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Original Article

Efficacy of Combination Therapy with Apigenin and Synovial Membrane-Derived Mesenchymal Stem Cells on Knee Joint Osteoarthritis in a Rat Model

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What's Known

- Mesenchymal stem cells have been widely used as a therapeutic approach in the cell-based therapy of osteoarthritis, with promising effects on cartilage regeneration.
- Apigenin acts as a free radical scavenger and anti-inflammatory agent.

What's New

- The co-injection of apigenin and mesenchymal stem cells to the knee will increase the success of osteoarthritis therapy.
- Apigenin effects are possibly mediated through the reduction of oxidative stress, suppression of inflammation, and promotion of production of extracellular matrix components.

Abstract

Background: Osteoarthritis (OA) is a degenerative joint disease that causes a variety of adverse health effects. Considering the need to identify additional effective therapeutic options for OA therapy, we investigated the effect of co-injection of apigenin and synovial membrane-derived mesenchymal stem cells (SMMSCs) on OA in male rats' knee joints.

Methods: The study was performed in 2019 at the Department of Pharmacology, Shiraz University of Medical Sciences, Shiraz, Iran. Anterior cruciate ligament transection (ACLT) was used to induce OA. For three weeks, male Sprague-Dawley rats (eight groups, n=6 each) were treated once-weekly with intra-articular injections of apigenin alone or in combination with SMMSC (three million cells), phosphate-buffered saline, or hyaluronic acid. After three months, the interleukin 1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), superoxide dismutase (SOD), and malondialdehyde (MDA) levels were measured in the cartilage homogenate. The expression of extracellular matrix (ECM) components including collagen 2a1, aggrecan, IL-1β, TNF-α, inducible nitric oxide synthase (iNOS), transcription factor SOX-9, and matrix metalloproteinases 3 and 13 were assessed using real-time polymerase chain reaction (RT-PCR) analysis. Radiological evaluation and histopathological assessment were used to evaluate the knees.

Results: Levels of TNF-α ($P=0.009$), MDA ($P0.001<$), and IL-1β ($P<0.001$) decreased and the level of SOD increased ($P=0.004$) in the apigenin 0.3 µM with SMMSCs group. RT-PCR analysis indicated that IL-1β in the apigenin 0.3 µM with SMMSCs group reduced significantly ($P<0.001$). This group also exhibited increased expression levels of SOX-9, collagen 2a1, and aggrecan ($P<0.001$).

Conclusion: Apigenin may have supplementary beneficial effects on cell therapy in a rat model of OA due to its possible effect on the reduction of oxidative stress, suppression of inflammation, and promotion of production of ECM components.

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Keywords ● Apigenin ● Synovial membrane ● Mesenchymal stem cells ● Osteoarthritis ● Inflammation

Introduction

Osteoarthritis (OA) is the leading cause of functional limitations and disability worldwide that adversely impacts patients'

quality of life. The disease is associated with degeneration of the cartilage, which causes chronic joint inflammation, leading to arthritis.[1] It has been shown that oxidative stress signaling pathways may play an important role in the pathogenesis of OA.[2] Inflammatory inducers initiate the production of inflammatory mediators such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, IL-8, adipokines, Toll-like receptor (TLR), and nitric oxide (NO).[3] IL-1β decreases the expression of type II collagen and increases the expression of nitric oxide-synthase (NOS), cyclooxygenase (COX)-2, and matrix metalloproteinases (MMPs).[4] These events accelerate destructive responses affecting extracellular matrix (ECM) components, catabolic activation, and apoptosis.[5]

There is no effective cure for OA, and available therapies mostly focus on managing symptoms. Regeneration of the damaged articular cartilage in OA has been a challenging topic.[2] Recently, mesenchymal stem/stromal cells (MSCs) have been introduced as the preferred alternative, since they do not have the drawbacks of other methods. The concept of using MSCs is based on the fact that synovial membrane-derived MSCs (SMMSCs) can differentiate into chondrocytes and regenerate the damaged regions due to various trophic factors. Several *in vivo* assessments (animal and human studies) confirmed the high potency of MSCs in cartilage repair.[3, 6, 7]

It has been shown that combination therapy could be a potential therapeutic approach to address OA symptoms.[8] Considering the role of a wide range of molecular mechanisms in the pathogenesis of OA, the use of combined agents that target these pathways is suggested.[9] For example, it has been shown that elevated oxidative stress markers are associated with the pathogenesis of OA. Hence, the use of antitoxin factors is effective in OA therapy.[9]

Flavonoids, a polyphenolic class of phytochemicals, were shown to control inflammatory arthritis due to their antioxidant, anti-inflammatory, and immunomodulatory properties.[10] Apigenin (4′,5,7-trihydroxyflavone) is a flavonoid found in fruits and vegetables with a wide spectrum of pharmacological activities including antioxidant, anti-inflammatory, and anti-cancer properties.[11] The anti-inflammatory activity of apigenin is also linked to its inhibitory effect on the production of cytokines such as IL-4, IL-5, or IL-13, and inhibition of NOS and COX2 enzymes in T and B cells.[12]

Considering the need to identify additional effective therapeutic options for OA treatment, we investigated the effect of the co-injection of apigenin and SMMSCs on OA in male rats' knee joints.

Materials and Methods

Male Sprague-Dawley rats (n=48), weighing 200±20 g and aged 10-12 weeks were obtained from the Central Animal House of Shiraz University of Medical Sciences (Shiraz, Iran). The rats were housed in a standard vivarium, fed a standard diet, and water ad libitum, and kept under standard conditions (12-hour light:dark cycle, temperature 20-25 ºC, and humidity 25-35%). Experimental protocols were according to the Guide for the Care and Use of Laboratory Animals. The study was approved by the Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran (code: IR.SUMS.REC.1397.228).

