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The Impact of Wharton's Jelly-derived Exosomes on the Production of Inflammatory Mediators from HIG-82 Synoviocytes

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ABSTRACT

Background: Osteoarthritis (OA) is the most common joint disease worldwide. Routine treatment options are limited, and total knee replacement surgeries often come with complications. In recent years, the use of biologics, such as Wharton's jelly (Wj) derived from the umbilical cord (UC), has gained popularity. While mesenchymal stem cells (MSCs) derived from Wj show promise in restoring articular cartilage, they also have some limitations. Recent studies have indicated that exosomes isolated from acellular Wj may offer advantages under certain conditions.

Objective: To investigate the anti-inflammatory properties of exosomes isolated from Wj in synoviocytes.

Methods: Decellularization of Wj was performed using sterile umbilical cords obtained from patients. Next, the exosomes were isolated from Wj using ultracentrifugation. After characterizing the exosomes, they were co-cultured with inflammatory synovial fibroblast cells (HIG-82) for 24 hours. Then, the gene expression levels and protein contents of some important inflammatory mediators including metalloproteinase-13 (MMP-13), cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) were measured in the cells using real-time PCR and ELISA tests, respectively.

Results: The expression levels of MMP-13, COX-2, and iNOS genes were significantly reduced in the cultured cells treated with exosomes compared to untreated cells. Moreover, the content of MMP-13, COX-2, and iNOS proteins were significantly lower in the supernatant of the cultured cells compared to the control.

Conclusion: Wj-derived exosomes exhibit notable anti-inflammatory properties, which can help mitigate inflammation in the synovial environment of joints. However, further research is required to fully understand their benefits and potential applications in treating osteoarthritis.

Keywords: Exosome, Inflammation, Osteoarthritis, Synoviocytes, Wharton's jelly

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INTRODUCTION

Osteoarthritis (OA), is an irreversible degenerative joint disease characterized by several major pathological features: increasing subchondral bone sclerosis, destruction of articular cartilage, synovial inflammation, and osteophyte development (1). It is the most common type of chronic arthritis that interferes with daily activities, especially in the elderly (2, 3). Current treatments are mostly palliative and do not fully prevent the progression of the disease (4).Therefore, invasive surgery such as arthroplasty or osteotomy remains the only treatment option. As a result, researchers have focused on developing innovative therapies for repairing damaged joint tissues (5, 6).

Recent investigations have emphasized the crucial role of immune cells in the development of osteoarthritis (7). Various inflammatory mediators including cytokines and chemokines, like IL8, and MCP-1, are released by chondrocytes and synoviocytes in the joints of patients with osteoarthritis (8). Moreover, synovial fibroblasts are considered another source of secreting pro-inflammatory cytokines and matrix-degrading enzymes in osteoarthritis (9). These inflammatory components adversely affect cell signaling pathways, gene expressions, and activities of the joint tissue (10), leading to the secretion of more inflammatory mediators and molecules. This ultimately changes the anatomical structures and physiological functions of the joint (11).

A previous study has demonstrated that interleukin (IL)-1β, a pro-inflammatory cytokine, plays a significant role in the pathogenesis of osteoarthritis by inhibiting the production of essential structural proteins, such as aggrecan and collagen type II. Moreover, the increase in IL-6 may indeed be the mechanism of action of IL-1 (12). The IL-1 released by human osteoarthritic cartilage is sufficient to increase IL-6 synthesis by chondrocytes (13). *In vitro,* human synoviocytes release IL-6 independently, with the process being further increased by the release of IL-1 and TNF-α. Levels of interleukin-6 have been associated with temporomandibular joint pain, and IL-6 and IL-1 work together to induce breakdown of cartilage collagen (14). Inflammation and cartilage deterioration have been linked to other cytokines, such as IL-6 and IL-17 (14). Importantly, there is evidence that these cytokines work together to promote cartilage breakdown and the release of inflammatory mediators (15). Furthermore, IL-1β can induce the synthesis of matrix metalloproteinases (MMPs), particularly MMP-1 and MMP-13, which promote the destruction of articular cartilage (16). Cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) are other targets of IL-1β that are up-regulated in osteoarthritis leading to an increase in the synthesis of nitric oxide (NO) (17), and prostaglandin E2 (PGE2), respectively (18). These mediators further enhance the secretion of more inflammatory factors and the degradation of extracellular matrix (ECM) (16, 19).

