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The Role of Extracellular Vesicles Derived from MicroRNA-146a–modified Mesenchymal Stem Cells in Modulating Inflammation in Experimental Glenohumeral Osteoarthritis

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ABSTRACT

Glenohumeral osteoarthritis (GOA) is characterized by chronic inflammation leading to joint damage. Extracellular vesicles (EVs) derived from mesenchymal stem cells (MSCs) are promising therapies because of their immunomodulatory functions. The anti-inflammatory effects of EVs from human Adipose-derived MSCs (hADSCs) overexpressing microRNA (miR)-146a were investigated in experimental GOA in this study.

hADSCs were transfected with a mimic negative control or miR-146a mimics. GOA was induced in C57/Bl6j mice, and subsequently, the animals were treated intra-articularly with phosphate-buffered saline, miR-146a EVs, or miR-control EVs. The expression of miR-146a and its targeted cytokines interleukin (IL)-4, IL-10, tumor necrosis factor-alpha (TNF-α), IL-17, and interferon-gamma (IFN-γ) were analyzed in the spleen of mice by enzyme-linked immunosorbent assay and in the articular cartilage by real-time polymerase chain reaction.

miR-146a EVs showed enrichment of miR-146a. In GOA mice, miR-146a EV treatment significantly reduced expression levels of inflammatory cytokines IFN-γ, IL-17, and TNF-α and increased the anti-inflammatory cytokine IL-10 and IL-4 compared to controls. miR-146a EV treatment raised the anti-inflammatory cytokines and reduced the pro-inflammatory cytokines of the spleen in treated mice.

This study demonstrates that EVs derived from hADSCs overexpressing miR-146a have enhanced anti-inflammatory potential in GOA by modulating cytokine expression and production. EVs engineered with inflammation-related miRNAs could be a cell-free therapeutic approach for GOA.

Keywords: Exosome; Extracellular vesicles; Glenohumeral; Mesenchymal stem cells; Osteoarthritis

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INTRODUCTION

Glenohumeral osteoarthritis (GOA) is defined by the deterioration of the subchondral bone and cartilage in the

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shoulder joint, leading to a reduction in the width of the glenohumeral joint.[1] The third most commonly affected large joint of the human body is the glenohumeral joint.[2] Determining the exact prevalence of glenohumeral osteoarthritis is challenging, but studies based on population samples indicate that 16.1% to 20.1% of adults aged 65 and older show radiographic signs of glenohumeral osteoarthritis.[3,4] Osteoarthritis symptoms encompass joint discomfort and restricted movement, while observable changes in radiology images involve the thinning of joint spaces, the development of osteophytes, the presence of periarticular cysts, and subchondral sclerosis.[5] Presently, there are no available treatments that can modify the course of osteoarthritis. Available interventions are restricted to providing relief from symptoms, typically achieved through the use of pain medications or joint injections, and ultimately surgical joint replacement for advanced stages of the disease.[6] The absence of treatments that modify the course of the disease underscores the importance of gaining a more comprehensive understanding of the underlying mechanisms of osteoarthritis. This knowledge is crucial for the development of effective therapies capable of impeding or preventing the progression of the condition. In the context of osteoarthritis, inflammation significantly contributes to the disease and is strongly implicated in disease initiation and progression.[7] Elevated levels of pro-inflammatory cytokines, including interleukin (IL)-1β and tumor necrosis factor (TNF)-α, are observed in the joints affected by osteoarthritis and are thought to drive cartilage breakdown through activation of matrix-degrading enzymes.[8]