The rats were randomly divided into eight groups (n=6 per group), and anterior cruciate ligament transection (ACLT)[13] was used to induce OA, except for the sham group. For three weeks, the rats were treated once-weekly with intra-articular injections of apigenin alone or in combination with either SMMSCs (three million cells), phosphate-buffered saline (PBS), or hyaluronic acid (H).

Sham: No intervention+treatment with PBS
OA (Negative control): OA+treatment with PBS
Positive control: OA+treatment with H
OA+treatment with MSCs
OA+treatment with apigenin 0.1 µM
OA+treatment with MSCs and apigenin 0.1 µM
OA+treatment with apigenin 0.3 µM
OA+treatment with MSCs and apigenin 0.3 µM

Induction of ACLT

The rats were intraperitoneally anesthetized using 40 mg/kg ketamine (KetaVed®, Vedco, St. Joseph, MO, USA) and 10 mg/kg xylazine (Rompun; Bayer AG, Leverkusen, Germany). After shaving and disinfecting the rats, the left medial parapatellar was incised to expose the knee joint. The joint cavity was revealed and the anterior cruciate was exposed through knee flexion. An anterior cruciate ligament (ACL) was detached and the rupture was confirmed using the drawer test. The cartilage surface was not damaged during the operation. In the sham group, ACL was exposed through a small medial parapatellar incision, the joint was washed with saline, and then the incision was sutured. Flunixin (2.5 mg/kg/day; Banamine®, Merck Animal Health USA) was subcutaneously injected daily for three days for postsurgical analgesia. The rats were given supplemental heat and closely monitored until full recovery from anesthesia. They were also monitored

daily for pain, infection, and other postoperative complications. All procedures were approved by the Animal Welfare Committee.

Preparation of SMMSCs

Preparation of SMMSCs was performed according to the previously reported procedure.[14]

Cell-Surface Marker Analysis

SMMSCs were confirmed by evaluating the expression of MSCs specific surface markers (including CD34, CD45, CD29, CD73) using RT-PCR analysis.[15] Cultured cells were harvested, and total RNA was isolated using an RNA extraction kit (Qiagen, USA) according to the manufacturer's protocol. At the end of the isolation process, the concentration of samples was determined with a spectrophotometer (Nanodrop; Thermo Fisher Scientific, Wilmington, DE, USA). RevertAid™ First Strand cDNA Synthesis kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used for cDNA synthesis. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed using ABI Biosystems™ StepOne™ and RealQ Plus 2x Master Mix Green (Ampliqon A/S, Odense, Denmark). The *β2M* housekeeping gene was used as the internal control of qPCR reactions. Reactions were amplified in a thermal cycler (Applied Biosystems, Thermo Fisher Scientific Inc., USA) with thermal conditions set at 94 °C for 10 min followed by 40 cycles of 15 second at 94 °C, 60 second at 58 °C, a final extension of 7 min at 72 °C, and melt curve analysis of 55~95 °C at 0.5 °C per five second. The analysis of real-time PCR data was performed using the $2^{-\Delta\Delta Ct}$ method[16] with target mRNA expression in each sample normalized against the endogenous control.

Differentiation Assay

To induce differentiation, SMMSCs were seeded into 6-well plates at a density of 5×10^4 per well, and the medium was replaced after 24 hours with either adipogenic, osteogenic (R&D Systems), or fresh media. After three weeks, SMMSCs were washed twice in PBS, fixed for 30 min in 10% formaldehyde, and then washed with water twice. Then, SMMSCs were stained with fresh Oil Red O solution (0.5%) or Alizarin Red S solution (1.4%) for 40 min at room temperature.

Intra-Articular Injection

Two months after surgery, animals were checked for the induction of the ACLT model using radiological imaging. For three weeks, the rats were treated once-weekly with intra-articular injections of apigenin (Sigma Aldrich, 10798-100MG) alone or in combination with either SMMSCs or PBS vehicle under general anesthesia. Injections were carried out in both knees in a volume of 50 µl using Hamilton syringes.

Assessment of Cell Viability with MTT Assay

MTT assay was used to evaluate the potential toxicity of apigenin on the cells. SMMSCs were plated into 96-well plates at a density of 5×10^3 per well. After 24 hours, the media were replaced with fresh media enriched with different concentrations of apigenin (0, 0.1, 0.2, 0.3, 0.4, and 0.5 µM). After 24, 48, and 72 hours, cell viability was measured by MTT assay. After incubation with MTT solution (5 mg/ml) at 37 °C and 5% CO_2 for four hours, the supernatant was discarded and dimethylsulphoxide (DMSO) was added to each well. Cell viability was calculated as the ratio of optical density (OD) of each concentration point, measured at 570 nm, relative to that of the negative control.

Radiography

Knee joints were examined under general anesthesia using a Dry Imager (Fujifilm DryPix 6000 smart Tokyo, Japan) to evaluate the severity of OA before and after treatment. Radiographic grading was based on previously published numerical rating scales.[17]

Knee Joint Specimen

After imaging, animals were sacrificed by cervical dislocation. Knee joints were exposed and dissected aseptically from superior and inferior tissues. Some specimens were fixed in 10% paraformaldehyde for histopathological study, and some others were immediately frozen in liquid nitrogen and stored at -80 °C for enzyme-linked immunosorbent assay (ELISA) and PCR analysis.

