DMM surgery, followed by further breading for 8 weeks. (a) The joint, osteophyte, and cartilage tissue were visualized using Safranin O staining. The OARSI score (b), osteophyte maturity (c), subchondral bone thickness (d), and osteophyte size (e) were quantified. The synovium (f) and synovitis (g) were represented. Statistical analysis wass performed using Mann-Whitney U test. C=cartilage, S=sclerosis, O=osteophyte. Scale bar: 100 μm

IV. DISCUSSION

OA is characterized by morphological changes in joint, including degeneration, fibrillation, and erosion of the cartilage surface. Osteophyte formation, which is characterized by bony outgrowth at the joint margins, often manifests in response to cartilage degeneration (Nuki 1999). Hypertrophic alterations in chondrocytes signal an increase in cell size and metabolic activity, and chondrocytes are the primary cellular components of cartilage (Pacifici et al., 1990). Maintaining cartilage homeostasis relies on a delicate balance between anabolic (SOX9, Aggrecan, COL2A1) and catabolic (Mmps and ADAMTs) factors. Anabolic factors contribute to the synthesis and maintenance of the ECM in cartilage, thereby ensuring structural integrity (Kim et al., 2015). In contrast, catabolic factors are involved in ECM degradation and turnover. Notably, increased catabolism or decreased anabolism contributes to the degradation of cartilage components and promotes the development and progression of OA (Mueller and Tuan 2011) (Firestein et al., 2012). Therefore, a comprehensive understanding of these molecular and cellular processes is necessary for the development of effective treatments for OA.

In the present study, JLS treatment upregulated the expression of Aggrecan, a crucial protein for cartilage organization and ECM stabilization, in chondrocytes (Kiani et al., 2002). Additionally, JLS treatment caused a decrease in the expression of Mmp13 and Adamts5. Mmp13, a member of the matrix metalloproteinase family, plays a critical role in cartilage degeneration, and a decrease in its expression inhibits of OA progression (Wang et al., 2013). Adamts5, a member of the Adamts family, is

involved in the cleavage of the Aggrecan core protein and contributes to ECM degradation (Abbaszade et al., 1999). Collectively, these findings suggest that JLS exerts positive effects on normal chondrocyte function, potentially influencing the maintenance of a healthy cartilage.

To elucidate the mechanism of JLS in OA, we examined the effects of JLS on the expression of anabolic and catabolic factors in chondrocytes. JLS treatment did not significantly affect the expression of key anabolic factors (SOX9 and COL2A1), indicating maintenance of cellular stability. However, JLS treatment caused a concentration-dependent increase in Aggrecan expression, indicating that JLS may positively regulate the synthesis of Aggrecan, an essential ECM component associated with cartilage health. Additionally, JLS treatment suppressed the expression the catabolic factors Mmp3 and Adamts5, indicating that JLS may prevent the degradation of ECM components. In contrast, JLS treatment did not significantly affect the expression of hypertrophy markers, such as Mmp9, Col10a1, and Runx2, indicating the absence of hypertrophic changes in chondrocytes. In contrast, DS treatment increased the expression of Col10a1, suggesting that DS may induce chondrocyte hypertrophic differentiation. Overall, these results suggest that JLS does not induce chondrocyte hypertrophic differentiation.

In the present study, the decision to choose 1% JLS concentration in the *in vivo* experiment was based on the recommended daily salt intake for mice and the WHO recommended daily salt intake for humans (World-Health-Organization 2012). Although JLS treatment did not induce any significant effect in the mouse model, further *in vivo* studies are necessary to elucidate the therapeutic effects of long-term

JLS administration in OA. Future studies should refine the conditions and concentrations of JLS to optimize its efficacy for therapeutic purposes in live organisms.

Cell viability assay showed that JLS, KS, and DS were not cytotoxic to chondrocytes at concentrations of 0.1–0.5%; however, JLS was cytotoxic at concentration > 0.5%. These results indicate that low concentrations of JLS are safe and well tolerated by the chondrocytes. Therefore, salt concentration was set at a maximum of 0.5% in subsequent experiments to preserving cellular health while exploring the potential impacts of the salts on chondrocyte function and behavior.

To evaluate the translational potential of these findings, an *in vivo* experiment was performed using a mouse model of DMM-induced OA. Several parameters, including the OARSI score, osteophyte size, synovitis score, subchondral bone thickness, and osteophyte size, were assessed. Despite the promising results of the *in vitro* experiments, JLS treatment did not significantly affect the examined parameter in the *in vivo* experiment. Overall, these results suggest that although JLS demonstrated positive effects on chondrocytes in a controlled *in vitro* environment, the complex *in vivo* conditions may introduce additional variables that may influence the overall effectiveness of JLS. However, we speculate that long-term treatment with JLS may exert promising effects in *in vivo* models. Several factors may contribute to the disparity between the *in vitro* and *in vivo* results, including the dosage of JLS, duration of exposure, and intricate interactions between the various elements present in the salt. Therefore, further exploration and refinement of the experimental conditions are necessary to comprehensively investigate the *in vivo* effects of JLS.

To gain a comprehensive understanding of the therapeutic potential of JLS in OA, future studies should investigate the interactions of JLS *in vivo*. This may involve adjusting the experimental parameters, exploring different concentrations, and considering the dynamic nature of OA progression. Collectively, these findings highlight the complexity of translating *in vitro* observations into *in vivo* outcomes and underscore the importance of continued research to elucidate the potential of JLS in OA treatment.

V. CONCLUSIONS

In this study, we examined the therapeutic effects and potential mechanisms of JLS in OA using *in vitro* and *in vivo* models. JLS treatment was not cytotoxic to chondrocytes at concentration ≤ 0.5%. Additionally, JLS treatment suppressed the degradation of ECM components by inhibiting the expression of Mmp3, Mmp13, and Adamts, but increased the expression of anabolic factors. Moreover, JLS treatment did not induce chondrocyte hypertrophic differentiation. Although not statistically significant, JLS treatment decreased OARSI score, osteophyte maturity, and subchondral bone plate thickness in mice with DMM-induced OA. Overall, these results suggest that JLS is promising for the treatment and prevention of OA. However, future studies should explore different concentrations of JLS to identify the optimal dosage for *in vivo* use. Moreover, human studies are necessary to evaluate the safety and efficacy of JLS to promote the clinical application of JLS in OA treatment.

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