Mesenchymal stem cells (MSCs) are stromal cells endowed with the capacity for self-renewal and the capability to undergo differentiation into various cell lineages.[9-11] These cells can be harvested from diverse tissues such as bone marrow, adipose tissue, umbilical cord, and other sources.[12-14] The ability of MSCs to travel to sites of inflammation, as well as their ability to release paracrine factors that mitigate inflammation and support tissue healing, positions MSCs as promising candidates for osteoarthritis (OA) therapy.[15] Several studies have demonstrated the chondroprotective potential of MSCs for OA therapy. MSCs can differentiate into chondrocytes and release paracrine markers which inhibit inflammation and stimulate cartilage repair.[16,17] Direct injection of MSCs into arthritic joints has been shown to alleviate OA symptoms in animal models by reducing cartilage degradation and subchondral bone changes.[18,19] A developing approach to boost the effectiveness of MSCs entails altering their gene expression by introducing microRNAs (miRNAs).[20] miRNAs belong to a class of noncoding RNAs that have the capacity to modulate gene expression through diverse mechanisms.[21] These single-stranded, short molecules (20–25 nucleotides) operate at the transcriptional level to control gene expression.[22] miR-146a plays a role in negatively regulating the inflammatory response by influencing the Nuclear factor kappa B (NF-κB), tumor necrosis factor receptor-associated factor 6 (TRAF6), and IL-1 receptor-associated kinase 1 (IRAK1) pathway which are crucial molecules involved in the downstream signaling of pro-inflammatory cytokines.[23] Earlier works indicated that miR-146a-5p derived from MSCs has a potential for anti-inflammatory properties and promotes tissue regeneration in animal models.[24] Interestingly, recent studies have shown that MSCs can convey miRNAs to several sites by extracellular vesicles (EVs).[25] These membrane-bound structures contribute to facilitating communications between cells and transporting biological molecules.[26] MSC-EVs can replicate numerous therapeutic effects observed in the parent MSCs.[27] This suggests that they could offer a cell-free therapeutic strategy for OA, potentially overcoming challenges associated with directly administering cells. However, the potential of MSC-EVs modified to overexpress specific microRNAs, such as miR-146a, for targeting inflammation in OA has not yet been explored.

The current study suggests that EVs released from human adipose-derived MSCs (hADSCs) modified with miR-146a (MSC-miR-146a-EVs) could serve as a promising and innovative therapeutic strategy for regulating inflammation and decelerating the progression of experimental GOA.

MATERIALS AND METHODS

All materials and reagents used in this study were obtained from reputable commercial suppliers. Gibco (UK) provided cell culture media and supplements. Enzymes such as collagenase type I purchased from Sigma Aldrich (USA). Kits for RNA isolation, cDNA synthesis, and real-time polymerase chain reaction (PCR) were sourced from Thermo Fisher Scientific (USA). Enzyme-linked immunosorbent assay (ELISA) kits to quantify cytokine levels were procured from eBioscience (USA).

Isolation of hADSCs

To obtain hADSCs, subcutaneous adipose tissues were acquired with donor consent. The tissues were then processed through washing, chopping, and digestion using collagenase type I at a concentration of 1mg/mL for a duration of 30 to 60 minutes. After centrifuging at 600*g* for 5 minutes, the cellular pellet was resuspended in Dulbecco's Modified Eagle's Medium (DMEM) with low concentration, supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS). The resultant stromal vascular fraction or digested tissue was cultured in T75 culture flasks, and cells adhering to the surface were passaged upon reaching 80% confluence.[28]

Preparation of hADSC-derived miR-146a

hADSCs underwent transfection with either a mimic negative control (mimic NC, a non-targeting scrambled oligonucleotide without a known human target) or miR-146a mimic using Lipofectamine 3000, following the provided guidelines. Briefly, when hADSCs reached around 80% confluence, they were placed in Opti-MEM medium and exposed to Lipofectamine 3000 reagent along with either the miR-146a mimic or mimic NC for 72 hours.

hADSC-derived miR-146a EVs Isolation

Transfected hADSCs were utilized for extracellular vesicle (EV) isolation. Upon reaching 80% to 90% confluence, the regular culture medium of the transfected hADSCs, containing FBS, was substituted with a medium free of serum, and the cells were cultured for 2 days. Subsequent to this, the culture medium was gathered, and EVs were obtained through the ultracentrifuge method. The initial centrifugation took place for 20 minutes at 500*g* at 4°C, followed by a subsequent step at 18,000*g* for 30 minutes. For isolating extracellular vesicles, using a 0.22 μm filter, the supernatant underwent filtration, then ultracentrifuged for 90 minutes at 110,000*g*, and finally suspended in PBS.[29]