ELISA

IL-1β, TNF-α, and superoxide dismutase (SOD) levels in the cartilage homogenate (SpeedMill PLUS; Analytik Jena, Germany) were measured using commercially available ELISA kits (Abcam, USA), according to the manufacturer's instructions. OD was measured at 450 nm using a microplate reader (POLARstar, BMGLabtech, Ortenberg, Germany). Data were normalized by detecting the protein concentration of samples using bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China).

Malondialdehyde (MDA) Measurement

MDA is a product of lipids peroxidation and is known as a marker of oxidative stress intensity.

MDA can be quantified based on its reaction with thiobarbituric acid, a chromogenic reagent.[18] Briefly, the fresh tissue homogenate was placed into a 1.5 ml centrifuge tube with 1.15% potassium chloride solution. Thiobarbituric acid reactive substance (TBARS) assay reagent (2000 µl) was then added to the tissue homogenate (500 µl) and the solution was heated in a boiling water bath for 45 min. After cooling, 2 ml n-Butanol was added to each tube. The homogenate was then centrifuged and the supernatant was examined using a Thermo Spectronic Genesys five microplate reader (Labequip, Ontario, Canada) for colorimetric assay (OD 532 nm).

RT-qPCR Assessment

Real-time PCR was conducted to quantify changes in the mRNA levels of different genes, including collagen 2a1, aggrecan, IL-1β, TNF-α, iNOS, SOX-9, MMP-3, and MMP-13. The *β2M* gene was used as a housekeeping gene. Briefly, cartilage tissue was collected from the femoral and tibial condyles of each knee joint and ground using a mortar and pestle. Total RNA was isolated and analyzed as described above. The sequence of specific primers is presented in table 1.

Histopathological Evaluation

Histopathological evaluation on full-thickness sagittal sections of the cartilage in the weight-bearing area of the medial femoral condyle was performed according to the previously reported procedure. The samples were graded as normal (0), slight reduction (1), moderate reduction (2), severe reduction (3), and no dye (4).[19, 20]

Statistical Analysis

Data were presented as mean±SEM of multiple repeats of the same experiment (n=6). Statistical analysis was performed using GraphPad Prism software (version 6.0) with one-way analysis of variance (ANOVA) followed by Tukey *post hoc* test and Kruskal-Wallis with Dunn's *post hoc* test. Statistical significance levels were $P<0.05$.

Results

The results of the MTT assay showed that apigenin caused a dose- and time-dependent reduction in viable SMMSCs at higher concentrations than 0.3 µM. Importantly, no toxic effect was observed after treatment with lower concentrations of apigenin (figure 1). Based on these results, apigenin concentrations of 0.1 µM (A0.1) and 0.3 µM (A0.3) were selected for subsequent experiments.

Based on radiography results, in all treated groups, a reduction in the global OA score compared to pre-treatment was observed. The OA+H, OA+MSCs, and OA+MSCs+A0.3 groups exhibited a significant reduction in the global OA score (figures 2 and 3).

The highest levels of TNF-α and IL-1β were observed in the OA group, and the lowest in the

Table 1: Primer sequences for reverse transcription polymerase chain reaction

Gene name	Accession number	Primer sequence (5'-3')	Size (bp)
CD34	NM_001107202.2	Forward: AGCCATGTGCTCACACATCA Reverse: CAAACACTCGGGCCTAACCT	257
CD45	NM_001109887.2	Forward: CCAAGAGTGGCTCAGAAGGG Reverse: CTGGGCTCATGGGACCATTT	450
CD90	NM_012673.2	Forward: GACCCAGGACGGAGCTATTG Reverse: TCATGCTGGATGGGCAAGTT	177
CD73	NM_021576.2	Forward: TGCATCGATATGGCCAGTCC Reverse: AATCCATCCCCACCGTTGAC	208
Col2a1	NM_012929.1	Forward: CCAGAGTGGAAGAGCGGAGAC Reverse: CAGTGGACAGTAGACGGAGGAAAG	286
AGG	NM_022190.1	Forward: CAGATGGCACCCTCCGATAC Reverse: CCACTGACACACCTCGGAAG	156
IL-1β	NM_031512.2	Forward: GGAGAGACAAGCAACGACAA Reverse: TTGTTTGGGATCCACACTCTC	123
TNF-α	NM_012675.3	Forward: CCCACGTCGTAGCAAACCAC Reverse: TAGGGCAAGGGCTCTTGATG	264
iNOS	NM_012611.3	Forward: GGATGTGGCTACCACTTTGA Reverse: CATGATAACGTTTCTGGCTCTTG	107
SOX-9	NM_080403.1	Forward: AGTCGGTGAAGAATGGGCAA Reverse: ACCCTGAGATTGCCCGGAG	161
MMP-3	NM_133523.3	Forward: ATGATGAACGATGGACAGATGA Reverse: CATTGGCTGAGTGAAAGAGACC	99
MMP-13	NM_133530.1	Forward: CAAGCAGCTCCAAAGGCTAC Reverse: TGGCTTTTGCCAGTGTAGGT	130

