Article

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The Role of Intra-Articular Delivery of BM-MSCs-Derived Exosomes in Improving Osteoarthritis: Implication of *circYAP1/miRNA-21/TLR7* Axis

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Background: Bone marrow-derived mesenchymal stem cells (BM-MSCs) have recently attracted great attention due to their crucial anti-inflammatory and regenerative properties. This work aims to examine the curative impact of intra-articular injection of BM-MSCs-derived exosomes in ameliorating osteoarthritis (OA) progression in rats and to explore the interaction between circular RNA of Yes-associated protein 1 (*circYAP1*) and microRNA-21 (*miRNA-21*) in the rat knee joints.
Methodology: Gene expression *circYAP1*, *miRNA-21*, toll-like receptor-7 (*TLR7*), aggrecan, and collagen type II were evaluated by quantitative reverse transcription polymerase chain reaction (qRT-PCR) in the rat articular tissues. In addition, the Enzyme-linked immunosorbent assay (ELISA) technique was used to estimate the level of the inflammatory markers interleukin 4 (IL-4), interleukin 10 (IL-10), interleukin 1β (IL-1β), and tumor necrosis factor-alpha (TNF-α); and the oxidative markers glutathione (GSH), malondialdehyde (MDA) and total reactive oxygen species (ROS). Histopathological examination using Hematoxylin and Eosin (H&E) staining of the rat articular tissue was also performed along with an estimation of the articular cartilage thickness.
Results: Our results showed that BM-MSCs-derived exosomes significantly elevated *circYAP1* gene expression level ($p < 0.05$) along with subsequent downregulation of *miRNA-21* and *TLR7* ($p < 0.05$). These effects impacted the inflammatory milieu of rat articular surfaces, where there was a significant reduction ($p < 0.05$) of the pro-inflammatory and oxidative markers with significantly increased production of the anti-inflammatory and antioxidative markers ($p < 0.05$). Marked elevation in aggrecan and collagen type II gene expression was also found in the treated groups ($p < 0.05$).
Conclusion: Those data suggest that BM-MSCs-derived exosomes have a crucial role in mitigating OA symptoms and pathology progression and might be regarded as an effective as well as acceptable treatment option for OA.

Keywords: osteoarthritis; inflammation; MSCs-derived exosomes; *circYAP1*; *miRNA-21*

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Introduction

Osteoarthritis (OA) is a prevalent chronic illness that causes disability and severe physical discomfort in the elderly. It is considered the fourth primary cause of disability globally [1]. OA primarily affects the knees, hips, feet, hands, and spine [2], and its symptoms include chronic pain, stiffness, limited mobility, and joint tenderness [3]. Various risk factors, like age, female sex, genetic role, oxidative stress, and obesity, raise the likelihood of developing OA [4].

The most prominent feature of OA is the articular cartilage defects caused by degeneration of the collagen fibrils and proteoglycans in the cartilage matrix. This triggers chondrocyte surface mechanoreceptors, resulting in the induction of intracellular signaling pathways like nuclear factor kappa-beta (NF-κB) or mitogen-activated protein kinase (MAPK) and the synthesis of catabolic and pro-inflammatory mediators. OA also involves altered cellular metabolism with elevation in the generation of antianabolic, pro-catabolic, and pro-inflammatory substances. The synovium becomes inflamed in the early and late stages of OA due to the effect of the cytokines, prostaglandin E2, reactive oxygen species (ROS), nitric oxide (NO), and neuropeptides which disrupt the cellular metabolism and alter the equilibrium between cartilage matrix restoration and degradation [5].

The pro-inflammatory cytokines like interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-alpha (TNF-α) are crucial in the pathophysiology and development of OA. High levels of IL-6 and IL-1β inhibit the expression of extracellular matrix (ECM) components in OA cartilage. TNF-α, on the other hand, induces the synthesis of matrix metalloproteinases (MMPs) and suppresses the expression of proteoglycan and ECM components in chondrocytes [6,7].

Several microRNAs (miRNAs) have been discovered to influence interleukin-mediated ECM breakdown. *miRNAs* are also implicated in pain pathways and, thus, in clinical symptoms [8]. The precise molecular processes driving microRNA-21 (*miRNA-21*)-mediated knee joint discomfort are yet unknown. Previous research has demonstrated that *miRNA-21-5p* has a crucial function in the pathogenesis of OA. It was found that upregulation of *miRNA-21-5p* results in the initiation and development of OA by targeting fibroblast growth factor 18 (FGF18) to stimulate extracellular matrix degradation. Additionally, antagomir injection prevented cartilage breakdown via pharmacological suppression of *miRNA-21-5p* [9]. In the same context, Zhang *et al*. [10] found that *miRNA-21* overexpression may reduce chondrogenesis by targeting chondrocyte growth differentiation factor-5 (GDF-5).

Hoshikawa *et al*. [11] established that extracellular *miRNA-21* produced from synovial tissue is thought to result in knee joint pain in the OA model via toll-like receptor-7 (*TLR7*) stimulation. The authors reported that the knee joint pain caused by intra-articular *miRNA-21* injection was prevented by a *TLR7* antagonist, indicating that *miRNA-21* has a cellular non-autonomous non-canonical function. Amelioration of knee pain by intra-articular injection of mutant *miRNA-21* lacking a *TLR7* specific sequence further implies that *TLR7* is involved in *miRNA-21*-induced knee pain. *TLR7* activation stimulates several intracellular signaling pathway molecules, like MAPKs, whose stimulation in the peripheral terminals of primary sensory neurons leads to the development of acute pain via post-translational protein modification. Furthermore, synovial *TLR7* activation leads to *c-Jun*, *NF-κB*, and *c-Fos* stimulation [12]. The stimulation of these signaling pathways promotes the generation of downstream cytokines, IL-1β, IL-6, and TNF-α in the primary sensory neurons [13].