Western Blot Analysis

For the characterization of the isolated EVs western blotting for CD9 and CD63 markers was performed. Extracted total protein was achieved employing Radio-Immunoprecipitation Assay (RIPA) lysis and extraction buffer procured. Protein concentrations were assessed using the bicinchoninic acid (BCA) method prior to separating equal quantities of protein on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The isolated proteins were subsequently moved onto polyvinylidene fluoride (PVDF) membranes. Following blocking with 5% non-fat milk, the membrane was allowed to incubate overnight at a temperature of 4°C with primary antibodies specifically targeted against CD63 and CD9, facilitating the recognition and binding of these antibodies to their respective antigens. Afterward, the membranes were subjected to an additional incubation period lasting 120 minutes at room temperature. During this stage, the membranes were exposed to a secondary antibody, specifically an anti-rabbit immunoglobulin G antibody that was conjugated with horseradish peroxidase. This strategic incubation aimed to optimize the detection and visualization of the proteins under investigation. The horseradish peroxidase-conjugated secondary antibody binds to the primary antibodies previously applied to the target proteins, amplifying the signal for subsequent detailed analysis and interpretation.

Glenohumeral Osteoarthritis Animal Model and Treatment Protocol

Thirty C57Bl/6j mice, aged 12 months, were accommodated in an environment where the temperature was carefully regulated at 24°C. The mice experienced controlled light/dark cycles and received sterile food and water, ensuring a pathogen-free setting. All procedures involving the animals were executed under approved protocols from the Third Hospital of Shijiazhuang, Shijiazhuang, Hebei Province, China. Every possible measure was taken to reduce any potential suffering experienced by the animals. To initiate experimental osteoarthritis (GOA), mice were anesthetized through an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). This anesthesia regimen was employed to ensure proper sedation and immobilization of the mice during the induction procedure. Their shoulder areas were then shaved and sterilized with alcohol or betadine. A small incision was made through the skin over the glenohumeral joint and the joint capsule was exposed via blunt dissection. A 30-gauge needle attached to a microinjection syringe was then used to penetrate the joint capsule and inject 3 to 5 μL of a 2% solution of monoiodoacetic acid intra-articularly into the glenohumeral joint space. Then the needle was gently withdrawn and following the procedure, the incision in the skin was securely sealed using either sutures or staples. The mice were allowed to recover from

anesthesia and were monitored for any signs of distress, providing analgesics as needed.[30]

Over 2 weeks, continuous monitoring was conducted to facilitate the development of GOA in the mice. After the establishment of GOA, the mice were assigned randomly to 3 distinct groups, each consisting of 10 mice. These groups included a control group, which received PBS, a miR-control EVs-treated group, and a miR-146a-EV–treated group that received 100 μg EVs. The treatment was administered into the glenohumeral joint space.

Splenocytes Preparation

Once the spleen was isolated, a cellular suspension was created in Roswell Park Memorial Institute (RPMI)-1640 medium by mechanically processing small tissue fragments through a 70 μm cell strainer. Subsequently, to lyse red blood cells, they were disrupted by exposure to an ammonium chloride buffer at a temperature of 4°C for 10 minutes. The cellular mixture underwent centrifugation at 300*g* for 5 minutes at 4°C, leading to the formation of a cell pellet. This pellet was subsequently subjected to 2 consecutive washes with RPMI to ensure the removal of any residual contaminants. The collected splenocytes were then reconstituted in complete RPMI, supplemented with 10% FBS and 100 μg/mL penicillin-streptomycin, to create an optimal environment for their subsequent use in experiments.

Cytokine Assay

The splenocytes were collected and placed in a 24-well culture plate, with each well containing 2 million cells. After 48 hours, supernatants containing secreted proteins were gathered from the wells. ELISA kits were utilized to quantify the concentrations of various cytokines present in the supernatants. These included TNF-α, transforming growth factor-beta (TGF-β), interferon-gamma (IFN-ɣ), IL-4, IL-10, and IL-17a. This analytical approach enabled precise measurements of these cytokines, providing valuable insights into the immune and inflammatory responses within the experimental context. The ELISA assays were done following the methods provided by the manufacturer. To ensure precision, samples underwent testing in triplicate measurements.