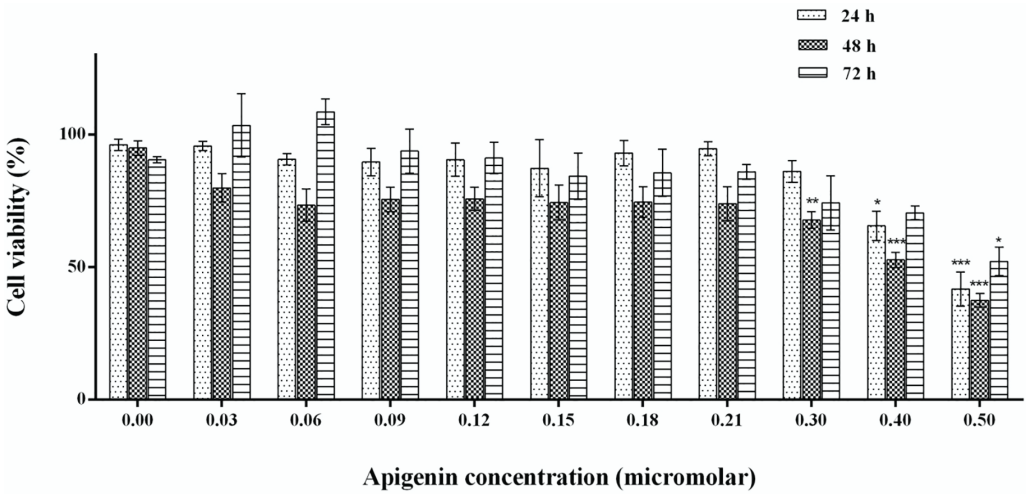

Figure 1: Cytotoxic effect of apigenin on SMMSCs examined using MTT assay. Data are expressed as the mean±SEM of six independent replicates. One-way analysis of variance (ANOVA) was used to evaluate significant differences between the groups. *P<0.05; **P<0.01; ***P<0.001

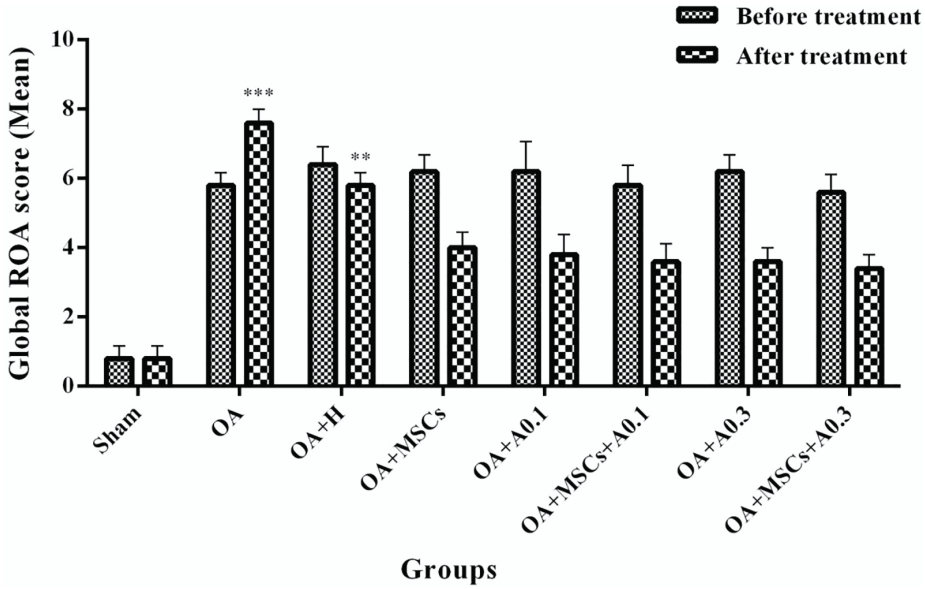

Figure 2: Global OA scores obtained from radiological assessment. Data are expressed as mean±SEM. Kruskal-Wallis with Dunn's *post hoc* test was used to evaluate significant differences between the groups. **P<0.01; ***P<0.001; H: Hyaluronic acid; MSCs: Mesenchymal stem cells; A: Apigenin

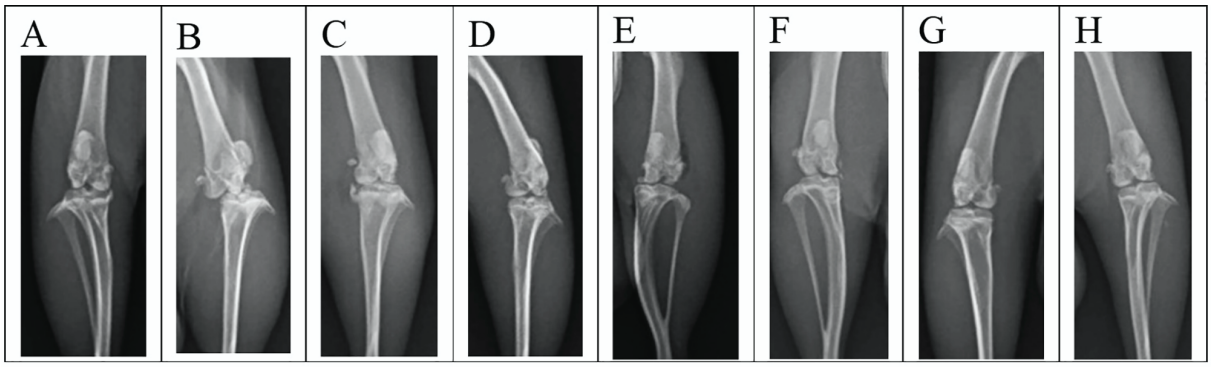

Figure 3: Radiographical assessment of the knee joint in the studied groups post-treatment. (H stands for Hyaluronic acid, MSCs for Mesenchymal Stem Cells and A for Apigenin.) A) Sham: Normal knee radiography, B) OA: Absent joint space width with severe femoral and tibial condyle osteophytes, C) OA+H: Reduced joint space width with severe femoral and tibial condyle osteophytes, D) OA+MSCs: Reduced joint space width with moderate femoral and tibial condyle osteophytes, E) OA+A0.1: Reduced joint space width with small femoral osteophyte, F) OA+MSCs+A0.1: Reduced joint space width with small femoral osteophyte, G) OA+A0.3: Reduced joint space width with small femoral osteophyte, H) OA+MSCs+A0.3: Normal joint space width with small femoral osteophyte