Wharton's jelly (Wj) is a type of mucous connective tissue that surrounds the blood vessels of the umbilical cord (20). Previous studies have shown that Wharton's jelly mesenchymal stromal cells (Wj-MSCs) exhibit anti-inflammatory activities that can stimulate matrix formation by damaged chondrocytes and synoviocytes, promoting cartilage restoration. This discovery has opened up new possibilities for the treatment of various tissue-degenerative diseases, such as OA, liver fibrosis, heart failure and spinal cord injuries (21). Nonetheless, factors such as maintaining biological activity, and logistical concerns, and the quantity of bioactive substances have limited the use of MSCs therapy in clinical settings (22). As a result, researchers are seeking to develop a cell-free approach that mimics the physiological effects of MSCs, leading to a surge in interest in their secretomes and exosomes (23, 24).

Exosomes are a type of extracellular nanovesicle with a diameter of 30 to 100 nm; surrounded by a lipid bilayer (25, 26). Since

exosomes are secreted by various types of cells, including stem cells, there has been increasing research conducted on exosomes as a method of intercellular communication in recent years. (27). These secretomes can maintain almost all the merits of the original cells, such as immunoregulation characteristics, minimal immunogenicity, and delivery of bioactive factors (22, 28, 29).

Since a large portion of the exosomes isolated from the umbilical cord's Wj originates from MSCs (30), the use of Wj is likely a valuable source of anti-inflammatory exosomes. This eliminates the time-consuming process of culturing MSCs and isolating exosomes, reducing the need for various equipment (31, 32). This study aimed to investigate the anti-inflammatory properties of exosomes directly isolated from Wj in synoviocytes.

MATERIALS AND METHODS

Decellularization of Wharton's jelly

Firstly, all patients were ethically informed about the study and signed the consent form. Cesarean sections were performed by surgeons in the Department of Gynecology and Obstetrics at Imam Reza Hospital, in Tabriz, Iran in order to obtain sterile umbilical cords. To wash them, each cord was split in half lengthwise, approximately 20 centimeters, first in a 2% antibiotic/antimycotic phosphate-buffered saline (PBS) solution and then in a 1% PBS solution. Following this, the cords were cut lengthwise, and the jelly was scraped out of the umbilical cord. To dissolve it, the jelly was added to 20 milliliters of 1× DMEM complete medium and vigorously resuspended. Subsequently, the jelly that had been resuspended in the medium was centrifuged for 10 minutes at 300 ×g, and the supernatant was collected to isolate exosomes (33).

Exosome Isolation

The exosomes were separated using a differential centrifugation procedure. To do this, the acellularized Wj was centrifuged at 2,500 ×g for 30 min to remove apoptotic cells. Next, the supernatant was collected and centrifuged again at 13,000 ×g for 1 hour. This step was carried out in Greiner 15-ml tubes to eliminate platelets and extracellular vesicles. The supernatant was transferred to a new tube and centrifuged at 100,000 ×g at 4 °C for 70 minutes (33). After centrifugation, the supernatant was discarded, and the pellet containing exosomes was resuspended in PBS, which was then stored at −80 °C for future use.

Characterization of Acellularized Wj-derived Exosomes

Dynamic Light Scattering (DLS)

The size of the isolated exosomes was measured using the ZetaSizer (Malvern Instruments Ltd, Malvern, UK), and the Bicinchoninic acid assay (BCA) was utilized to determine the exosomes' total protein content (Thermo Scientific, Rockford, USA).

Electron Microscopy

In summary, the samples intended for examination under a scanning electron microscope (SEM) were treated with a solution containing 2% glutaraldehyde in cacodylate buffer. They were then washed in buffer and dehydrated using a series of graded ethanol solutions. Next, the samples were coated with gold and evaluated in an FEI XL30 SEM.

