Arch Biol Sci. 2024;76(4):399-407 https://doi.org/10.2298/ABS240630030G

Mesenchymal stem cell-derived extracellular vesicles enriched with miR-124 exhibit anti-inflammatory effects in collagen-induced arthritis

Zhonghua Guo[1], Mengyuan He[2], Dongliang Shi[1], Zhongbo Zhang[1], Lisha Wang[1], Bowen Ren[1], Yunfei Wang[1], Junjie Wang[1], Shaoxiang Yang[1] and Hongchao Yu[3,*]

[1]*Department of Orthopedics, Henan Provincial Hospital of Traditional Chinese Medicine*, Zhengzhou City, Henan Province, 450002, China

[2]*Graduate student Henan University of Traditional Chinese Medicine*, Zhengzhou City, Henan Province, 450000, China

[3]*Department of Orthopedics, Affiliated Hospital of Shaanxi University of Traditional Chinese Medicine*, Xianyang City, Shaanxi Province, 712000, China

***Corresponding author**: Hy13468505752@outlook.com

Received: June 30, 2024, 2024; **Revised**: July 3, 2024; **Accepted**: July 8, 2024; **Published online**: October 9, 2024

Abstract: Rheumatoid arthritis (RA) is a chronic autoimmune disorder characterized by joint inflammation, leading to pain, swelling, stiffness, and joint damage. Mesenchymal stem cell-derived extracellular vesicles (MSC-EVs) hold significant promise as therapeutic agents owing to their potent paracrine activities. This study investigated the anti-inflammatory effects of human umbilical cord-derived MSC (hUCSC)-EVs overexpressing miR-124 in collagen-induced arthritis (CIA). hUCSCs were transfected with miR-124, and hUCSC-EVs were isolated and characterized. Arthritis was induced in mice by collagen injection, followed by intravenous administration of miR-124 EVs, miR-control, or vehicle. Cytokine levels, including interleukin (IL)-6, IL-1β, tumor necrosis factor (TNF)-α, IL-10, and transforming growth factor (TGF)-β, were measured in the joints using real-time PCR by enzyme-linked immunosorbent assay (ELISA). miR-124-hUCSC-EVs showed enrichment of miR-124. In arthritic mice, intravenous treatment with miR-124 EVs significantly reduced the clinical score, increased anti-inflammatory cytokines IL-10 and TGF-β, and lowered inflammatory cytokines IL-6, TNF-α, and IL-1β in the joints. This study shows that miR-124 EVs have anti-inflammatory activity in arthritis by suppressing pro-inflammatory cytokines through miR-124 overexpression. MSC-EVs overexpressed with inflammation-modulating miRNAs offer a promising cell-free approach for treating inflammatory arthritis.

Keywords: mesenchymal stem cells, extracellular vesicles, exosome, arthritis, cytokine

INTRODUCTION

Rheumatoid arthritis (RA) is characterized as a systemic autoimmune condition linked to an enduring inflammatory response that has the potential to harm not only joints but also non-joint organs such as the heart, kidneys, lungs, digestive system, eyes, skin, and nervous system [1]. RA is a persistent inflammatory condition observed globally and affecting about 5 out of 1000 adults. The prevalence of the disease is higher in women, occurring 2 to 3 times more frequently than in men, and it can manifest at any age [2]. The treatment regimen employs the pyramid approach, commencing with bed rest and nonsteroidal anti-inflammatory drugs. As the disease advances, effective disease-modifying anti-rheumatic drug (DMARD) therapy is added to the treatment plan [3]. Currently, there is no cure for RA, and treatments are aimed at relieving symptoms and limiting joint damage. Stem cell-based therapies have emerged as a promising approach for treating RA due to their immunomodulatory and regenerative properties [4].

Mesenchymal stem cells (MSCs) are progenitor cells characterized by self-renewal and multipotency, possessing the capability to differentiate into various cell types of mesodermal origin, such as chondrocytes, osteocytes, and adipocytes [5,6]. MSC-based therapy is a promising alternative to existing approaches in regenerative medicine [7]. The proposed mechanism

suggests that MSCs in tissue repair involve their paracrine activity, achieved through the exocytosis of a secretome that includes extracellular vesicles (EVs) and released factors. This secretome comprises growth factors and cytokines [8,9]. MSCs, in particular, have shown efficacy in attenuating RA through their secretome, which includes EVs [10]. However, the therapeutic potential of MSCs can be further enhanced by genetic modification to overexpress anti-inflammatory microRNAs (miRNAs) [11].

miRNAs are small non-coding RNA molecules that regulate gene expression posttranscriptionally [12,13]. Dysregulated miRNA expression is implicated in various diseases, including RA [14]. Previous studies indicate that miR-124 functions as an anti-inflammatory miRNA by suppressing pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) [15]. It achieves this by directly binding to the 3' untranslated regions of the signal transducer and activator of transcription 3 (STAT3), a crucial activator of IL-6, thus inhibiting the IL-6/STAT3 signaling pathway [16]. Moreover, miR-124 might indirectly inhibit other cytokines by targeting downstream signaling elements like nuclear factor-kappa B (NF-κB) [17]. We investigated the anti-inflammatory effects of EVs secreted from miR-124-overexpressed human umbilical cord MSCs (hUCSCs) (miR-124-EVs) in RA.