Epigenetic regulation of miRNAs, including circular RNAs (circRNAs), has a critical function in the pathogenesis of osteoarthritis. circRNAs are a type of RNA molecule derived from host gene mRNAs, containing a distinctive loop pattern without 5′ or 3′ endpoints. circRNAs are very resistant to exonucleases in contrast to their host genes. circRNAs are commonly involved in the regulation of the genes affecting the course of different illnesses, such as osteoarthritis, because of a variety of miRNA binding sites [14,15].

The dual luciferase reporter test done by Huang *et al*. [16] demonstrated that the circular RNA of Yes-associated protein 1 (*circYAP1*, also known as *hsa_circ_0024093*, a recently identified kind of circRNA, negatively regulates *miRNA-21*, thereby silencing its pro-inflammatory effect.

By investigating the molecular processes of dysregulation of circRNAs and miRNAs inside cartilage microenvironments, researchers may be able to uncover the pathophysiology of OA and develop novel techniques for future OA therapy. Despite the fact that the European Society for Clinical and Economic Aspects of Osteoporosis and Osteoarthritis (ESCEO) treatment algorithm suggests chronic symptomatic slow-acting osteoarthritic medications like glucosamine sulphate (GS) and chondroitin sulphate (CS) as primary treatment for knee osteoarthritis [17], yet mesenchymal stem cells (MSCs) therapy has proven its worth in ameliorating OA symptoms due to their ability of self-renewal, immune modulation and differentiation into cartilage cells. The regenerative, chondroprotective, and anti-inflammatory properties of MSCs could be attributed to their paracrine system [18].

Exosomes extracted from MSCs have been implicated in the etiology of OA and have been proven to be crucial media for cell-to-cell communication in joint cartilage tissue [19]. Extensive profiling has revealed that the exosomes generated from joint tissues and fluids are abundant in non-coding RNAs [20], especially circRNAs, miRNAs, and long non-coding RNAs (lncRNAs). These compounds could be loaded preferentially into exosomes and shuttled

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between stromal cells and chondrocytes [21]. Furthermore, the percentage of these cargos differs according to the donor cell type and the OA stage, implying that their characteristics might reflect the disease state [22].

In the current work, the authors evaluated the therapeutic impact of intra-articular injection of bone marrow-derived mesenchymal stem cells (BM-MSCs)-derived exosomes in ameliorating OA progression. The study also explored the interaction between *circYAP1* and *miRNA-21* in the rat knee joints in the context of exosome treatment.

Materials and Methods

Experimental Animals

The animal house of the Kasr Al-Ainy Hospital served as the source for all the animals. The study protocol (approval number: CU-III-F-60-23) was granted by Cairo University's Institutional Animal Care and Use Committee (IACUC). Forty male Wistar rats 8 to 10 weeks in age and 150–250 g in weight from an inbred strain were used in this investigation. The animals were kept in typical stainless-steel cages with unrestricted accessibility to water and food *ad-libitum* in typical environmental settings. The U.S. National Institute of Health's worldwide standards for the care and usage of laboratory animals were followed. The animals were reared in an aseptic environment in compliance with the IACUC standards.

Experimental Design

The rats were randomized into five groups, eight rats each. Randomization of groups was done through the random table method. One control group and the other four groups experienced artificially induced OA after being anesthetized by 45 mg/kg of 3% intra-peritoneal pentobarbital sodium. A 50 µL injection of 3 mg monosodium iodoacetate (MIA), obtained from Aladdin (Shanghai, China), diluted in sterile normal saline at a concentration of 60 g/L, was administered at a single dose into the right knee joint [23]. After 1-week adaptive breeding, the rats were randomly subdivided into:

Group I: Control group (healthy negative control rats).

Group II: OA rats (positive control).

Group III: OA rats treated with oral GS (200 mg/kg/day), once daily for 10 consecutive weeks [24,25].

Group IV: OA rats treated with oral GS (400 mg/kg/day), once daily for 10 consecutive weeks [24,25].

Group V: OA rats treated with intra-articular injection of BM-MSCs-derived exosome solution (100 µg) into the right knee joint, once a week for 8 consecutive weeks [26].

Rats were euthanized by rapid cervical decapitation at the end of the experimental period.

Preparation of Bone Marrow-Derived Mesenchymal Stem Cells

BM-MSCs were isolated and cultured following previously established protocols [27].

For the purpose of collecting bone marrow, 8-week-old male rats' femurs and tibiae were flushed with Dulbecco's Modified Eagle Medium (DMEM) (D5796, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (F6178, Sigma-Aldrich). The cells were placed over Ficoll-Hypaque (F8016, Sigma-Aldrich) in sterile conical tubes at a ratio of 2:1. After that, the cells were centrifuged. Following their isolation, the nucleated cells were resuspended in a full culture media supplemented with 1% penicillin-streptomycin (P4333, Sigma-Aldrich). For the primary culture, the cells were incubated for 5% humidified CO_2 at 37 °C for 2 weeks with replacement of the media every 4 days.