Western Blot

Exosomal lysates containing 30 μg of protein were subjected to electrophoresis using a 10% SDS-PAGE gel. The separated proteins were then transferred onto nitrocellulose membranes from Millipore, USA. Next, the blots were subjected to overnight incubation with primary antibodies (CD9, CD81, CD63) obtained from Abcam, located in Cambridge, MA, USA. After an additional 2 h of incubation, the secondary antibodies—goat anti-rabbit horseradish peroxidase IgG (diluted 1:10,000) (System Biosciences,

Palo Alto, CA) were added. The blots were identified using Chemiluminescence (ECL) technology from Santa Cruz, CA, USA. Glyceraldehyde3-phosphate dehydrogenase (GAPDH) expression was used as a control.

Cell Culture

Rabbit synovial fibroblast cells (HIG-82) (from Pasteur Institute, Tehran, Iran) were cultured in DMEM F-12 medium (Hyclone, Utah, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Paisley, UK) and 1% penicillin–streptomycin (Gibco, Paisley, UK). The cells were then incubated at 37°C with 5% CO2. After achieving theexperimental conditions, HIG-82 synovial cells, which exhibit a fibroblast-like morphology, were treated with 10 ng/mL (34) of the inflammatory cytokine IL-1β to induce inflammation in the HIG-82 cells. 24 h post-stimulation, the cultured cells were co-cultured with 50 µg/ml exosomes (35) for an additional 24 h at 37°C in an atmosphere containing 5% CO2.

Gene Expression Assay

The expression of MMP13, COX-2, and iNOS was examined using real-time polymerase chain reaction (PCR). Cultured cells were harvested and washed with a PBS solution. First, total RNA was extracted from the cells using the Qiagen Mini kit (Germantown, MD, USA) following the manufacturer's instructions. Next, 1 µg of RNA was converted into complementary DNA (cDNA) using the SuperScript III reverse transcription kit from Invitrogen. Using the SYBR Green PCR SuperMix (BioRad, Hercules, CA, USA) and the specific primers listed in Table 1, cDNA products were analyzed using real-time PCR. The gene expression results were normalized to that of GAPDH.

ELISA Analysis

The ELISA tests were conducted to assess the levels of MMP13, COX2, and iNOS proteins in the culture supernatants. The ELISA kits from Biolegend in San Diego, CA, USA were used, following the methods indicated by the manufacturer. The colorimetric absorbance at a wavelength of 450 nm was determined using the SpectraMax® Gemini™ EM Microplate. The protein concentrations were then evaluated using SoftMax Pro 7 software.

Statistical Analysis

All experiments were performed in triplicate and descriptive statistics were presented as mean ± SD. the normal distribution of scale variables was assessed using the Kolmogorov–Smirnov test. Two-by-two comparisons were calculated using t-test analysis and GraphPad Prism 8.0 (GraphPad Software, Inc., La Jolla, CA, USA). A P-value less than 0.05 was considered significant.

RESULTS

The Characteristics of Wj-derived Exosomes

Characteristic examination indicated that the average size of isolated exosomes

Table 1. Rabbit primer pairs sequences

Gene	Primer	Sequence (5′→3′)
MMP-13	Forward	TGGTCTTCTGGCTCACGCTT
	Reverse	ACTCTTGCCGTTGTAGGTGT
iNOS	Forward	GACGTCCAGCGCTACAATATCC
	Reverse	GATCTCTGTGACGGCCTGATCT
COX-2	Forward	CGGATTCTACGGTGAAAACTGC
	Reverse	GACGATGTTCCAGACTCCCTTG
GAPDH	Forward	TGACGACATCAAGAAGGTGGTG
	Reverse	GAAGGTGGAGGAGTGGGTGTC

MMP-13: Matrix metalloproteinase 13, iNOS: Inducible nitric oxide synthase, COX-2: Cyclooxigenase 2, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

was 76.1 (±10.57) nm which falls within the normal range of exosome sizes reported previously (36) (Figure 1a). The exosomes were visualized by electron microscopy and showed a size range of less than 150 nm with a spheroid morphology (Figure 1b). Moreover, key exosome markers such asCD9, CD63 and CD81 were confirmed using an immunoblot test (Figure 1c).