MATERIALS AND METHODS

Ethics statement

The study was approved by the ethical committee of Henan Provincial Hospital of Traditional Chinese Medicine (Second Affiliated Hospital of Henan University of Traditional Chinese Medicine) with certificate number 1439-01. The research was conducted following the principles of the Declaration of Helsinki and in compliance with local regulatory requirements. Written informed consent was obtained from all participants. All animal experiments were performed per the ARRIVE guidelines and regulations.

hUCSCs isolation and characterization

Three umbilical cords were obtained from healthy pregnant women undergoing cesarean section. MSCs were isolated using the previously established protocol [18]. The collected samples were placed in phosphate-buffered saline (PBS) and transported to the laboratory, where they underwent PBS washing and were cut into 5-cm^2 segments. These segments were longitudinally sliced, removing blood vessels in the process. The prepared segments were then transferred to 75-cm^2 flasks and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). The flasks were incubated at 37°C in a humidified atmosphere with 5% CO_2 and left undisturbed for 7 days. Following the initial week-long incubation, the medium was changed for the first time and subsequently every 3-4 days. After 2 weeks, the supernatant was removed, and the adherent cells were allowed to expand. The growth medium underwent renewal every three days. To facilitate passaging, cells were detached using trypsin/EDTA once cultures reached 80% confluence, and subsequently, they were replated in new flasks.

Before use in experiments, the multi-differentiation potential of hUCSCs was evaluated by inducing their differentiation into adipocytes and osteoblasts. To induce adipogenesis, hUCSCs were cultured with 10 ng/mL insulin and 1×10^{-8} M dexamethasone. For osteogenic induction, hUCSCs were cultured with 50 μg/mL L-ascorbic acid-2 phosphate, 10 mM glycerol 2-phosphate disodium salt, and 1×10^{-8} M dexamethasone. After three weeks of culturing, the hUCSCs were washed, fixed, and stained with Alizarin Red for 5 min or with Oil Red O for 30 min to observe differentiation. hUCSCs were examined using flow cytometry to analyze distinct surface markers, showing positive results for CD73 and CD90, and negative results for CD34 and CD45.

Transfection of hUCSCs with miR-124

hUCSCs underwent transfection with either a mimic negative control (mimic NC) or a miR-124 mimic (GenePharma, Shanghai, China) using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's protocol. Namely, hUCSCs at about 80% confluency were cultured in Opti-MEM medium and incubated with Lipofectamine 3000 reagent for 72 h, together with either the miR-124 mimic or mimic NC.

hUCSCs-derived miR-124 EV isolation and characterization

EVs were obtained from the transfected hUCSCs by ultracentrifugation following a previously outlined method [19]. In brief, transfected hUCSCs were cultured for 48 h in media containing EV-free FBS. EV-free FBS was prepared through a series of centrifugation steps at 4°C, including spins at 400 ×g for 20 min, 20,000 ×g for 30 min, and 110,000 ×g for 7 h, followed by filtration using a 0.22-μm filter [20]. The culture medium was then collected and subjected to sequential centrifugation steps: first at 300 ×g for 10 min, then at 2000 ×g for 10 min, followed by 10,000 ×g for 30 min, and finally at 100,000 ×g for 70 min.

Anti-CD63 (1:1000) and anti-CD9 (1:2000) antibodies were used to assess the isolated exosome by Western blot analysis. Total proteins were extracted using the radio-immunoprecipitation assay (RIPA) lysis and extraction buffer (KeyGen Biotechnology, Nanjing, China). Protein concentrations were determined using the bicinchoninic acid (BCA) method. Equal protein amounts were separated by sodium dodecyl sulfate (SDS)- polyacrylamide-gel electrophoresis (PAGE), transferred to polyvinylidene difluoride (PVDF) membranes (EMD Millipore Corp., Burlington, MA), and then incubated overnight at 4°C with primary antibodies against CD9 and CD63 (Abcam, USA). Subsequently, the membranes were blocked with bovine serum albumin (BSA) at a concentration of 5% (v/v) and incubated with a horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (Abcam, USA) for 120 min at room temperature. Band intensities were analyzed using Image-Pro Plus 6.0 software and standardized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a loading control.

Collagen-induced arthritis animal model and treatment protocol

Thirty male, 7 to 10-week-old DBA/1 mice were housed in a carefully controlled environment with a temperature set at 24°C. The mice were subjected to controlled light/dark cycles and provided with sterile food and water, creating a pathogen-free setting. For CIA induction, Hooke Kit™ Chicken Collagen/CFA Emulsion (EK-0210) was used following the manufacturer's protocol. In summary, mice were subjected to intradermal immunization at the tail base with chicken type II collagen (CII) emulsified in complete Freund's adjuvant (CFA). After 21 days, a secondary challenge involved injecting CII in incomplete Freund's adjuvant. Mice were checked for signs of CIA every other day, starting on day 14 after immunization. The CIA in mice was scored using a scale of 0 to 16 (0 to 4 for each paw, adding the scores for all 4 paws), using the following criteria: 0 – normal paw; 1 – one toe inflamed and swollen; 2 – mild swelling of entire paw; 3 – entire paw inflamed and swollen; 4 – very inflamed and swollen paw or ankylosed paw. For macroscopic assessment of arthritis, the thickness of each affected hind paw was measured daily with microcalipers (Kroeplin GmbH, Schlüchtern, Germany). After establishing CIA, mice were randomly divided into three groups, each comprising 10 mice. These groups included a control group that received PBS, a miR-control EV-treated group, and a miR-124-EV-treated group, with each receiving a dose of 100 μg EVs. The treatment was administered intravenously on day 14. The mice were killed on day 42.

Splenocyte preparation

CIA mice were euthanized on the 42nd day after disease induction. The splenocytes were extracted using a 70-μm cell strainer. After red blood cell (RBC) lysis, the splenocytes were cultured in RPMI-1640 media supplemented with 10% FBS and 1% penicillin/streptomycin.