Gene Expression Analysis in the Articular Cartilage

hADSC-EV RNA extraction was completed according to TRIzol manufacturer protocols. For miR-146a expression quantification, A quantity of 1 microgram of RNA underwent reverse transcription to generate complementary DNA (cDNA) utilizing a miRcute miRNA First-Strand cDNA Synthesis Kit. The assessment of relative miR-146a expression levels was conducted using the ABI 7500 Real-Time PCR system applying the $2^{-\Delta\Delta Ct}$ method with the miRcute miRNA qPCR Detection Kit. The expression of various cytokines within articular cartilage was examined 3 weeks post-induction. Isolation of RNA from the tissue and its concentration was established using a NanoDrop Spectrophotometer. The process of generating cDNA was carried out by utilizing 1 μg of total RNA and employing the cDNA Reverse Transcription Kit. The purity of the resulting cDNA was confirmed using NanoDrop. Real-time RT-PCR was conducted in triplicate for 5 genes: *IL10*, *IL4*, *IFNG*, *TNFA*, and *IL17A*. An internal control gene (β-actin) was applied for normalization. Gene expression thresholds were calculated using the relative quantification method, denoted as $2^{-\Delta\Delta CT}$, following the standard protocol. The mRNA expression fold, relative to the control, was considered for further analysis.

Statistical Analysis

A comparison among groups with normal distribution was conducted using a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test, while the Kruskal–Wallis test was employed for groups with non-normal distribution. The presentation of all data included mean values along with standard deviation, and statistical significance was established at p values below 0.05. Graphical representations were generated using GraphPad Prism 8 software. The statistical analyses were executed using IBM SPSS Statistics.

RESULTS

Characterization of hADSC-EVs

Subsequently, hADSCs were transfected with a miR-146a mimic and negative control. EVs were isolated from the hADSCs supernatants 72 hours post-transfection. To characterize these EVs, Western blot analysis was employed to detect the surface markers of EVs (CD9 and CD63). The data revealed the expression CD9 and CD63 in the EVs, confirming the successful isolation from transfected hADSCs (Figure 1).

hADSCs Packaged miR-146a into Secreted EVs

As shown in Figure 2, qRT-PCR results indicated that the expression levels of miR-146a were approximately 2.5 folds higher in EVs derived from miR-146a–transfected hADSCs compared to those from hADSCs transfected with miR-con. These findings underscore the efficient packaging of miR-146a into secreted exosomes by MSCs (Figure 2).

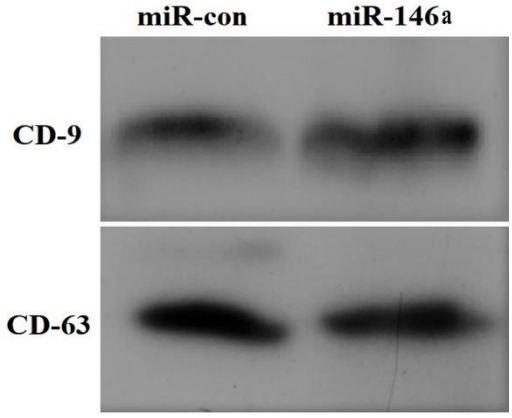

Figure 1. Characterization of human adipose tissue stem cell derived extracellular vesicles (hADSC-EVs). The Western blot was used to assess the CD9 and CD63 expressions in hADSC-EVs extracellular vesicles obtained from human umbilical cord stem cells transfected with either miR-146a or MicroRNA control (miR-con).

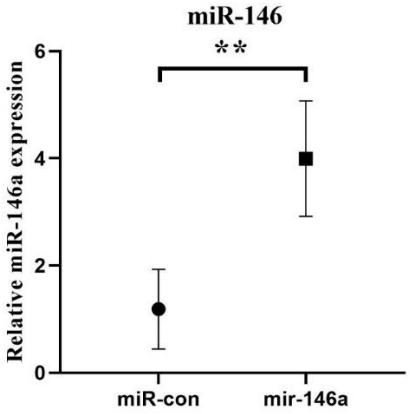

Figure 2. Human adipose tissue stem cell (hADSCs) package miR-146a within the secreted extracellular vesicles (EVs). The miR-146a expression in human adipose tissue stem cell-derived extracellular vesicles (hADSC-EVs) transfected with either miR-146a or MicroRNA control (miR-con) was evaluated using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The data are presented as means±standard deviation (SD), and significance levels are denoted as ** when $p<0.01$.

miR-146a EVs Regulated the Expression of Pro- and Anti-inflammatory Cytokines

To explore the impact of miR-146a on cytokine expression, we extracted the articular cartilage from the experimental groups 3 weeks after induction. Subsequently, we examined the expression of mentioned cytokines using RT-PCR.