The Expression Levels of MMP-13, iNOS and COX-2 Genes

The expression levels of the MMP-13, iNOS and COX-2 genes were investigated in HIG-82 cells treated with exosomes. As shown in Figure 2, after treating the inflammatory HIG-82 cells with exosomes, the expression levels of the MMP-13 gene significantly decreased compared to the untreated cells (P=0.0154). Moreover, the fold changes in expression of the iNOS gene significantly decreased in the cells treated with exosomes compared to the untreated cells (P=0.0013). Additionally, the expression levels of the COX-2 gene in treated inflammatory HIG-82 cells with exosomes significantly declined compared to the control (P=0.0179). (Figure 2a, b, c, and Table 2)

Secretion Levels of MMP-13, iNOS and COX-2 Molecules

To evaluate the secretion levels of MMP-13, iNOS and COX-2 molecules in the supernatants of cultured HIG-82 cells, the ELISA method was used (Figure 3). As shown, the amount of the MMP-13 molecule significantly decreased in exosome-treated cells compared to untreated cells (P=0.0321). Furthermore, the secretion of the iNOS molecule significantly diminished in the exosome-treated HIG-82 cells compared to the control (P=0.0298). Similarly, the secretion level of the COX-2 molecule significantly declined in the supernatant of exosome-treated cells compared to the untreated cells (P=0.0437) (Figure 3a, b, c, and Table 2).