Cytokine assay

Splenocytes were acquired and cultured at a density of 2×10^6 cells per well in a 24-well culture plate. After 48 h of stimulation with 10 μg/mL chicken collagen, supernatants containing secreted proteins were retrieved from the wells. Cytokine concentrations, including TNF-α, transforming growth factor-beta (TGF-β), IL-6, IL-1β, and IL-10, in the supernatants were measured using enzyme-linked immunosorbent assay (ELISA) kits obtained from eBioscience in San Diego, California. All assays were performed following the manufacturer's protocol, and for heightened accuracy, each sample underwent testing in triplicate measurements.

RNA extraction and real-time PCR

hUCSC-EVs underwent RNA extraction using TRIzol as per the manufacturer's instructions. miR-124 expression was quantified by reverse-transcribing 1 μg of RNA to cDNA with a MiRcute miRNA First-strand cDNA synthesis kit. The ABI 7500 Real-Time PCR system and MiRcute miRNA qPCR detection kit were utilized for relative quantification via the $2^{-\Delta\Delta Ct}$ method. To assess the mRNA levels of cytokines, total RNA was extracted from the joint tissue (articular cartilage). This was achieved using TRIzol reagent following the manufacturer's instructions. Subsequently, reverse transcription from total RNA to cDNA and quantitative real-time PCR were conducted using the Takara PrimeScript RT Master Mix and SYBR Green Premix, respectively. The results were analyzed by the $2^{-\Delta\Delta Ct}$ method, presented as fold changes, and normalized to GAPDH.

Statistical analysis

A comparison between groups displaying normal distribution was performed through one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. The Kruskal-Wallis test was utilized for groups with non-normal distribution. The data were presented as mean values with standard deviations, and statistical significance was assessed at P values <0.05. Graphical representations were created using GraphPad Prism 8 software. The statistical analyses were carried out using IBM SPSS Statistics.

RESULTS

Characterization of hUCSCs

Flow cytometric analysis identified specific cell surface antigens expressed by hUCSCs, showing positivity for CD73 and CD90 and absence of expression for CD34 and CD45. Furthermore, morphological examination of hUCSCs displayed a fibroblastic appearance with an adherence characteristic. After adipogenic induction, Oil Red O staining demonstrated the formation of lipid droplets in the cells after 21 days. Similarly, osteogenesis was evaluated using Alizarin Red staining, indicating the presence of calcium deposits following a 21-day incubation (Fig. 1).

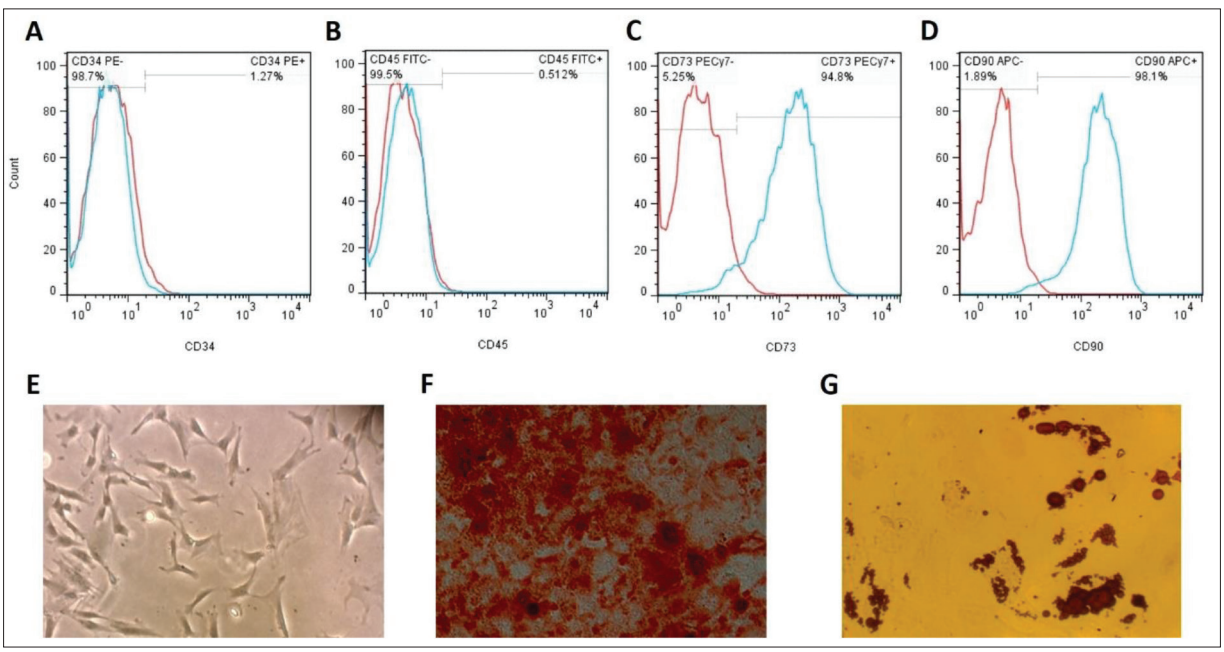

Fig. 1. Characterization of hUCSCs. Flow cytometric analysis of hUCSCs revealed the absence of CD34 expression (**A**) and CD45 (**B**) and the expression of CD73 (**C**) and CD90 (**D**). Morphologically, hUCSCs displayed a fibroblastic appearance with an adherence characteristic (**E**). Osteogenesis was confirmed by Alizarin Red staining, indicating the presence of calcium deposits (**F**). Adipogenic induction led to the formation of lipid droplets, as shown by Oil Red O staining (**G**). hUCSC – human umbilical cord stem cell.