The treatment with miR-146a EVs resulted in a mark reduction in mRNA expression levels of IL-17A, TNF-α, and IFN-γ compared to miR-con EVs. Significant reduction in mRNA expression levels of IL-17A, TNF-α, and IFN-γ was also observed in miR-146a EVs and miR-con EVs-treated groups compared to the control group (Figure 3).

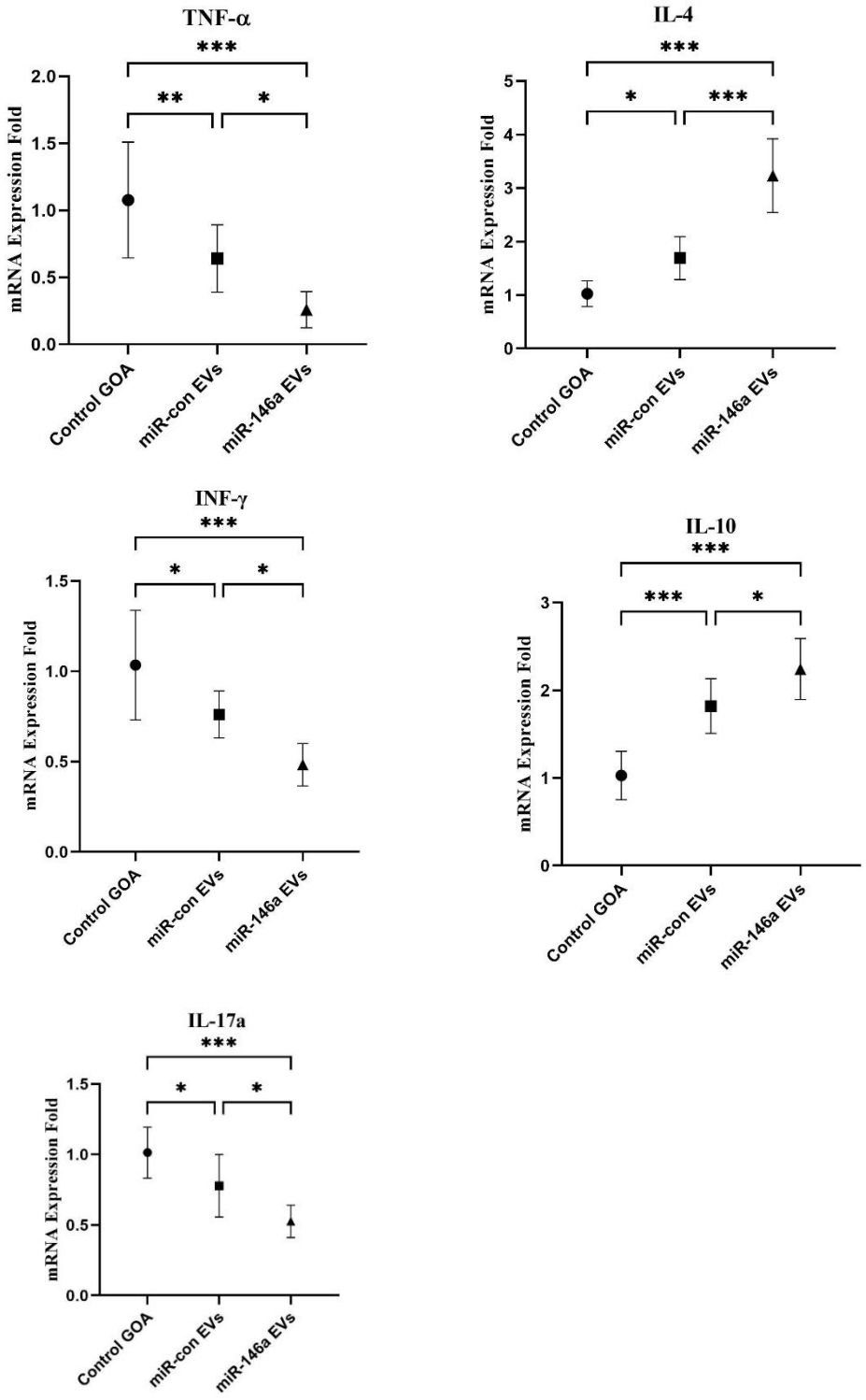

Figure 3. The differences in the expression of genes related to pro- and anti-inflammatory cytokines in the joint tissue of the experimental groups were examined. The results are shown as the mean ± standard deviation (SD). Statistical significance is denoted as *** $p<0.001$, ** $p<0.01$, and * $p<0.05$. EVs, extracellular vesicles; TNF: tumor necrosis factor; GOA: glenohumeral osteoarthritis.