OA+MSCs+A0.3 group (P<0.001 and P<0.001, respectively). The level of TNF-α in all apigenin and apigenin+MSCs groups was less than in the OA+H and OA+MSCs groups, and its level decreased with each increase in apigenin concentration (figures 4A and 4B). On the other hand, the lowest anti-oxidative activity of SOD was observed in the OA group. Injection of stem cells with apigenin at a concentration of 0.3 µM led to the highest increase in the level of anti-oxidative activity of SOD (figure 4C).

MDA level, as a biomarker of lipid peroxidation, was highest in the OA group (figure 4D). A significant decrease in MDA level was observed in the OA+MSCs, OA+MSCs+A0.1 (P=0.022), and OA+MSCs+A0.3 (P=0.009) groups. The results also showed that co-injection of apigenin (0.1 and 0.3 µM) and SMMSCs reduced MDA level to levels lower than of the OA group.

Relative expressions of IL-1β, TNF-α, iNOS, MMP-3, MMP-13, collagen 2a1, aggrecan, and SOX-9 genes are shown in figure 5. The expression level of TNF-α, MMP-3, MMP-13, IL-1β, and iNOS genes was highest in the OA group than all other groups. The expression level of these genes, known for their role in inflammatory signaling pathways, was significantly reduced in the OA+MSCs, apigenin, and OA+MSCs+apigenin groups (P<0.001). Importantly, it was observed that

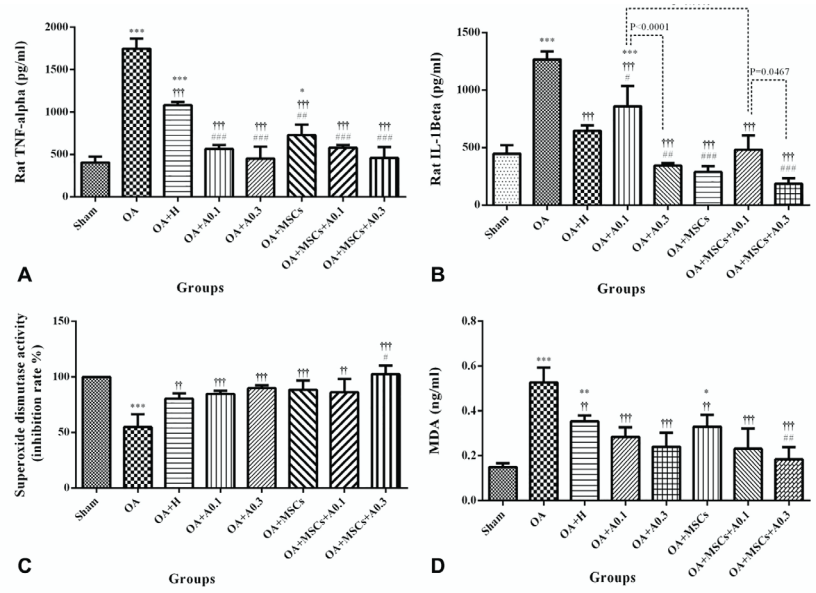

Figure 4: The measured levels of TNF-α (A), IL-1β (B), SOD (C), and MDA (D) in cartilage tissue are illustrated. One-way analysis of variance (ANOVA) followed by the Tukey *post hoc* test was used to evaluate significant differences between groups. Significant differences from the Sham group: *P<0.05, **P<0.01, ***P<0.001; Significant differences from the OA group: ††P<0.01, †††P<0.001; Significant differences from the H group: #P<0.05, ##P<0.01, ###P<0.001; H: Hyaluronic acid, MSCs: Mesenchymal stem cells, A: Apigenin

