

miR-10a Delivered via MSC-derived Extracellular Vesicles Modulates Inflammation in a CCl₄-induced Liver Fibrosis Model

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ABSTRACT

Liver fibrosis is a significant global health issue characterized by an abnormal accumulation of extracellular matrix proteins, that disrupts normal liver architecture and function. Mesenchymal stem cells (MSCs) show therapeutic potential by releasing extracellular vesicles (EVs) containing regulatory microRNAs like miR-10a.

This study evaluates miR-10a-enriched human umbilical cord MSC (hUCMSC)-EVs in a CCl₄-induced model of liver fibrosis, focusing on inflammatory marker modulation. Liver fibrosis was induced in experimental animals using CCl₄ administration. MSCs were isolated from the human umbilical cord and loaded with either a miR-10a mimic or a control sequence through Lipofectamine 3000. EVs were then isolated from the culture media of both miR-control and miR-10a-modified MSCs. The therapeutic potential of these miR-10a-loaded EVs was assessed by treating the CCl₄-induced fibrosis model with these vesicles. The efficacy of the treatment was evaluated by measuring two anti-inflammatory markers interleukin (IL)-10 and IL-4 and three pro-inflammatory markers (tumor necrosis factor- α (TNF- α), IL-6, and interferon- γ (IFN- γ)) using enzyme-linked immunosorbent assay (ELISA) and real-time polymerase chain reaction (PCR) techniques.

The administration of miR-10a-loaded MSC-EVs resulted in a significant modulation of inflammatory markers. Our results revealed an increase in the levels of anti-inflammatory cytokines (IL-10 and IL-4) and a concurrent decrease in pro-inflammatory cytokines (TNF- α , IL-6, and IFN- γ) in the treated group compared to the control group.

The study demonstrates the therapeutic potential of MSC-EVs encapsulating miR-10a in ameliorating CCl₄-induced liver fibrosis. By modulating the balance between pro-inflammatory and anti-inflammatory cytokines, miR-10a-loaded EVs show promise as a targeted treatment approach for liver fibrosis.

Keywords: Extracellular vesicle; Cytokine; Mesenchymal stem cells

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INTRODUCTION

Fibrosis is a pathological condition that arises from an abnormal tissue regeneration process triggered by chronic injury. This condition results in the persistent activation of fibroblasts, ultimately leading to irreversible damage to the affected tissues.¹ Liver fibrosis involves an abnormal increase in extracellular matrix proteins, especially collagen, and is frequently linked to long-term liver conditions. This condition can result in advanced liver fibrosis, which has the potential to develop into cirrhosis, and may progress to liver failure and increased pressure in the portal vein system, often necessitating a liver transplant.² At present, there are no Food and Drug Administration (FDA)-approved anti-fibrotic medications for the treatment of liver fibrosis. Current treatment approaches focus mainly on targeting the root causes of chronic liver damage and attempting to halt or delay disease advancement.³

Mesenchymal stem cells (MSCs) are one of the most commonly utilized cell types in the field of regenerative medicine. Many investigations have demonstrated the positive effects of therapies utilizing MSCs in addressing a variety of health conditions, such as diabetes, cardiac ischemia, neurological disorders, and diseases affecting bone and cartilage.⁴ MSCs primarily exert their effects through the secretion of paracrine factors, which include growth factors, cytokines, chemokines, and bioactive lipids that influence immune responses and facilitate tissue remodeling.^{5,6} They also release microvesicles known as extracellular vesicles (EVs), which contain proteins and functional microRNAs (miRs) that enable communication intercellular via membrane fusion or receptor-mediated uptake. Compared with whole-cell therapies, using EV-based treatments reduce the risks associated with ectopic tissue formation and tumor development.⁷

miRs are short, noncoding RNA molecules that do not encode proteins.⁸ Aberrant miR expression has been linked to the development and progression of various diseases, including liver fibrosis.⁹ In mouse liver tissue, the anti-fibrotic miR-10a is highly expressed. In hepatic fibrosis, the administration of miR-10a mimics significantly increases cell proliferation. Furthermore, by altering the transforming growth factor- β (TGF- β)/Smad signaling pathway, miR-10a reduces hepatic fibrosis.¹⁰ However, miR mimics are quickly degraded in biological environments and require repeated administered to maintain their effects.¹¹ By

encapsulating synthetic miR-10a mimics within the protective membrane of MSC-EVs, a stable delivery system is created that allows for extended expression of the miR in the target tissue microenvironment.¹²

The primary goal of this study is to determine the therapeutic effect of miR-10a encapsulated in the human umbilical cord MSC-EVs on the CCl₄-induced liver fibrosis in rats, with particular emphasis on modulation of the inflammatory microenvironment. Liver fibrosis constitutes an important public health issue due to the lack of widely accepted treatment options. This project aims to address this clinical challenge by utilizing the anti-inflammatory activities of miR-10a. Achieving the desired therapeutic effects by decreasing inflammatory cytokines while enhancing the anti-inflammatory mechanisms to slow the fibrosis progression, while improving the stability and bioavailability of miR-10a through EV encapsulation.