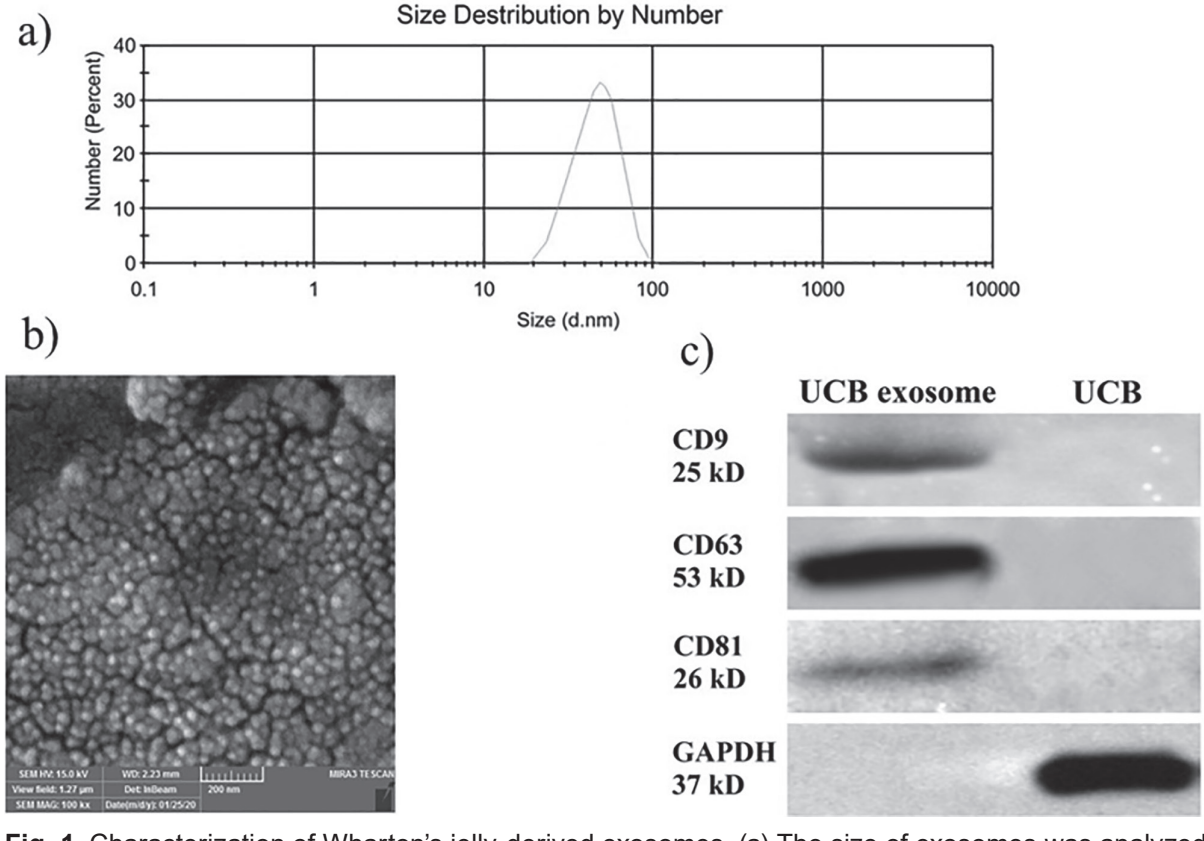

Fig. 1. Characterization of Wharton's jelly-derived exosomes. (a) The size of exosomes was analyzed using DLS. (b) Scanning electron microscope (SEM) image. (c) Surface markers of exosomes were identified using western blot. UCB: Umbilical Cord Blood, DLS: Dynamic Light Scattering.