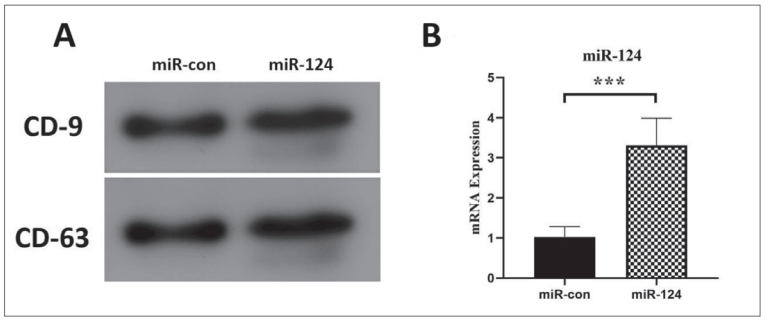

Fig. 2. hUCSCs package miR-124 into secreted EVs. **A** – Expressions of CD9 and CD63 in EVs derived from miR-124- or miR-con-transfected hUCSCs were evaluated by Western blotting. **B** – Expression of miR-124 in EVs derived from miR-124- or miR-con-transfected hUCSC was assessed by qRT-PCR. Values are presented as means±SD. *** P<0.001. EVs – extracellular vesicles; hUCSC – human umbilical cord mesenchymal stem cell; miR – miRNA

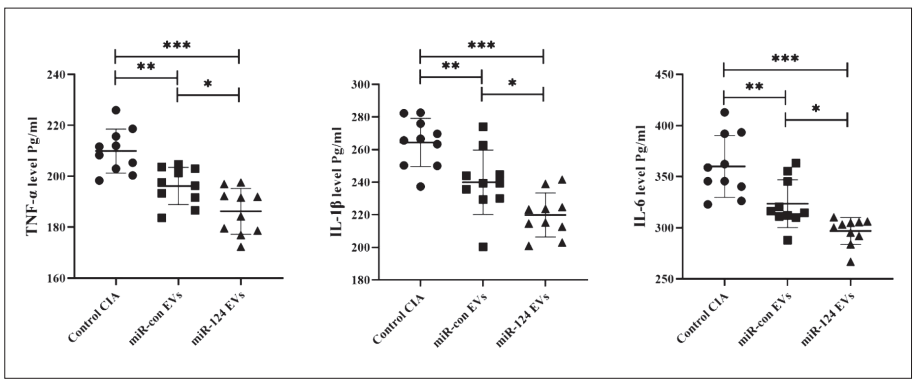

Fig. 3. Comparison of the clinical score between miR-124-EV-, miR-con-EV-treated, and CIA control mice (10 mice in each group). The data is presented as the mean±standard deviation (SD). Statistical significance is denoted as *P<0.05.

Characterization of hUCSC-EVs

The hUCSCs were transfected with a miR-124 mimic and a negative control. EVs were obtained from the supernatants of these hUCSCs 72 h after transfection. For the characterization of hUCSC-EVs, Western blot analysis detected exosomal markers, including CD9 and CD63, confirming their EV nature. The results indicated the presence of CD9 and CD63 in the EVs, validating their successful isolation from transfected hUCSCs (Fig. 2A).

hUCSCs packaged miR-124 into secreted EVs

As illustrated in Fig. 2B, the qRT-PCR outcomes revealed a 3-fold increase in the expression levels of miR-124 in EVs originating from hUCSCs transfected with miR-124, in contrast to those derived from hUCSCs transfected with miR-con. These results underscore the effective encapsulation of miR-124 into EVs released by hUCSCs.

EVs containing miR-124 decreased the clinical score in CIA mice

The first clinical sign of CIA was observed 14 days after immunization. Our results showed that EVs containing miR-124 significantly lowered the clinical score compared to control CIA mice (P=0.049). No significant differences between the EVs containing miR-124 and miR-con groups and between miR-con and control CIA mice were observed (Fig. 3).

EVs containing miR-124 influenced the release of both pro-inflammatory and anti-inflammatory cytokines by splenocytes

Splenocytes were extracted from CIA mice to evaluate the influence of EVs containing miR-124 on immune cell functionality. Following a 48 h incubation period with chicken collagen, the concentrations of pro-inflammatory cytokines IL-6, IL-1β, and TNF-α, as well as anti-inflammatory cytokines IL-10 and TGF-β in the culture supernatant, were measured using ELISA. EVs containing miR-124 significantly decreased the release of the pro-inflammatory cytokines IL-6, IL-1β, and TNF-α by splenocytes compared to EVs not containing miR-124 (miR-con) (P=0.042, P=0.026, and P=0.033, respectively) (Fig. 4). Additionally, miR-124 EVs significantly increased the release of the anti-inflammatory cytokines IL-10 and TGF-β compared to miR-con EVs (P=0.011 and P=0.03, respectively). Comparable results were noted when assessing the groups treated with miR-124 EVs and miR-con, in comparison to the control group (Fig. 5). These results demonstrate that miR-124 containing EVs can influence splenocytes to shift their cytokine profile from pro-inflammatory to anti-inflammatory.