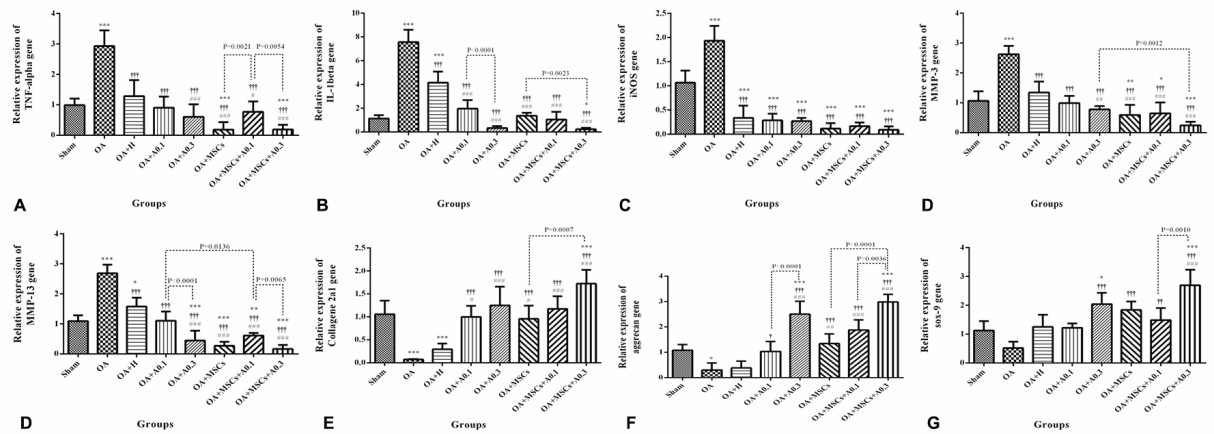

Figure 5: The figure shows the relative expression of TNF-α (A), IL-1β (B), iNOS (C), MMP-3 (D), MMP-13 (E), collagen 2a1 (F), aggrecan (G), and SOX-9 (H) genes. One-way analysis of variance (ANOVA) followed by the Tukey *post hoc* test was used to evaluate significant differences between the groups. Significant differences from the Sham group: *P<0.05, **P<0.01, ***P<0.001; Significant differences from the OA group: †P<0.05, ††P<0.01, †††P<0.001; Significant differences from the H group: #P<0.05, ##P<0.01, ###P<0.001; H: Hyaluronic acid; MSCs: Mesenchymal stem cells; A: Apigenin

apigenin at a concentration of 0.3 μM reduced the levels of these genes more significantly than in the OA+MSCs group. On the other hand, the expression levels of SOX-9, collagen 2a1, and aggrecan were lowest in the OA group, whereas they increased after treatment with either apigenin or MSCs, and with apigenin and SMMSCs. Interestingly, at 0.3 μM, apigenin acted as a strong inducer of the expression of these genes.

The results of the histopathological evaluation of different groups including surface, matrix, cell distribution, cell population viability, subchondral bone, and cartilage mineralization are depicted in table 2. Histopathologic scoring for all parameters was closer to the sham group in the cell-treated and MSCs+apigenin treated groups. The result of safranin staining is presented in figure 6. Histopathological assessment (H&E) and safranin staining indicated that the OA+MSCs+A0.3 group showed a better result and a near normal articular cartilage (figures 7 and 8).

Discussion

The co-injection of apigenin and SMMSCs had a positive effect on rats with knee OA. After injecting this compound into the knee joint of rats, a significant decrease in the MDA, IL-1β, and TNF-α levels in the gene or its product, and an increase in SOD and aggrecan levels were observed. To the best of our knowledge, this is the first study that demonstrated the beneficial effects of combination therapy with apigenin and SMMSCs in knee OA.

Nowadays, there is a growing interest in the use of natural products in the treatment of OA. Among such products, apigenin is the main candidate due to its important biological activities.[21] In this study, we took an innovative approach to assess the effect of apigenin on OA in the knee joint of a rat model when combined with cell therapy. It is known that OA is a disease associated with synovial inflammation and increased oxidative stress markers. We hypothesized that the potential anti-oxidant and anti-inflammatory properties of apigenin could enhance the efficacy of cell therapy. Therefore, we used apigenin and SMMSCs as a combination therapy.

OA is a progressive degenerative disorder of the articular cartilage affecting the entire joint,

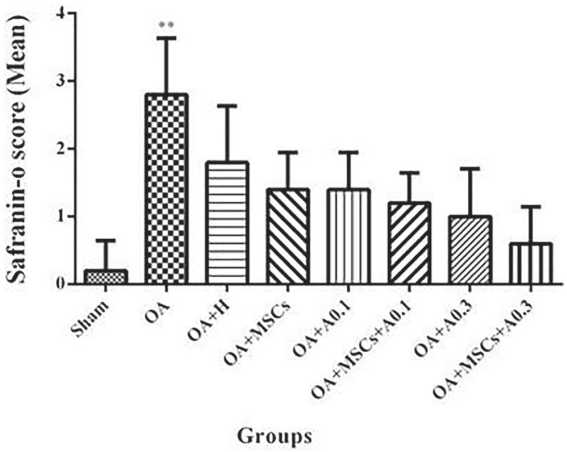

Figure 6: Safranin-O score was measured on the cartilage in the different groups. Data are expressed as mean ±SEM. Kruskal-Wallis test with Dunn's *post hoc* test was used to evaluate significant differences. Significant differences from the Sham group: ¨P<0.01; H: Hyaluronic acid; MSCs: Mesenchymal stem cells; A: Apigenin

Table 2: Histopathological classification of the severity of the osteoarthritis lesion according to the modified International Cartilage Research Society (ICRS) visual histological assessment scale

Group	OA	Sham		OA+H		OA+MSCs		OA+A0.1		OA+MSCs+ A0.1		OA+A0.3		OA+MSCs+ A0.3	
	Mean± SEM	Mean± SEM	P	Mean± SEM	P	Mean± SEM	P	Mean± SEM	P	Mean± SEM	P	Mean± SEM	P	Mean± SEM	P
Surface	1.00± 0.63	3.00± 0.00	0.0040	2.00± 1.00	0.1470	3.00± 0.00	0.0040	2.25± 0.75	0.0470	3.00± 0.00	0.0040	3.00± 0.00	0.0020	3.00± 0.00	0.0020
Matrix	1.00± 0.26	3.00 ± 0.00	0.0010	2.00± 0.00	0.3940	2.67± 0.33	0.0110	2.25± 0.48	0.0400	2.67± 0.33	0.0110	2.50± 0.29	0.0200	3.00± 0.00	<0.001
Cell distribution	1.33± 0.21	3.00± 0.00	0.0010	1.67± 0.33	0.6940	2.67± 0.33	0.0160	1.75± 0.25	0.5900	3.00± 0.00	0.0010	2.25± 0.25	0.1270	3.00± 0.00	<0.001
Cell population viability	1.00± 0.45	3.00± 0.00	0.0020	2.33± 0.33	0.2560	3.00± 0.00	0.0020	2.50± 0.50	0.0160	3.00± 0.00	0.0010	2.50± 0.50	0.0160	3.00± 0.00	<0.001
Subchondral bone	1.17± 0.31	2.67± 0.33	0.0120	2.00± 0.58	0.2010	2.67± 0.33	0.0120	2.25± 0.25	0.1000	2.67± 0.33	0.0120	2.25 ± 0.25	0.1000	2.75± 0.25	<0.001
Cartilage mineral-ization (calcified cartilage)	0.50± 0.50	3.00± 0.00	0.0010	3.00± 0.00	0.0010	3.00± 0.00	0.0010	1.50± 0.87	0.1600	3.00± 0.00	0.0010	2.25± 0.75	0.0140	3.00± 0.00	<0.001