After the development of big colonies and at 90% confluence, the cultures underwent two PBS washes (P5493, Sigma-Aldrich). After trypsinizing the cells for five minutes at 37 °C using 0.25% trypsin (T1426, Sigma-Aldrich) in 1 mL EDTA (E6758, Sigma-Aldrich), the cells were centrifuged for 20 minutes at 1200 rpm. The cell pellets were suspended and incubated in an incubator at 37 °C. The Mycoplasma test detected no infection.

Flow Cytometry for Detection of Surface Markers in the BM-MSCs

The isolated BM-MSCs were characterized for the expression of the cluster of differentiation (CD) surface markers CD34, CD29, and CD105 using a flow cytometer (NAVIOS EX 10-color, Beckman Coulter, Brea, CA, USA). The third-passage MSCs were digested in 0.25% trypsin (T1426, Sigma-Aldrich), and a single-cell suspension was produced.

Following two PBS buffer washes, the cells were adjusted to 1×10^6 cells/mL concentration. The cells were then treated for 30 minutes at 4 °C with the monoclonal antibodies FITC-CD34 (RAM34, AB_2043837), FITC-CD29 (HMB1-1, AB_763478), and FITC-CD105 (MEM226, AB_1071115) (Invitrogen, Waltham, CA, USA). The cells were then twice washed with PBS and then resuspended. Monoclonal isotype control antibodies were used to identify the background markers. The flow cytometry was utilized to assess the expression of the mesenchymal surface markers and the positivity (%) of the antigens.

Extraction and Characterization of Exosomes Derived From BM-MSCs

Exosomes generated from MSCs were extracted using the techniques mentioned before [27].

The exosomes were isolated from the third passage of stem cells. When the third-passage stem cells achieved 80% confluence, the media was removed and DMEM was added. The culture media was then centrifuged at 10,000 ×g for

Table 1. Primer sequences of the studied genes.

Gene symbol	Primer sequence from 5′- 3′
miRNA-21	(F) 5′ AGCTTATCAGACTGATGTTG 3′ (R) 5′ GAACATGTCTGCGTATCTC 3′
circYAP1	(F) 5′ GCAAGAACTGCTTCGGCAGGTCCT 3′ (R) 5′ GTTTATATAGTAAATTTCTCCATC 3′
β-actin (housekeeping gene)	(F) 5′ TCACCCACACTGTGCCCATCTATGA 3′ (R) 5′ CATCGGAACCGCTCATTGCCGATAG 3′
TLR7	(F) 5′ GTTTTACGTCTACACAGTAACTCTCTTCA 3′ (R) 5′ TTCCTGGAGGTTGCTCATGTTTT 3′
U6 snRNP (small nuclear ribonucleoprotein)	(F) 5′ TGCTTCGGCAGCACATATAC 3′ (R) 5′ AGGGGCCATGCTAATCTTCT 3′
Aggrecan	(F) 5′ CTTGGGCAGAAGAAAGATCG 3′ (R) 5′ GTGCTTGTAGGTGTTGGGGT3′
Collagen type II	(F) 5′ ATGACAATCTGGCTCCCAACACTGC 3′ (R) 5′ GACCGGCCCTATGTCCACACCGAAT 3′

F, Forward primer; R, Reverse primer. *miRNA-21*, microRNA-21; *circYAP1*, circular RNA of Yes-associated protein 1; *TLR7*, toll-like receptor-7.

15 minutes at 4 °C, and the supernatant was obtained. To extract exosomes, the collected supernatant was ultracentrifuged at 100,000 ×g for 1 hour at 4 °C. After that, the exosomes were once more suspended in 200 µL of pre-chilled PBS and kept for later usage at –80 °C. After washing, the isolated exosomes were fixed with 2.5% glutaraldehyde for 2 hours to facilitate characterization. The exosomes were then ultracentrifuged and suspended in 100 µL of human serum albumin (HSA).

Ten µL of the exosome suspension was dropped on carbon-coated copper grids with a pore size of 2 nm, and the grids were left to stand at room temperature for three to five minutes. The exosomes were negatively stained with 3% aqueous phosphor-tungstic acid for one minute. After blotting, the transmission Electron Microscope (TEM) (JEOL, JEM-2100, Tokyo, Japan) was used to identify the exosomes as round or oval vesicles with membranous structures and was also used to capture images of the exosomes on the copper grids.

At the end of the experimental period, the serum samples were withdrawn from the rat tail veins to assess the inflammatory and oxidative biomarkers. Euthanasia was done through 100% V/min CO_2 due to its quick onset of action. While the proximal tibia specimens were obtained and fixed with 4% paraformaldehyde for histological evaluation, the distal femur knee joint tissues were separated and preserved at –80 °C for genetic research.

Estimation of Gene Expression of circYAP1, miRNA-21, TLR7, Aggrecan, and Collagen Type II by Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

The GeneJET RNA Purification Kit (#K0702, Thermo Fisher Scientific, Inc., Waltham, MA, USA) was applied to obtain the total RNA from the rat tissues. The quality of the RNA was checked using a NanoDrop® 1000 spectrophotometer (NanoDrop Technologies, Inc. Wilmington, NC, USA). Then, complementary DNA (cDNA) was generated using the High-Capacity cDNA Reverse Transcription Kits (Cat.# 4368814, Thermo Fisher Scientific, Inc., Waltham, MA, USA). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to amplify the genes *circYAP1*, *miRNA-21*, *TLR7*, aggrecan, and collagen type II through SYBR Premix Ex TaqTM II (Cat.# RR39WR, Perfect Real Time, TaKaRa Bio Inc. Osaka, Japan). Gene Runner Software (Cat.# 5.0.69 Beta; Hastings Software Inc., Hastings, NY, USA) was utilized to create the PCR primers from RNA sequences found in GenBank (Table 1).