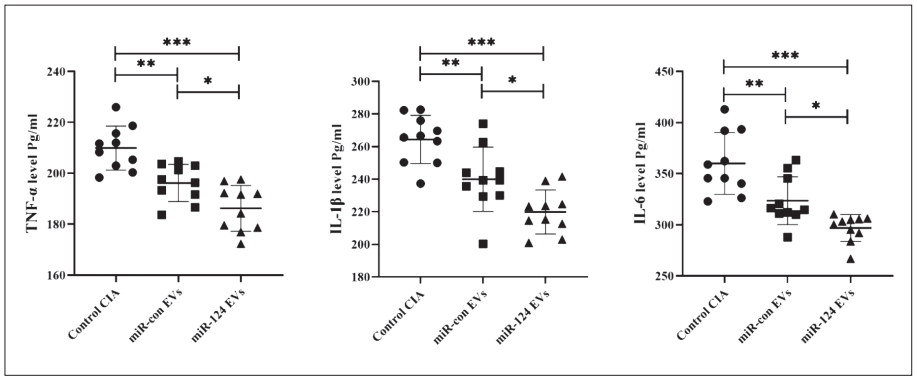

Fig. 4. Release of proinflammatory cytokines by splenocytes (10 mice in each group). The data is presented as the mean±standard deviation (SD). Statistical significance is denoted as ***P<0.001, **P<0.01, and *P<0.05. hUCSC-EV – human umbilical cord mesenchymal stem cell-extracellular vesicle, IL – interleukin, SD – standard deviation, TNF – tumor necrosis factor.

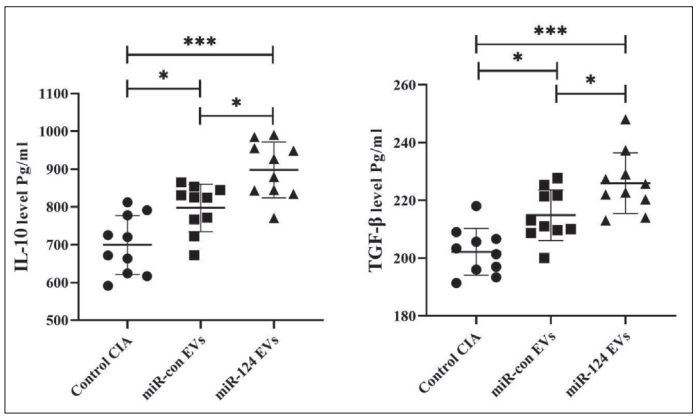

Fig. 5. Release of anti-inflammatory cytokines by splenocytes (10 mice in each group). The data is presented as the mean±standard deviation (SD). Statistical significance is denoted as ***P<0.001, **P<0.01, and *P<0.05. hUCSC-EV – human umbilical cord mesenchymal stem cell-extracellular vesicle, IL – interleukin, SD – standard deviation, TGF – transforming growth factor-beta.

EVs containing miR-124 played a regulatory role in modulating the expression of pro-inflammatory and anti-inflammatory cytokines in joint tissue

EVs containing miR-124 significantly downregulated the expression of the pro-inflammatory cytokines IL-6, IL-1β, and TNF-α in joint tissue compared to miR-con EVs (P=0.0018, P=0.015, and P=0.0019, respectively). IL-6 expression was reduced by 32% at the mRNA levels. IL-1β expression was decreased by 21% at the mRNA levels. TNF-α mRNA levels were also downregulated by 25%. A notable decrease in the mRNA expression levels of IL-6, IL-1β and TNF-α was evident in both miR-124 EV- and miR-con EV-treated groups when compared to the control group; IL-6 mRNA decreased by 52%, IL-1β mRNA by 44%, and TNF-α mRNA by 55%, and IL-6 mRNA decreased by 29%, IL-1β mRNA by 27%, and TNF-α mRNA by 40% (Fig. 6). Additionally, EVs containing miR-124 strongly upregulated expression of the anti-inflammatory cytokines IL-10 and TGF-β compared to miR-con EVs (P=0.0002 and P=0.003, respectively). IL-10 mRNA levels increased by 32%, and TGF-β mRNA expression was elevated by 19%. A noteworthy elevation in the mRNA levels of IL-10 and TGF-β was evident in both miR-124 EV- and miR-con EV-treated groups compared to the control group; IL-10 mRNA increased by 75% and TGF-β mRNA by 95%, and IL-10 mRNA increased by 33% and TGF-β mRNA by 65% (Fig. 7). These changes in cytokine profiles suggest miR-124 within EVs plays an important role in shifting the profile of cytokines towards an anti-inflammatory phenotype by modulating both pro- and anti-inflammatory cytokine expression at the transcriptional levels.

DISCUSSION

EVs enriched with miR-124 have been shown to have anti-inflammatory effects in CIA [21]. These EVs play a crucial role in the pathogenesis of RA and osteoarthritis (OA), influencing processes such as extracellular matrix degradation, cell-to-cell communication, and inflammation modulation [22, 21]. In particular, miR-124a, a key regulator of proliferation and monocyte chemoattractant protein-1 (MCP-1) secretion, is significantly decreased in RA synoviocytes, and its induction suppresses the production of cyclin-dependent kinase 2 (CDK-2) and MCP-1 proteins [23]. This suggests that EVs enriched with miR-124 could potentially be used as a therapeutic target for RA and OA. This study demonstrates the potential therapeutic efficacy of EVs derived from miR-124-modified hUCSCs in the treatment of RA. The results showed that EVs containing miR-124 significantly decreased the clinical score in comparison to control CIA mice. Also, miR-124 containing EVs