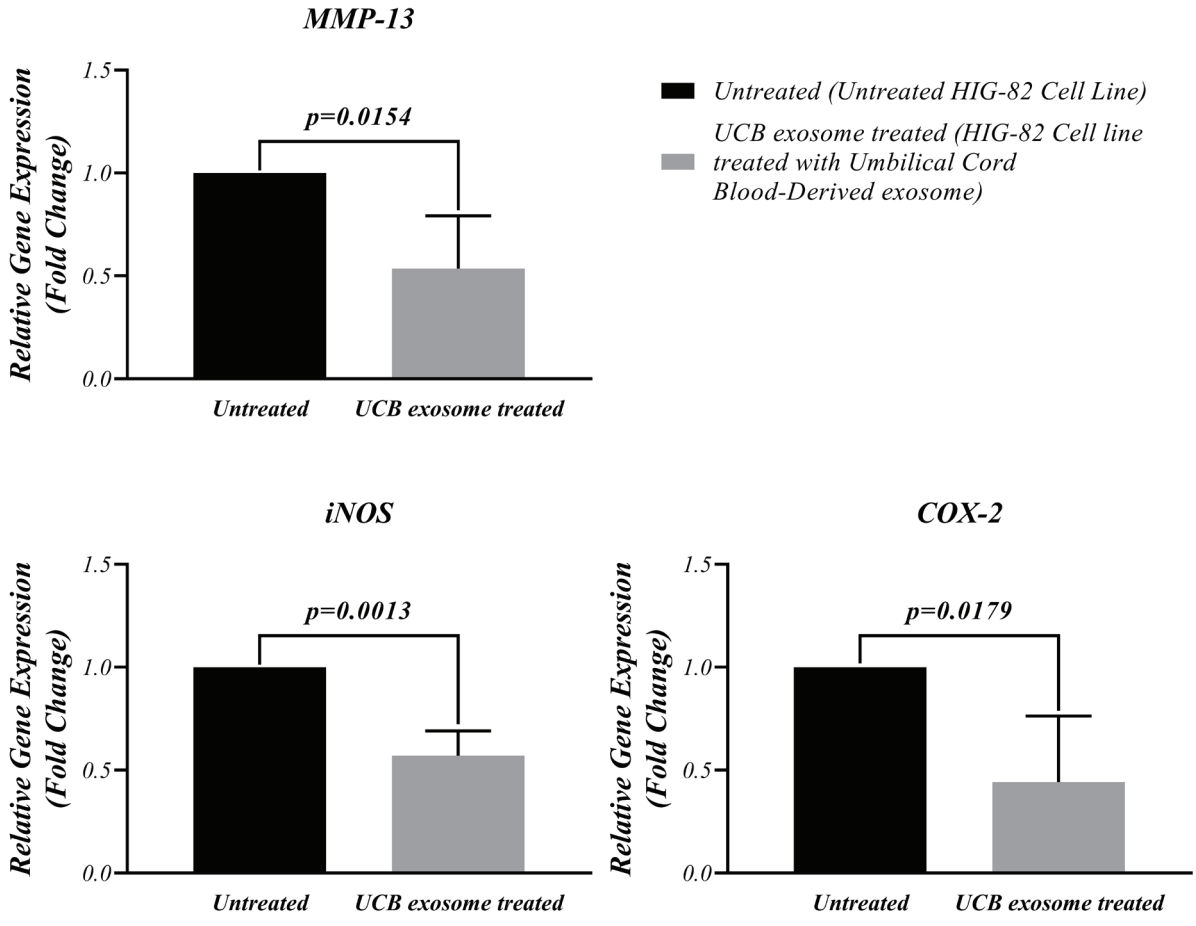

Fig. 2. Relative mRNA expression levels of *MMP-13*, *iNOS* and *COX-2* genes. The fold changes in *MMP-13, iNOS,* and *COX-2* gene expressions in inflammatory HIG-82 cells after treatment with Wj-derived exosomes for 24 hours, compared to untreated cells. PCR amplification was normalized against *GAPDH* expression. The data are presented as mean ± SD. MMP-13: metalloproteinase-13, COX-2: cyclooxygenase-2, iNOS: inducible nitric oxide synthase, UCB: Umbilical Cord Blood.

Table 2. MMP-13, iNOS, and COX-2 expression patterns in HIG-82 cell line treated with WJ-derived exosomes

Target	Untreated cells (mean±SD)	Treated cells with exosomes (mean±SD)	*P*-value
Relative Gene Expression (Fold Change)			
MMP-13	1±0	0.5360±0.2558	0.0154
iNOS	1±0	0.5700±0.1204	0.0013
COX-2	1±0	0.4420±0.3217	0.0179
ELISA (Cell supernatant)			
MMP-13 (pg/mL)	2184±608.8	1315±391.5	0.0321
iNOS (ng/mL)	11.20±3.493	6.200±1.924	0.0298
COX-2 (ng/mL)	3.280±0.8927	2.020±0.7596	0.0437

MMP-13: Matrix metalloproteinase-13, iNOS: Inducible nitric oxide synthase, COX-2: Cyclooxigenase-2

DISCUSSION

Synovial fibroblasts are a major source of pro-inflammatory cytokines and matrix-degrading enzymes in the progression of osteoarthritis (9). In this study, we aimed to investigate the use of Wj-derived exosomes to reduce the production of key inflammatory mediators, such as MMP-13, iNOS, and COX-2, in synovial fibroblast cell HIG-82.