The following were the PCR reaction's conditions: 95 °C for 5 minutes, followed by 40 cycles at 95 °C for 15 seconds, and one minute at 60 °C. The data were quantitatively analyzed utilizing the $2^{-\Delta\Delta Ct}$ technique normalized to *U6* small nuclear ribonucleoprotein (snRNP) and *β-actin*.

Estimation of Serum Levels of Inflammatory Cytokines by Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was used for the estimation of TNF-α (Cat.# E-EL-R2856), IL-1β (Cat.# E-EL-R0012), IL-4 (Cat.# E-EL-R0014), and IL-10 (Cat.# E-EL-R0016), based on the manufacturer's instructions (Elabscience Biotechnology, Austin, TX, USA). The ROS (#CSB-EL020063RA), glutathione (GSH) (#E02G0233), and malondialdehyde (MDA) (#E02M0023) were evaluated as per the manufacturer's instructions (Wuhan EIAab Science, Wuhan, China) using an ELISA plate reader (Stat Fax 2200, Awareness Technologies, Los Angeles, CA, USA). The samples were done in triplicates.

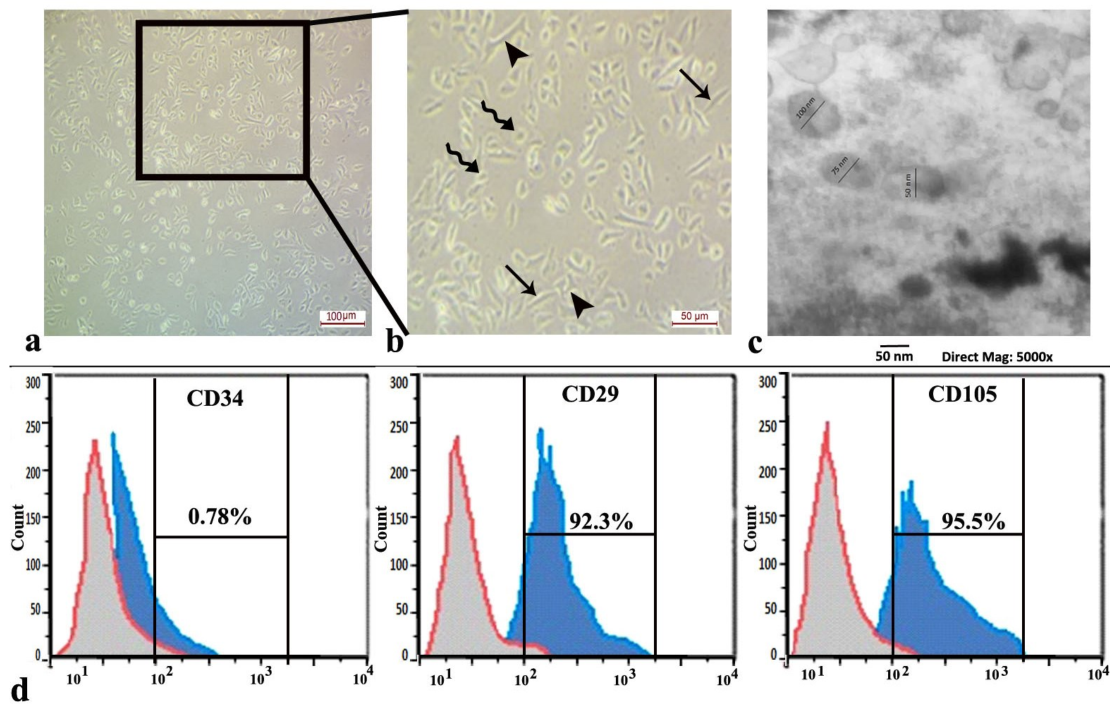

Fig. 1. Characterization of BM-MSCs and exosomes. (a) Morphology of BM-MSCs in culture at 21 days showing adhesive cells in culture with about 70–80% confluence of cells. (b) Higher magnification shows BM-MSCs appearing as branched (arrowhead), spindle, and fusiform-shaped cells (arrows) with few spheroids and polyhedral forms (spiral arrows). The photos were presented by an inverted microscope. (c) Ultrastructure of BM-MSC-derived exosomes by transmission electron microscopy. The exosomes show the characteristic oval or spheroid double-membrane bound morphology with a 50–100 nm diameter. (d) Flow cytometric analysis of cultured MSCs (Negative for CD34, and positive for CD29 and CD105). BM-MSCs, bone marrow-derived mesenchymal stem cells.

Histopathological Examination of Osteoarthritis

After careful dissection, the right knee joints were cleaned of any adhering tissue and preserved in 0.1 M phosphate-buffered saline (PBS) with 4% paraformaldehyde solution (P885233, Macklin, Shanghai, China). For decalcification, the knee joints were immersed in 20% w/v ethylenediamine tetraacetic acid (EDTA; 6381-92-6, Loba Chemie Pvt. Ltd., Mumbi, India) for six weeks at 4 °C. Every three days, the decalcifying solution was changed. The knee joints were then dehydrated via an ethanol series at room temperature, cleaned in xylene, set in paraffin, sliced into 5-µm-thick sections, mounted on glass slides, and stained for two minutes with hematoxylin staining solution (KGA223, Keygen Biotech, Nanjing, China). Any excess was removed using an alcohol and hydrochloric acid mixture for one second. This was followed by a 25-second eosin stain. The sections were then rinsed with water and dehydrated using ascending grades of alcohol, xylol, and balsam concentrations [28]. The thickness of the articular cartilage was estimated using a light microscope (Olympus BX51, Olympus, Tokyo, Japan) [29].