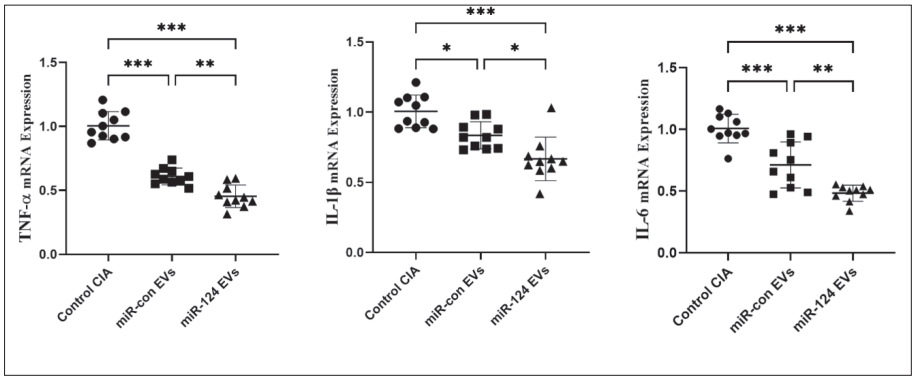

Fig. 6. Variations in expression of proinflammatory cytokine genes in the joint tissue of (10 mice in each group). The data is presented as the mean±standard deviation (SD). Statistical significance is indicated as ***P<0.001, **P<0.01, and *P<0.05. hUCSC – human umbilical cord mesenchymal stem cell, EV – extracellular vesicle, IL – interleukin, SD – standard deviation, TNF – tumor necrosis factor.

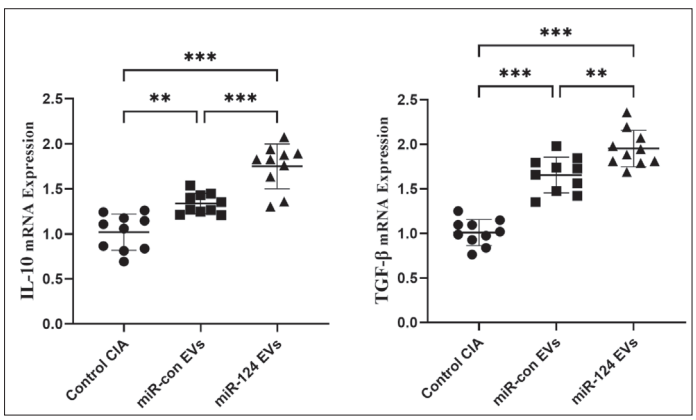

Fig. 7. Variations in expression of anti-inflammatory cytokine genes in the joint tissue (10 mice in each group) were analyzed. The data is presented as the mean±standard deviation (SD). Statistical significance is indicated as ***P<0.001, **P<0.01, and *P<0.05. hUCSC – human umbilical cord mesenchymal stem cell, EV – extracellular vesicle, IL – interleukin, SD – standard deviation, TGF – transforming growth factor-beta.

exerts potent anti-inflammatory effects and regulates cytokine expression *in vitro* using splenocytes and *in vivo* in a mouse model of CIA.

The characterization of hUCSCs confirms that they possess standard MSC properties. Isolation of EVs from the supernatant of miR-124-transfected hUCSCs revealed their encapsulation of efficient levels of miR-124. This suggests Lipofectamine 3000 can effectively load specific miRNAs into MSC-secreted EVs for targeted delivery. Previous studies have also reported successful miRNA packaging into EVs following MSC transfection [24,25].

The results demonstrate that EVs containing miR-124 decreased arthritis severity in a mouse model of CIA compared to control CIA mice. CIA was first observed 14 days after immunization. The clinical scoring system takes into account joint swelling and erythema and allows quantification of arthritis severity. Mice that received EVs containing miR-124 had significantly lower clinical scores than control CIA mice, showing reduced arthritis severity. Interestingly, mice that received EVs not containing miR-124 (miR-con group) still exhibited lower clinical scores than control CIA mice, though not to the same degree as the miR-124 EVs group. However, there was no statistically significant difference in clinical scores between the miR-124 EV group and the miR-con group. This suggests that EVs may exert some protective effects independently of their miR-124 cargo; loading EVs with miR-124 further enhances this protection and decreases arthritis severity in the CIA mouse model of RA.

The *in vitro* analysis of EV effects on splenocytes provides insights into mechanisms of action. miR-124 EVs significantly reduced secretion of pro-inflammatory cytokines IL-6, IL-1β, and TNF-α, while augmenting anti-inflammatory IL-10 and TGF-β levels. This modulation shifts the cytokine milieu from inflammatory to regulatory. miR-124 is known to inhibit IL-6 and TNF-α production through binding to 3'UTRs of the region of STAT3 and inhibits IL-6/STAT3 signaling [26,27]. Additionally, miR-124 may indirectly suppress other cytokines by targeting downstream signaling components like NF-κB [26]. The ability of EVs to markedly influence cytokine profiles in immune cells establishes their therapeutic functionality.

At the tissue level, miR-124 EVs also downregulated the expression of pro-inflammatory cytokines and upregulated anti-inflammatory cytokines in arthritic joints, mirroring splenocyte findings. Cytokine

regulation can be attributed to the direct targeting of mRNAs by miRNAs delivered via EVs into joint cells [28]. The concerted *in vitro* and *in vivo* cytokine modulation suggest that miR-124 EV-mediated suppression of inflammation occurs through posttranscriptional repression of multiple pro-inflammatory factors.

While MSC therapy holds promise for RA, challenges remain regarding safety, efficacy, and standardization [29]. This study indicates that transfected MSCs overexpressing anti-inflammatory miRNAs before EV harvesting could help optimize their therapeutic activity. Specifically, the immunomodulatory and regenerative functions of MSCs can be augmented through genetic modification to produce EVs enriched for potent anti-rheumatic miRNAs like miR-124. Compared to direct cell administration, EVs offer distinct advantages as they are non-immunogenic, less oncogenic, and easier to quality-control [30].