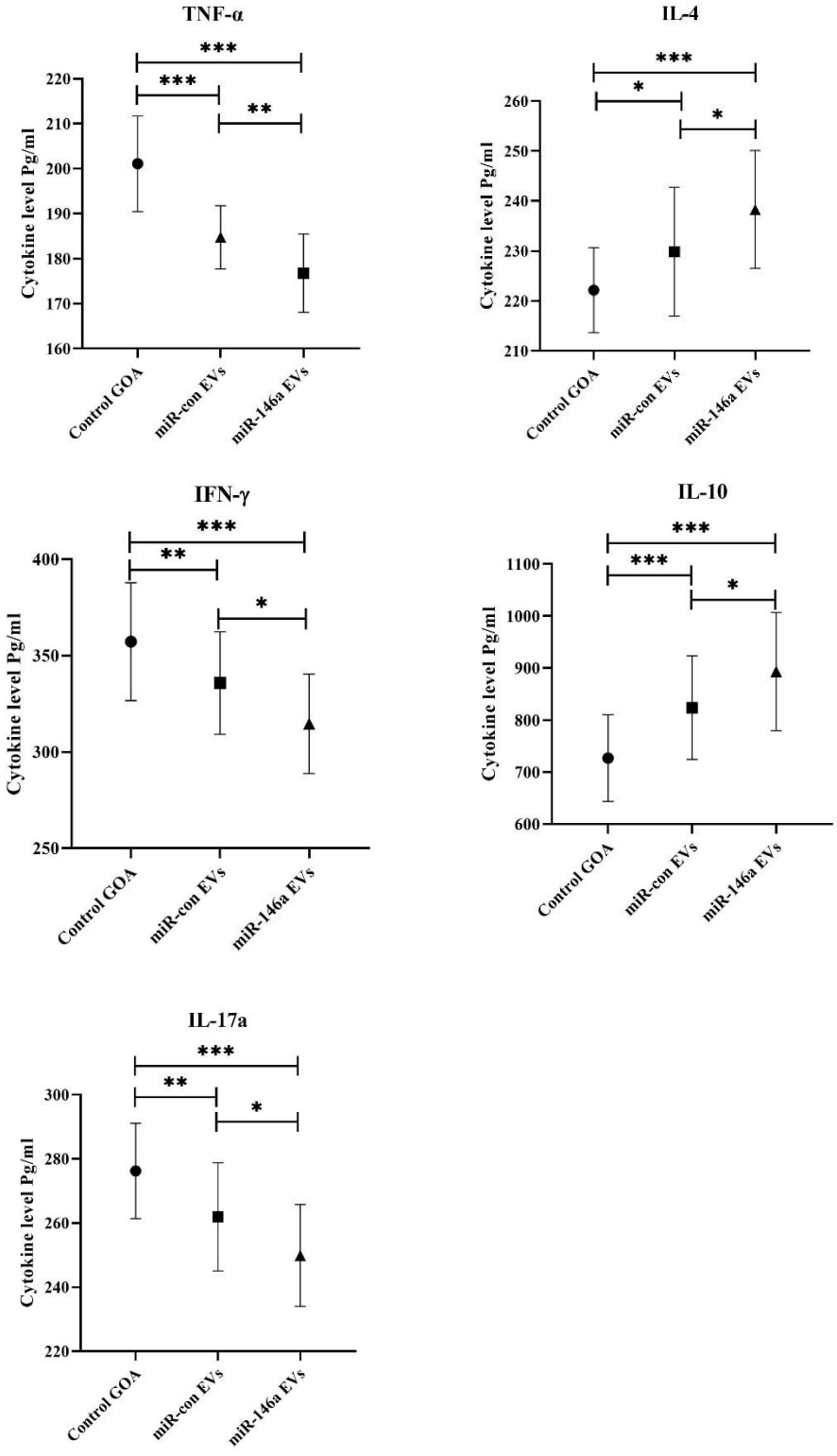

Figure 4. The investigation included evaluating the secretion of pro- and anti-inflammatory cytokines by splenocytes in the experimental groups. The results are expressed as the mean±standard deviation (SD). Statistical significance is indicated as ***p<0.001, ** p<0.01, and * p<0.05. EVs: extracellular vesicles; TNF: tumor necrosis factor, Glenohumeral osteoarthritis (GOA)

DISCUSSION

The findings from the present investigation offer encouraging indications that EVs derived from hADSCs modified to overexpress miR-146a have enhanced therapeutic potential for targeting inflammation in GOA. A significant finding was that treatment with hADSC-miR-146a-EVs significantly downregulated the expression of IFN-γ, TNF-α, and IL-17A both locally in the articular cartilage and systemically based on splenocyte cultures. This coincided with an increase in the anti-inflammatory cytokines IL-4 and IL-10. These collective changes of cytokine milieu induced by hADSC-miR-146a-EVs treatment likely contribute to their anti-inflammatory and chondroprotective effects in experimental GOA.

The molecular mechanisms underlying these observations can be attributed to miR-146a's acknowledged function as a suppressor of the NF-κB signaling cascade.[31] NF-κB has a central role in regulating inflammatory intermediaries implicated in OA pathology.[32] By suppressing IRAK1 and TRAF6, which are crucial upstream components of the NF-κB cascade, miR-146a is believed to attenuate NF-κB stimulation and consequent generation of pro-inflammatory cytokines.[33] Our findings are well aligned with previous studies demonstrating miR-146a's potential to inhibit inflammation in various disease models through modulation of this pathway.[34]

A notable novelty of this study is the investigation of MSC-EVs engineered with disease-modifying miR-146a for OA therapy. While the chondroprotective actions of MSCs themselves are increasingly recognized, practical limitations exist for their clinical use including low retention and survival after administration.[19,35] EVs provide an attractive cell-free alternative that could circumvent such hurdles by transferring bioactive mediators from parent MSCs to target cells.[36] The results herein indicate that hADSC-miR-146a-EVs maintain the immunomodulatory properties of their parent MSCs, as evidenced by changes in cytokine profiles and presumably downstream pathways. This supports the concept that MSC-EVs can serve as an efficient cell-free delivery system.