Statistical Methods

The statistical program for the social sciences (SPSS) version 28 (#28.0.1.1, IBM Corp., Armonk, NY, USA) was utilized to code and input the data. The data were represented as mean and standard deviation. Analysis of variance (ANOVA) with multiple comparisons post hoc Bonferroni test was applied to compare the groups [30]. *p*-values of less than 0.05 were regarded as statistically significant.

Results

Characterization of BM-MSCs and Exosomes

The MSCs were distinguished by their colony-forming unit and fibroblast spindle shape (Fig. 1a,b). Transmission electron microscopy was utilized to determine the exosomes based on their size (50–100 nm) and cup-shaped spheroidal morphology (Fig. 1c). MSCs' phenotypic FACS analysis revealed that the surface markers CD29 (92.3%) and CD105 (95.5%) were positive, whereas CD34 (0.78%) was negative (Fig. 1d).

Effect of GS and Exosomes on circYAP1, miRNA-21, and TLR7 Gene Expression

Fig. 2 represents the results of our experiment investigating the effect of glucosamine sulfate (GS) and exosomes on gene expression levels in osteoarthritis (OA). Specifically, we measured the expression levels of *circYAP1* due

Table 2. Inflammatory and oxidative stress biomarkers in the assessed groups.

	Control	OA	Glucosamine sulfate 200	Glucosamine sulfate 400	Exosomes
TNF-α (pg/mg protein)	27.77 ± 5.25	148.03 ± 15.34 *	87.07 ± 8.46 $^{*\#}$	54.43 ± 8.61 $^{*\#\$}$	43.9 ± 8.36 $^{\#\$}$
IL-1β (pg/mg protein)	20.77 ± 5.22	189.07 ± 20.64 *	95.33 ± 8.77 $^{*\#}$	68.23 ± 8 $^{*\#\$}$	39.33 ± 7.23 $^{\#\$@}$
IL-4 (pg/mg protein)	82.77 ± 4.92	18.17 ± 2.64 *	50.87 ± 4.71 $^{*\#}$	61.2 ± 6.24 $^{*\#\$}$	74.97 ± 7.68 $^{\#\$@}$
IL-10 (pg/mg protein)	50.27 ± 12.06	17.87 ± 3.59 *	36.1 ± 6.08 $^{*\#}$	46.7 ± 8.7 $^{\#}$	53.9 ± 6.72$^{\#\$}$
ROS (pg/mg protein)	21.17 ± 2.91	96.07 ± 7.11 *	54.07 ± 6.08 $^{*\#}$	48.8 ± 5.62 $^{*\#}$	28.23 ± 3.94 $^{\#\$@}$
GSH (nmol/mg protein)	1.69 ± 0.14	0.44 ± 0.2 *	0.84 ± 0.1 $^{*\#}$	1.27 ± 0.13 $^{*\#\$}$	1.38 ± 0.17 $^{*\#\$}$
MDA (nmol/mg protein)	0.37 ± 0.09	1.62 ± 0.14 *	0.97 ± 0.14 $^{*\#}$	0.59 ± 0.16 $^{\#\$}$	0.6 ± 0.14 $^{\#\$}$

Values are presented as mean ± standard deviation (SD). Statistically significant relative to the corresponding value in: * vs control group, $^{\#}$ vs OA group, $^{\$}$ vs glucosamine sulfate (200 mg/kg/day) group, $^{@}$ vs glucosamine sulfate (400 mg/kg/day) group ($p < 0.05$) (n = 8). OA, osteoarthritis; TNF-α, tumor necrosis factor-alpha; IL-4, interleukin 4; IL-10, interleukin 10; IL-1β, interleukin 1β; ROS, reactive oxygen species; GSH, glutathione; MDA, malondialdehyde.

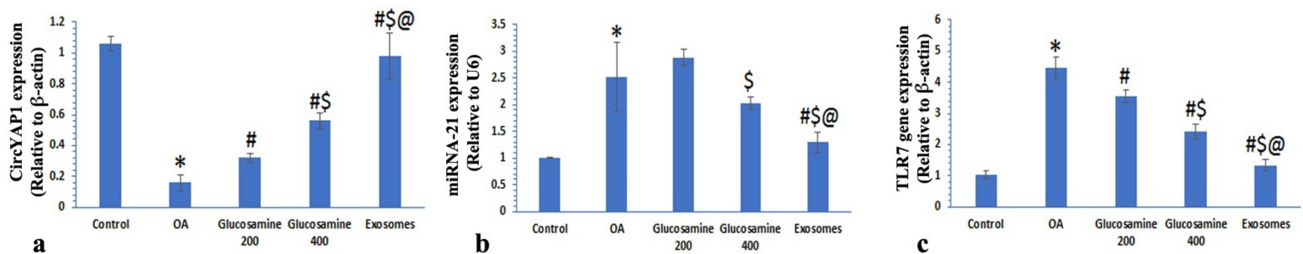

Fig. 2. Comparison between (a) *circYAP1*, (b) *miRNA-21*, and (c) *TLR7* gene expression in all assessed groups. Values are presented as mean ± SD. statistically significant relative to the corresponding value in: * vs control group, $^{\#}$ vs OA group, $^{\$}$ vs glucosamine sulfate (200 mg/kg/day) group, $^{@}$ vs glucosamine sulfate (400 mg/kg/day) group ($p < 0.05$) (n = 8). *circYAP1*, circRNA of Yes-associated protein 1; *miRNA-21*, microRNA-21; TLR7, toll-like receptor-7.