Some limitations exist, such as the inability to fully characterize the miRNA and protein cargo in EVs. Further investigations are warranted to identify all relevant miR-124 targets and elucidate comprehensive mechanisms of action. It is also unclear whether therapeutic benefits are sustained long-term post-treatment. Larger animal studies of the dose response, ideal administration protocols, and whether effects preclude disease relapse would strengthen translational research.

CONCLUSIONS

This research demonstrates that EVs secreted from miR-124-modified hUCSCs mitigate arthritis via modulation of cytokine networks. Significant decreases in pro-inflammatory cytokines and increases in anti-inflammatory cytokines were observed *in vitro* and *in vivo*. Together, the findings substantiate the anti-inflammatory properties of miR-124 EVs and highlight their potential as a cell-free therapeutic avenue for RA. While further optimizations are required, these results provide support for the continued development of miRNA-loaded MSC-EVs as tailored therapeutics capable of systemically reprogramming dysregulated immunity in inflammatory joint diseases.

Funding: 1. Funding for the 2021 Henan Province Traditional Chinese Medicine Top Talents Training Project [Yuwei Traditional Chinese Medicine Letter (2021) No. 15]. 2. Henan Province Traditional Chinese Medicine Science Research Special Project (2023ZY2097).

Author contributions: ZG and MH contributed equally to this work. ZhG; writing – original draft preparation, methodology, investigation, conceptualization, MH and DS; data curation, formal analysis, writing – review and editing, ZhZh, LW, BR; methodology, software, data curation, review and editing, YW, JW, SY; methodology, resources, formal analysis, validation, HY; supervision, writing – review and editing, project administration. All authors have read and agreed to the published version of the manuscript.

Conflict of interest disclosure: The authors declare no conflicts of interest.

Data availability: Data underlying the reported findings have been provided as a raw dataset available here: https://www.serbiosoc.org.rs/NewUploads/Uploads/Guo%20et%20al_Raw%20Dataset.pdf

REFERENCES

1. Conforti A, Di Cola I, Pavlych V, Ruscitti P, Berardicurti O, Ursini F, Giacomelli R, Cipriani P. Beyond the joints, the extra-articular manifestations in rheumatoid arthritis. Autoimmun Rev. 2021;20(2):102735. https://doi.org/10.1016/j.autrev.2020.102735
2. Myasoedova E, Crowson CS, Kremers HM, Therneau TM, Gabriel SE. Is the incidence of rheumatoid arthritis rising?: results from Olmsted County, Minnesota, 1955-2007. Arthritis Rheum. 2010;62(6):1576-82. https://doi.org/10.1002/art.27425
3. Burmester GR, Pope JE. Novel treatment strategies in rheumatoid arthritis. Lancet. 2017;389(10086):2338-48. https://doi.org/10.1016/S0140-6736(17)31491-5
4. Vohra M, Sharma A, Bagga R, Arora SK. Human umbilical cord-derived mesenchymal stem cells induce tissue repair and regeneration in collagen-induced arthritis in rats. J Clin Transl Res. 2020;6(6):203.
5. Qin L, Liu N, Bao C-l-m, Yang D-z, Ma G-x, Yi W-h, Xiao G-z, Cao H-l. Mesenchymal stem cells in fibrotic diseases—the two sides of the same coin. Acta Pharmacol Sin. 2023;44(2):268-87. https://doi.org/10.1038/s41401-022-00952-0
6. Azimzadeh M, Mahmoodi M, Kazemi M, Hakemi MG, Jafarinia M, Eslami A, Salehi H, Amirpour N. The immunoregulatory and neuroprotective effects of human adipose derived stem cells overexpressing IL-11 and IL-13 in the experimental autoimmune encephalomyelitis mice. Int Immunopharmacol. 2020;87:106808. https://doi.org/10.1016/j.intimp.2020.106808
7. Sharma RR, Pollock K, Hubel A, McKenna D. Mesenchymal stem or stromal cells: a review of clinical applications and manufacturing practices. Transfusion. 2014;54(5):1418-37. https://doi.org/10.1111/trf.12421
8. Skalnikova H, Motlik J, Gadher SJ, Kovarova H. Mapping of the secretome of primary isolates of mammalian cells, stem cells and derived cell lines. Proteomics. 2011;11(4):691-708. https://doi.org/10.1002/pmic.201000402
9. Dezfuly AR, Safaee A, Amirpour N, Kazemi M, Ramezani A, Jafarinia M, Dehghani A, Salehi H. Therapeutic effects of human adipose mesenchymal stem cells and their paracrine