Data are expressed as mean±SEM. The scores were analyzed using Kruskal-Wallis analysis. Significant differences from the OA group; H: Hyaluronic acid; MSCs: Mesenchymal stem cells, A: Apigenin

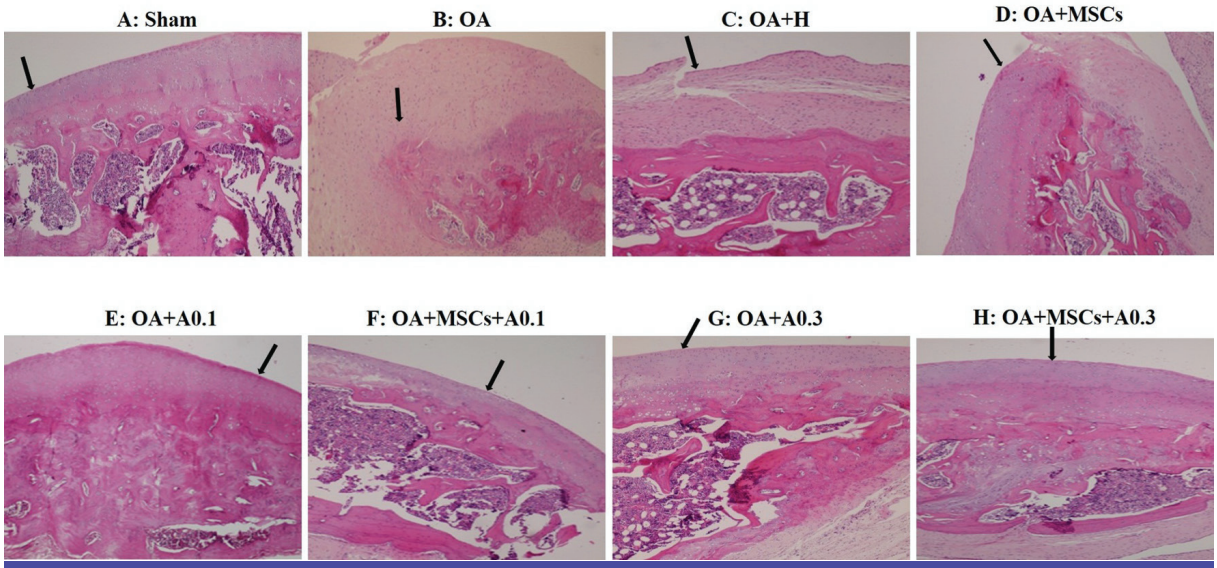

Figure 7: H&E staining was used for histopathological assessment (×200). A: Normal articular cartilage; B: Only fibrocartilaginous tissue with disorganized cell distribution; C: Fibrocartilaginous tissue with irregular surface; D-H: The thickness of hyaline cartilage and cell distribution in the articular surface gradually increased from group D to group G; H: Hyaluronic acid; MSCs: Mesenchymal stem cells; A: Apigenin