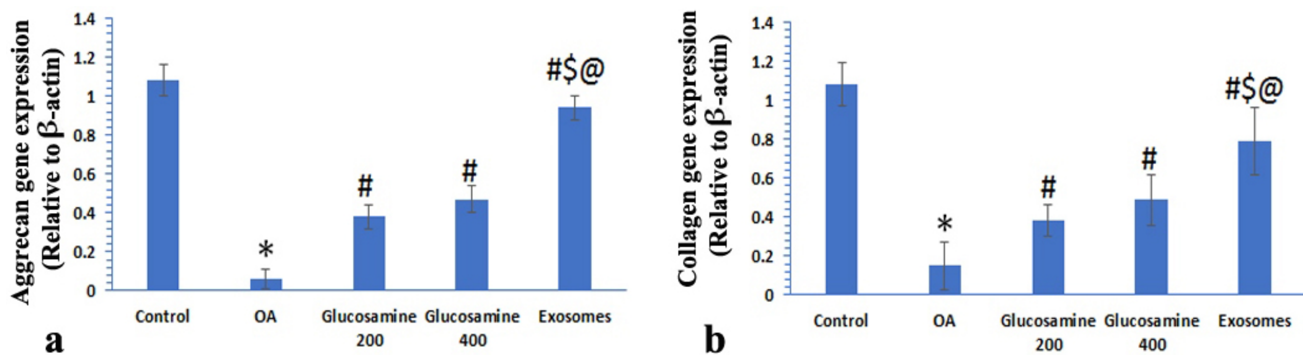

Fig. 3. Comparison between aggrecan and collagen type II Gene Expression in all assessed groups. (a) Aggrecan gene expression (Relative to β-actin), (b) collagen type II gene expression (Relative to β-actin). Values are presented as mean ± SD. Statistically significant relative to: * vs control group, $^{\#}$ vs OA group, $^{\$}$ vs glucosamine sulfate (200 mg/kg/day) group, $^{@}$ vs glucosamine sulfate (400 mg/kg/day) group ($p < 0.05$) (n = 8).

to its role in promoting osteogenic differentiation and attenuating osteoporosis. We also assessed *miRNA-21* as a novel crucial regulator of OA pathogenesis by promoting cartilage degradation by targeting the *TLR7* genes. Our results showed significant downregulation of *circYAP1* with subsequent upregulation of both *miRNA-21* and *TLR7* gene expression in the OA group (p-value < 0.05). These results were reversed in all treated groups, with the best results shown in the exosomes-treated group (p-value < 0.05) (Fig. 2).

Assessment of Inflammatory and Oxidative Stress Biomarkers

The present results revealed significant elevation in IL-1β, TNF-α, total ROS, and MDA levels in the OA group. This was accompanied by a substantial reduction in IL-4, IL-10, and GSH levels ($p < 0.05$). Treatment with Exosomes and GS (200 & 400 mg/kg/day) compensated for this inflammatory and oxidative status with a significant reduction in IL-1β, TNF-α, total ROS, and MDA, along with

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a significant increase in levels of IL-10, IL-4, and GSH, with the best results obtained in the Exosomes-treated group (Table 2).

Effect of Treatment on Aggrecan and Collagen Type II Gene Expression

Our results revealed a significant decrease in the gene expression of the cartilage matrix protein, collagen type II, and the proteoglycan, aggrecan in the OA group (p-value < 0.05). A significant increase in the aggrecan and collagen type II mRNA levels was observed in the GS (200 & 400 mg/kg/day)- and Exosomes-treated groups, with the highest levels observed in the Exosomes-treated groups (Fig. 3).

Histopathological Examination of the Articular Surface among Different Studied Groups

Hematoxylin and Eosin (H&E)-stained articular tissues of the rats treated with BM-MSCs-derived exosomes showed marked improvement and reduction of osteoarthritis grading, while those treated with GS 200 & 400 mg/kg/day showed mild to moderate improvement, respectively with a significant increase in the mean arbitrary length of a cartilaginous thickness (Fig. 4).

Discussion

Our research demonstrated that intra-articular injection of BM-MSCs-derived exosomes has a remarkable therapeutic effect on OA, as evidenced by the molecular, biochemical, and histopathological findings of the study, with more superior anti-inflammatory and anti-oxidative effects than the conventional treatment glucosamine sulfate. We hypothesized that the role of exosomes in ameliorating OA progression could be related to the potential epigenetic regulation of *miRNA-21/TLR7* signaling pathway through their pool of cargos, including different types of non-coding RNAs. This study demonstrated significant downregulation of the *circYAP1*, with consequent upregulation of its target, *miRNA-21*, and the downstream *TLR7* in OA. Treatment with intra-articular injection of BM-MSCs-derived exosomes showed restoration of the normal levels of *circYAP1* mRNA, with subsequent down-regulation of *miRNA-21* and, consequently, *TLR7*. This relationship highlights the sponge-like effect of *circYAP1* that leads to *miRNA-21* sequestration. These findings are aligned with Xue *et al*. [31], who demonstrated the crucial role of *circRNAs* in the pathophysiology of OA. The authors reported that dysregulation of *circRNAs* in the OA synovial tissue mediates abnormal chondrocyte proliferation, synovial inflammation, cartilage degradation, bone injury, and improper immunological responses.