agents on sodium iodate induced retinal degeneration in rats. Life Sci. 2022;300:120570. https://doi.org/10.1016/j.lfs.2022.120570
10. Liu H, Li R, Liu T, Yang L, Yin G, Xie Q. Immunomodulatory effects of mesenchymal stem cells and mesenchymal stem cell-derived extracellular vesicles in rheumatoid arthritis. Front Immunol. 2020;11:1912. https://doi.org/10.3389/fimmu.2020.01912
11. Wei W, Huang Y, Li D, Gou H-F, Wang W. Improved therapeutic potential of MSCs by genetic modification. Gene Ther. 2018;25(8):538-47. https://doi.org/10.1038/s41434-018-0041-8
12. Hammond SM. An overview of microRNAs. Adv Drug Deliv Rev. 2015;87:3-14. https://doi.org/10.1016/j.addr.2015.05.001
13. Jafarinia M, Farrokhi MR, Ganjalikhani Hakemi M, Cho WC. The role of miRNAs from mesenchymal stem/stromal cells-derived extracellular vesicles in neurological disorders. Human Cell. 2023;36(1):62-75. https://doi.org/10.1007/s13577-022-00813-2
14. Renman E, Brink M, Ärlestig L, Rantapää-Dahlqvist S, Lejon K. Dysregulated microRNA expression in rheumatoid arthritis families—a comparison between rheumatoid arthritis patients, their first-degree relatives, and healthy controls. Clin Rheumatol. 2021;40:2387-94. https://doi.org/10.1007/s10067-020-05502-9
15. Sun Y, Li Q, Gui H, Xu D-P, Yang Y-L, Su D-F, Liu X. MicroRNA-124 mediates the cholinergic anti-inflammatory action through inhibiting the production of pro-inflammatory cytokines. Cell Res. 2013;23(11):1270-83. https://doi.org/10.1038/cr.2013.116
16. Zhang J, Lu Y, Yue X, Li H, Luo X, Wang Y, Wang K, Wan J. MiR-124 suppresses growth of human colorectal cancer by inhibiting STAT3. PloS One. 2013;8(8):e70300. https://doi.org/10.1371/journal.pone.0070300
17. Angelopoulou E, Paudel YN, Piperi C. miR-124 and Parkinson's disease: A biomarker with therapeutic potential. Pharmacol Res. 2019;150:104515. https://doi.org/10.1016/j.phrs.2019.104515
18. Hassan G, Kasem I, Soukkarieh C, Aljamali M. A simple method to isolate and expand human umbilical cord derived mesenchymal stem cells: using explant method and umbilical cord blood serum. Int J Stem Cells. 2017;10(2):184-92. https://doi.org/10.15283/ijsc17028
19. Théry C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Curr Protoc Cell Biol. 2006;30(1):3.22. 1-3.. 9. https://doi.org/10.1002/0471143030.cb0322s30
20. Ahmadvand Koohsari S, Absalan A, Azadi D. Human umbilical cord mesenchymal stem cell-derived extracellular vesicles attenuate experimental autoimmune encephalomyelitis via regulating pro and anti-inflammatory cytokines. Sci Rep. 2021;11(1):11658. https://doi.org/10.1038/s41598-021-91291-3
21. Withrow J, Murphy C, Liu Y, Hunter M, Fulzele S, Hamrick MW. Extracellular vesicles in the pathogenesis of rheumatoid arthritis and osteoarthritis. Arthritis Res Ther. 2016;18:1-12. https://doi.org/10.1186/s13075-016-1178-8
22. Fu H, Hu D, Zhang L, Tang P. Role of extracellular vesicles in rheumatoid arthritis. Mol Immunol. 2018;93:125-32. https://doi.org/10.1016/j.molimm.2017.11.016
23. Nakamachi Y, Kawano S, Takenokuchi M, Nishimura K, Sakai Y, Chin T, Saura R, Kurosaka M, Kumagai S. MicroRNA-124a is a key regulator of proliferation and monocyte chemoattractant protein 1 secretion in fibroblast-like synoviocytes from patients with rheumatoid arthritis. Arthritis Rheum. 2009;60(5):1294-304. https://doi.org/10.1002/art.24475
24. Li C, Li X, Zhao B, Wang C. Exosomes derived from miR-544-modified mesenchymal stem cells promote recovery after spinal cord injury. Arch Physiol Biochem. 2020;126(4):369-75. https://doi.org/10.1080/13813455.2019.1691601
25. Li D, Zhang P, Yao X, Li H, Shen H, Li X, Wu J, Lu X. Exosomes derived from miR-133b-modified mesenchymal stem cells promote recovery after spinal cord injury. Front Neurol. 2018;12:845. https://doi.org/10.3389/fnins.2018.00845
26. Yang J, Xu C, Wu M, Wu Y, Jia X, Zhou C, Zhang X, Ge S, Li Z, Zhang L. MicroRNA-124 inhibits hepatic stellate cells inflammatory cytokines secretion by targeting IQGAP1 through NF-κB pathway. Int Immunopharmacol. 2021;95:107520. https://doi.org/10.1016/j.intimp.2021.107520
27. Wang J, Zhang X, Han J, Zhou P, Yu X, Shen Z, Mao R, Lu M, Huang Y, Zhang J. MicroRNA-124 expression in Kupffer cells modulates liver injury by targeting IL-6/STAT3 signaling. Antiviral Res. 2023;211:105510. https://doi.org/10.1016/j.antiviral.2022.105510
28. Asirvatham AJ, Magner WJ, Tomasi TB. miRNA regulation of cytokine genes. Cytokine. 2009;45(2):58-69. https://doi.org/10.1016/j.cyto.2008.11.010
29. Papadopoulou A, Yiangou M, Athanasiou E, Zogas N, Kaloyannidis P, Batsis I, Fassas A, Anagnostopoulos A, Yannaki E. Mesenchymal stem cells are conditionally therapeutic in preclinical models of rheumatoid arthritis. Annals Rheum Dis. 2012;71(10):1733-40. https://doi.org/10.1136/annrheumdis-2011-200985
30. Maumus M, Rozier P, Boulestreau J, Jorgensen C, Noël D. Mesenchymal stem cell-derived extracellular vesicles: opportunities and challenges for clinical translation. Front Bioeng Biotechnol. 2020;8:997. https://doi.org/10.3389/fbioe.2020.00997