Regarding the translation of these findings, further investigations are still warranted. While cytokine analyses in this study demonstrated functional impacts systemically and locally, additional characterization of intracellular signaling and matrix changes in cartilage are needed to fully elucidate anti-OA mechanisms. Studies incorporating multiple time points and dose responses would also help optimize delivery protocols. Safety deserves consideration as well, though MSC-EVs appear well-tolerated based on existing literature. Lastly, comparative analyses with biochemical and molecular features of human OA are important to strengthen clinical relevance.

In summary, the present findings offer proof of concept that MSC-derived EVs modified with inflammation-targeting miR-146a hold promise as a noncellular approach for GOA treatment. With additional optimization and validation, this therapeutic strategy could represent an innovative option for modulating dysfunction in OA while avoiding the challenges of directly transplanting cells. Ultimately, advancing our understanding of miR-146a-mediated EV effects may have broader implications for controlling pathological inflammation in various musculoskeletal conditions.

In conclusion, the current study demonstrates that EVs derived from hADSCs overexpressing miR-146a have enhanced anti-inflammatory potential in GOA by modulating miRNA targets and cytokine production both locally and systemically. These MSC-EVs engineered with inflammation-related miRNAs have the potential to be a highly effective treatment strategy that does not require the presence of cells for regulating joint inflammation and decelerating disease progression in GOA. However, additional research is necessary to comprehensively understand how they work and confirm their clinical significance and safety before they can be used as a treatment option.

STATEMENT OF ETHICS

All procedures involving the animals were executed under approved protocols from the Third Hospital of Shijiazhuang, Shijiazhuang, Hebei Province, China.

FUNDING

No funds have been received for this research project.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Not applicable.

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