In the same context, it is established that exosomes secreted by MSCs protect the articular cartilage from damage via lncRNA-miRNA, circRNA-miRNA, and miRNA-mRNA co-expression networks in osteoarthritis [32]. In accordance with our results, the relationship between *circYAP1* and *miRNA-21* was examined in an acute kidney injury model and revealed that upregulation of *circYAP1* expedited cell growth via sponging *miRNA-21-5p*. This resulted in decreased production of inflammatory and ROS markers. The authors also showed that *circYAP1* stimulated the PI3K/AKT/mTOR pathway, thereby protecting the HK-2 cells from ischemia/reperfusion damage [16]. Another study by Huang Y *et al*. [33] revealed that *circYAP1* reduced osteoporosis by increasing YAP1 protein levels and activating the Wnt/-βcatenin signaling pathway.

Consistent with our results, Hoshikawa *et al*. [11] discovered that extracellular overexpression of *miRNA-21* secreted from synovial tissue mediates OA. The *miRNA-21* ligand is the *TLR7*, which has a GU-rich motif required for pattern recognition by *TLRs*. In an OA rat model, a single intra-articular injection of a *miRNA-21* inhibitor or *TLR7* antagonist offered long-term pain reduction [11].

Earlier research has demonstrated that miRNAs can promote the release of inflammatory cytokines, which in turn would trigger autoimmune disease. In SLE and rheumatoid arthritis, endogenous *TLR7* ligands have been demonstrated to upregulate the T cell polarizing cytokines IFN-α, IL-1β, and IL-6. *miRNA-21* also stimulated the expression of IL-17 and IL-23 in psoriatic arthritis [34]. Our study confirmed those results, where *miRNA-21* upregulation in the OA group was accompanied by a significant increase in the pro-inflammatory and oxidative markers (TNF-α, IL-1β, MDA, and ROS), and a significant reduction in the anti-inflammatory and antioxidant markers (IL-4, IL-10, and GSH).

The results of the GS-treated groups in our study also support the previous studies illustrating the high effectiveness of glucosamine in improving OA and confirming that this efficacy is achieved via inhibition of the inflammatory markers through a *TLR4-NF-kB*-dependent pathway [35]. Another study by Conrozier T and Lohse T [36] revealed that Glucosamine improved the symptoms, the prognosis, and the concurrent persistent inflammation and oxidative stress associated with OA.

Our results also support the previous studies illustrating the role of umbilical cord MSCs (UC-MSCs) intra-articular injection in improving OA. The MSCs improved OA by inhibiting the expression of the pro-inflammatory cytokines and the cartilage-degrading enzymes [37]. Another study elucidated that MSCs-derived exosomes enhanced osteoarthritis in a mouse model by promoting the osteogenic differentiation of the cartilage cells and enhancing the anti-oxidant Nrf2/HO-1 signaling pathway [38].

A comparable anti-inflammatory activity was also reported with the BM-MSCs-extracellular vesicles (EV) which acted as mediators of chondrocyte homeostasis. Application of EV to OA chondrocytes decreased the production of the pro-inflammatory interleukins (IL-1α, IL-6, IL-1β, IL-17, and IL-8), inhibited the *NF-κB/TNF-α* signaling