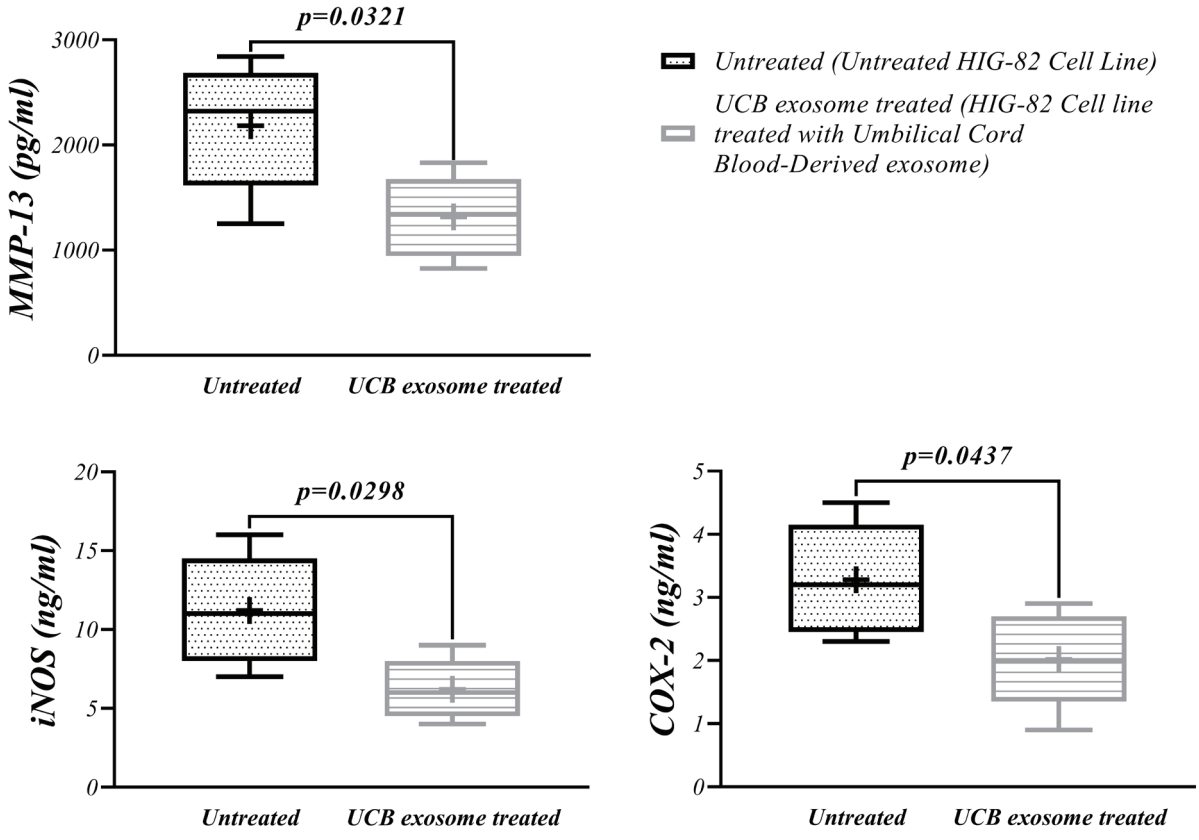

Fig. 3. Secretion levels of *MMP-13*, *iNOS*, and *COX-2* factors. The secretion of *MMP-13, iNOS* and *COX-2* proteins in the supernatants of inflammatory HIG-82 cells co-cultured with Wj-derived exosomes for 24 hours compared to untreated cells. The data are presented as mean ± SD. MMP-13: metalloproteinase-13, COX-2: cyclooxygenase-2, iNOS: inducible nitric oxide synthase, UCB: Umbilical Cord Blood.

Our data revealed that treatment of these cells with the isolated exosomes led to a significant reduction of MMP-13, COX-2, and iNOS compared to the control.

Overall, it has been demonstrated that excessive expression of iNOS plays a key role in several inflammatory conditions particularly OA (37). Indeed, iNOS which generates NO is mechanistically associated with the pathological consequences of OA. This occurs through disturbing ECM homeostasis and the balance of cytokine expression, ultimately resulting in oxidative damage and chondrocyte apoptosis (38). Moreover, overproduction of NO results in cartilage damage by increasing MMP activity and decreasing aggrecan and collagen synthesis (39). It has been reported that the collagenase activity of MMP-13 can degrade type II collagen, which is the primary collagen in articular cartilage (39, 40). consistent with these findings, elevated levels of MMP-13 have been observed *in vitro* and in animal model of osteoarthritis (41, 42). In parallel, numerous studies have shown that the level of MMP-13 level is significantly increased in patients with osteoarthritis (43-46). According to a previous study, COX breaks down arachidonic acid to form PGE2, which is a major mediator of inflammation (47). The isoform COX-2 is encoded by PTGS2 and is stimulated by several growth factors and cytokines (48, 49). Attur et al. demonstrated that chondrocytes in cartilage explants express COX-2, leading to the production of PGE2. This, in turn, inhibits the synthesis of proteoglycan and accelerates the destruction of aggrecan and type II collagen by increasing the activity of MMP-13 in individuals with osteoarthritis (50).