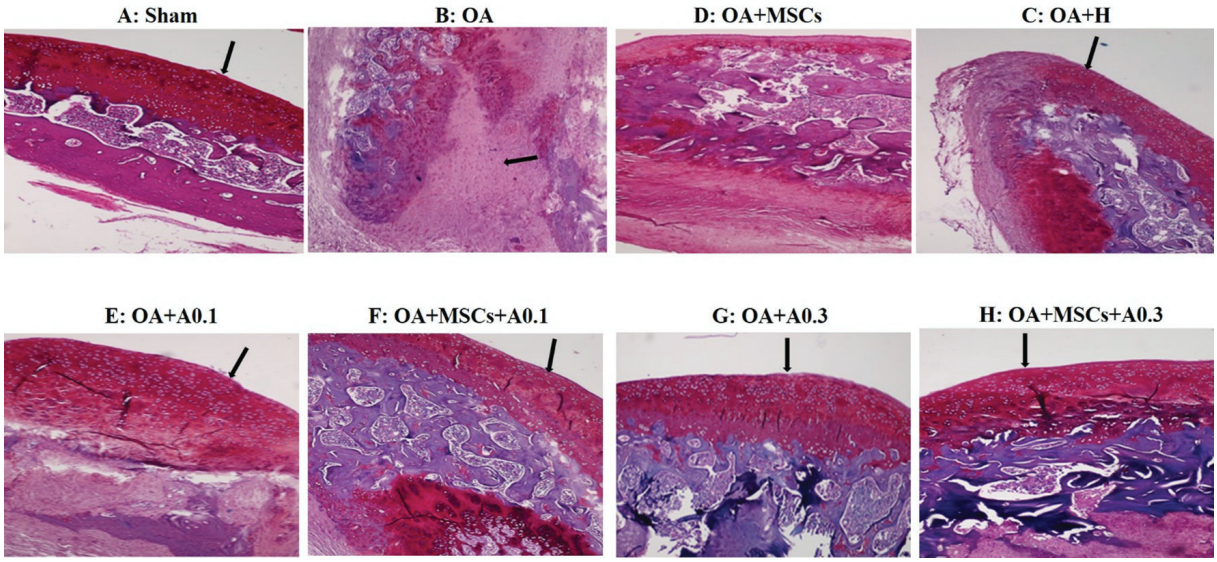

Figure 8: Safranin staining (×200) was used for histopathological evaluation.A) Normal articular cartilage with uniform safranin staining; B) Loss of safranin staining due to mainly fibrocartilage tissue; C) Reduced articular surface with decreased Safranin staining; D-H) The healing of hyaline articular surface gradually improved from group D to group G as shown by safranin staining; H: Hyaluronic acid; MSCs: Mesenchymal stem cells; A: Apigenin

characterized by inflammatory pathways in the synovium tissue and tendon.[22] In normal conditions, chondrocytes regulate the balance between the synthesis and degradation of ECM components. However, in a pathological state, homeostasis is not maintained, leading to increased levels of degradation pathways.[23]

One of the challenges in OA therapy is the restoration of the affected articular cartilage. OA can be the result of dysfunction in a population of MSCs.[24] Abnormal changes in normal MSC populations in the joint were found to lead to reduced proliferative capacity, differentiation ability, and increased degeneration reactions.[25] A recent clinical study suggested MSCs as an effective cell-based treatment for cartilage regeneration.[26] MSCs are shown to generate new cartilage and stimulate its formation by the resident chondrocytes or other cells in the joint and inhibit joint inflammation.[27] However, there are still concerns about stem cell therapy in terms of the correct dosage, timing of intervention, and the type, mode, and delivery route of MSCs.[28, 29]

In the present study, SMMCSs were used for the treatment of OA. MSCs can be isolated from different tissues including bone marrow, synovium, periosteum, skeletal muscle, and adipose tissue. It has been reported that the

origin of MSCs is an important factor affecting the quality of OA cell therapy. Important criteria of synovial-MSCs such as high chondrogenic capacity and high regenerative potential showed the prominence of this type of MSCs.[30] To compare the effect of apigenin alone and in combination with SMMCSs, the positive control group in our study was treated with hyaluronic acid (H). Intra-articular injection of H can reduce pain in comparison with conventional OA treatments such as nonsteroidal anti-inflammatory drugs (NSAID); the mechanism of which remains unclear. The potential of H to attenuate the production of inflammatory mediators and induce the proliferation of chondrocytes has been shown.[31]

Our results showed that the co-injection of apigenin and SMMSCs reduced the inflammation more effectively than in other groups treated with apigenin, SMMCSs, or H. The combination therapy significantly decreased IL-1β and MDA levels and increased the SOD level. A previous study indicated that apigenin possesses strong anti-oxidant, anti-inflammatory, and anti-cancer properties as well as anti-MMP effect.[32] In line with our findings, Park and colleagues showed that apigenin acts as a chondroprotective agent *in vivo* when administered intra-articularly.[33] The chondroprotective activity of apigenin is possibly mediated through its inhibitory effect on the expression level of IL-1β, as an important pro-inflammatory cytokine, as well as on MMPs activity.[34] The same result was obtained in the gene expression level, where the expression level of IL-1β significantly decreased in the OA+MSCs+apigenin groups in comparison with the OA+MSCs and OA groups. The IL-1β produced by chondrocytes contributed to the pathogenesis of OA through the induction of MMPs expression and thus stimulated the progression of OA.[35] Furthermore, it has been reported that apigenin decreases the expression of IL-1β by inhibiting transcription factor NFκB.[36]

The results showed that the expression level of SOX-9, collagen 2a1, and aggrecan genes in the OA+MSCs+apigenin groups was higher than that of the OA and OA+MSCs groups. SOX-9 is a transcription factor known for its important role in cellular development, especially in regulating chondrocyte development. It is also an important factor in regulating the expression of ECM genes, including collagen type II and aggrecan.[37] Furthermore, it has been shown that SOX-9 is a negative regulator of cartilage degeneration enzymes at the early stage of human OA.[37] Therefore, the positive effect of apigenin on the expression level of these genes could be due to its possible role in cartilage protection. On the other hand, chondrocytes suppress oxidants by producing antioxidant enzymes. Depletion of SOD can lead to accumulated amounts of ROS and, consequently, cell dysfunction and cell death.[38] Our results showed that the SOD level in OA rats significantly decreased whereas co-injection with apigenin and SMMSCs increased SOD.

One of the events associated with OA is lipid peroxidation. We demonstrated that apigenin combined with SMMSCs can reduce the MDA level, as a marker of lipid peroxidation. Although this has already been reported in several pathological conditions,[39] our study is the first to use the application of MDA level in OA cell therapy.

The main limitation of the study was the lack of safety examinations for the injected compound *in vivo*. While we checked the possible toxicity of apigenin *in vitro*, it is recommended that future studies assess its cytotoxic effect *in vivo*.

Conclusion

Co-treatment with apigenin and SMMCSs improved knee OA therapy outcome. Apigenin may have supplementary beneficial effects on cell therapy in a rat model of OA due to its ability to reduce oxidative stress, suppress inflammation, and promote the production of ECM components. Further research into the exact mechanism of this protective effect is recommended.

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