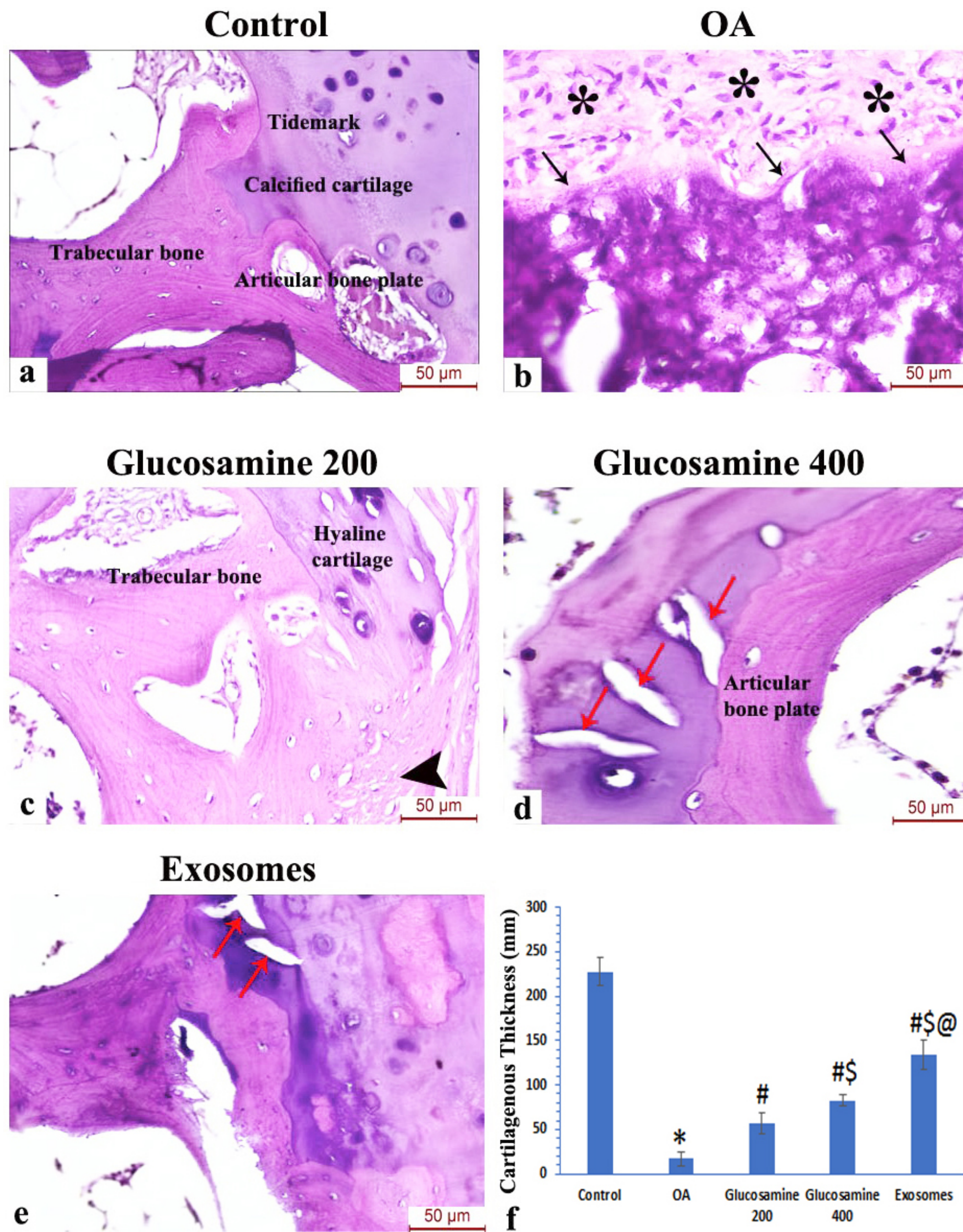

Fig. 4. Histopathological examination of rat joint tissues of different groups. (a) Normal Control: Osteoarthritis Research Society International (OARSI) grade 0. Hyaline articular cartilage with smooth cartilage surface. The matrix and associated chondrocytes are organized in appropriately oriented, well-ordered layers. No enlargement/distortion of chondrons nor proliferative changes of chondrocytes (mean arbitrary thickness 227 mm overlying the articular bone plate). (b) Osteoarthritis group: OARSI grade 6. The unmineralized hyaline cartilage is completely eroded. The articular surface is composed of irregularly deformed bone plates due to microfractures (arrows) occupied by reparative fibrocartilage (*) associated with bone repair. (c) Model treatment with glucosamine sulphate (GS) (200 mg/kg/day): OARSI grade 4: Focal erosion and loss of Cartilaginous matrix replaced by fibrous tissue (arrowhead). (d) Model treatment with GS (400 mg/kg/day): OARSI grade 3: vertical fissures in cartilaginous matrix extending into the mid and deep zone (red arrows) indicating a better protective effect on cartilaginous matrix. (e) Exosomal therapy: OARSI grade 3: vertical fissures in cartilaginous matrix extending into the mid and deep zone (red arrows) (Hematoxylin and Eosin (H&E), 400×). The mean arbitrary thickness (134 mm) is higher than treatment with GS 200 and GS 400 (mg/kg/day) indicating a better protective effect on the cartilaginous matrix. (f) Mean arbitrary length of cartilaginous thickness in mm per 10 high power fields (HPF). Values are presented as mean ± SD. Statistically significant relative to the corresponding value in: * vs control group, # vs OA group, $ vs glucosamine sulfate (200 mg/kg/day) group, @ vs glucosamine sulfate (400 mg/kg/day) group ($p < 0.05$) (n = 8).

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and promoted chondrocyte proliferation and glycosaminoglycans (GAGs) deposition [39]. This is consistent with our results, where we illustrated a significant increase in the expression of aggrecan and collagen type II in the BM-MSCs-derived exosomes treated group.

The improvement in the histopathological architecture of the rats' articular tissue of the exosome-treated group is concordant with the previous results of Zhu *et al.* [5] who illustrated the effectiveness of induced pluripotent stem cells-derived exosomes (iMSC-Exosomes) and synovial MSCs-derived exosomes in improving the cell count, immunohistochemistry and the histopathological features of OA. The therapeutic effects of the pluripotent MSC-Exosomes showed better results in promoting the proliferation and migration of chondrocytes [5,40].

Future studies investigating the protective role of other circular and micro RNAs on the pathogenesis of OA are highly required. Also, testing other osteoarthritis models using different modes of administration of exosomes would give more comprehensive evidence about the protective role of exosomes in the context of osteoarthritis. Employing specific knock-out models for target genes can better confirm the specific genes implicated in osteoarthritis and/or regulated by exosomes.

Conclusion

Intra-articular administration of BM-MSCs-derived exosomes could be a potentially efficient and recent line for treating OA, as proved by the enhanced histopathological picture of the rats' joint tissues and the improved biochemical and molecular markers. Our study highlighted the crucial role of BM-MSCs-derived exosomes in modulating the *circYAP1*/*miRNA-21*/*TLR7* axis, causing alleviation of inflammation and stimulation of chondrocyte proliferation. The exact molecular mechanisms of action warrant further investigation.

Availability of Data and Materials

Data are available upon reasonable request from the corresponding author.

Author Contributions

DM, MS, and DS designed the research study. DM and DS performed the research. AH, DE, ME, NA, HE, and AS aided in the experimental methodology. MA helped in validation and funding acquisition. SE and BA performed manuscript writing, reviewing, editing, methodology and validation. All authors have made critical revisions to the manuscript. All authors have read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of this work.

Ethics Approval and Consent to Participate

The animal study protocol was approved by Cairo University's Institutional Animal Care and Use Committee (IACUC) (approval number: CU-III-F-60-23).

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Conflict of Interest

The authors declare no conflict of interest.

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