Numerous studies have confirmed that Wj-MSCs affect articular cells, such as synovial fibroblasts, which are important players in synovitis (51, 52). Co-treatment of Wj-MSCs

with synovial fibroblasts from OA patients revealed a significant decrease in the expression levels of inflammatory factors and matrix-degrading enzymes along with an increase in the synthesis of aggrecan, collagen II, and other cartilage matrix proteins in the fibroblasts (53-55). Therefore, Wj-MSCs have been employed as a potential therapeutic option in arthritic disorders due to their immunomodulatory features and chondrogenic capacities (52). In addition to the positive characteristics of the cells themselves, it has been observed that extracellular vesicles secreted by MSCs can mimic the immunomodulatory and tissue regeneration capabilities of the cells, playing a key role in treatment approaches (56). Jiang et al., showed that hWj-MSC-exosomes can enhance the growth of chondrocytes and promote the tendency of macrophages toward M2 phenotype polarization. This, in turn, inhibits the inflammatory response and improves osteochondral regeneration in a rabbit knee osteochondral defect repair model (57).

As MSCs are surrounded by the ECM components in the UC (30), we investigated whether there are beneficial factors in the ECM of cells that contribute to the reduction of inflammatory conditions observed in osteoarthritis. Our results showed that treating inflammatory synovial fibroblasts HIG-82 with exosomes derived from Wj led to a decrease in the levels of gene expression and molecular secretion of MMP-13, iNOS and COX-2 compared to the untreated cells. Exosomes may contain various molecules that can induce anti-inflammatory effects. For example, one important molecule believed to play a key role in the pathophysiology of OA is Wnt. In a study by Mao et al. it was demonstrated that in an OA mouse model, exosomes produced from human MSCs that overexpressed microRNA (miR)-92a-3p facilitated chondrogenesis, preserved the functionality of articular chondrocytes, and prevented the degradation of cartilage (58). Furthermore, bone marrow MSC-derived exosomes were able to target HDAC3 and activate the STAT1/NF-κB p65 signaling pathway. This activation prevented chondrocyte pyroptosis (59). Moreover, the therapeutic properties of exosomes for osteoarthritis are significantly influenced by long non-coding RNAs (lncRNAs). Exosomal lncRNA MEG-3 can increase the production of collagen type II and prevent IL-1β-induced senescence and death in chondrocytes, potentially preserving the chondrocyte phenotype (60). Liu et al. found that MSC-Exos significantly reversed IL-1β-induced chondrocyte growth suppression and apoptosis induction. They identified lncRNA KLF3-AS1 as the crucial effective molecule in this process. Additionally, MSC-exosomes decreased MMP-13 and Runx2 levels, while increasing aggrecan and collagen type II alpha 1 chain (Col2a1) expression (61). Moreover, lncRNA KLF3-AS1 reduced chondrocyte damage by acting as a miR-206 sponge to enhance GIT1 expression in chondrocytes (62). Despite the lack of literature on the anti-inflammatory effects of Wj-derived exosomes, Bakhtyar et al., have shown that acellular gelatinous Wj derived from the human UC improves skin wound healing both *in vitro* and *in vivo* (63). This highlights the anti-inflammatory properties of Wj. For the first time, our results show that exosomes derived from Wj have a powerful capability to be involved in restricting synovial inflammation and the progressive destruction of the osteocytes environment. Moreover, accurate identification of the contents of exosomes in future studies can help determine the anti-inflammatory mechanisms of exosomes in various diseases and be used as therapeutic targets.

CONCLUSION

Inflammation plays a key role in the pathogenesis of osteoarthritis. The release of early-stage inflammatory cytokines, such asIL-1β, leads to the up-regulation of collagenase like MMP-13, as well as iNOS and COX-2 which degrade the ECM. Wj-

derived exosomes have the potential to reduce the levels of MMP-13, iNOS, and COX-2 gene expressions, as well as protein contents in IL-1β-induced inflammatory HIG-82 cells. This finding paves the way for further research into the benefits of Wj-derived exosomes in the treatment of osteoarthritis.

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AUTHORS' CONTRIBUTION

AF and LA designed the study. AF and MH performed all the experiments and wrote the manuscript. AA and MS aggregated the data. SP and AM analyzed the data. All authors read and approved the final manuscript

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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