MATERIALS AND METHODS

Isolation of hUCMSCs

Sections of umbilical cord, approximately 20 cm in length, were collected from full-term deliveries of healthy mothers who had given informed consent. The samples were rinsed with phosphate-buffered saline (PBS) and cut into 5-cm segments, which were then minced into 1–3 mm³ pieces. These tissue fragments were digested for 1 hour at 37 °C with collagenase type II and 0.125% trypsin in PBS. To terminate the enzymatic reaction, 10% fetal bovine serum (FBS) was added. Cells were seeded into T75 flasks containing Dulbecco's Modified Eagle Medium (DMEM) and incubated for 48 hours at 37 °C in a humidified atmosphere with 5% CO₂. The mixture was then filtered and centrifuged for five minutes at 1500 rpm. The collected cells were then resuspended in low-glucose DMEM (L-DMEM) supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. The culture medium was replaced every 3 days to maintain optimal growth conditions. When cell cultures reached 80% confluence, they were passaged using trypsin/EDTA and transferred to new flasks.

Characterization of hUCMSCs

To confirm the identity of human umbilical cord mesenchymal stem cells (hUCMSCs), an extensive characterization was conducted on cells from passages 3 to 5. Surface marker expression was assessed using flow

cytometry, revealing positive expression of CD90 and CD105, while CD34 and CD45 were not detected. The stemness of these cells was further confirmed by assessing their multi-lineage differentiation capability. To induce adipogenesis, hUCMSCs were cultured in adipogenic culture media supplemented with 10 ng/mL of insulin and 1×10^{-8} M dexamethasone for 14 days, with Oil Red O staining performed to validate the formation of lipid droplets. Osteogenic differentiation of hUCMSCs was performed using osteogenic medium supplemented with 50 µg/mL L-ascorbic acid-2-phosphate, 10 mM β-glycerophosphate, and 1×10^{-8} M dexamethasone for 21 days, with mineralization assessed via Alizarin Red staining.

hUCMSCs Transfection with miR-10a

To boost the therapeutic potential of hUCMSCs, a modification was conducted using a non-viral transfection technique. Human umbilical cord mesenchymal stem cells at passages 3 to 5 were cultured in standard growth medium until they reached approximately 80% confluency. At this point, the medium was switched to Opti-MEM, a reduced-serum formulation optimized for transfection using cationic lipids. Transfection was carried out using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, , USA), introducing either a miR-10a mimic or a negative control sequence (GenePharma, Shanghai, China) into the cells. The transfection process was carried out for 72 hours under standard culture conditions, allowing the cells to take up the material.

EV Isolation from Transfected hUCMSCs

A comprehensive protocol was used to isolate EVs from transfected hUCMSCs.¹³ After transfection, hUCMSCs were cultured until they reached about 85%–90% confluence. The medium was then changed to one supplemented with EV-depleted FBS to guarantee that collected extracellular vesicles originated solely from the cells themselves. This EV-depleted FBS was prepared using a thorough process involving ultracentrifugation and filtration. To prepare EV-depleted FBS, the serum underwent sequential centrifugation at 4 °C: first at 500g for 20 minutes, followed by 18,000g for 30 minutes, and finally at 120,000g for 7 hours. The resulting supernatant was then filtered through a 0.22 µm membrane. After each step, the pellet at the bottom was discarded, and the supernatant was kept. This supernatant was next

subjected to ultracentrifugation at 100,000g twice, with each spin lasting 70 minutes. The final pellet was collected and resuspended in 1 mL of sterile PBS.

Characterization of EVs Isolated from Transfected hUCMSCs

To confirm the identity of EVs isolated from transfected hUCMSCs, the surface markers of the EVs were analyzed through flow cytometry, which confirmed the presence of a standard EV marker, CD63. Furthermore, total protein concentration was determined using a bicinchoninic acid (BCA) assay to normalize the EV yield and maintain consistent dosing in subsequent experiments.

CCl₄-induced Liver Fibrosis Model and Treatment Protocol

To assess the therapeutic potential of miR-10a-enriched EVs, an in vivo liver injury model was created using thirty adult male C57BL/6 mice aged 8–10 weeks. The mice were sourced from a certified laboratory animal supplier and maintained under standard conditions, including controlled temperature, humidity, and a regulated light-dark cycle. All experimental procedures adhered to Animal Research: Reporting of In Vivo Experiments (ARRIVE) animal care and guidelines. Liver damage was triggered through intraperitoneal injections of a carbon tetrachloride (CCl₄) and olive oil mixture (1:4 ratio), given at 0.6 mL/kg body weight twice weekly over a period of 4 weeks.

Once liver fibrosis was confirmed, the mice were randomly divided into three groups of ten each: a control group receiving PBS, a group treated with miR-control EVs, and a group administered miR-10a-EVs. Administrations were done intravenously 5 weeks after the first intraperitoneal injections of CCl₄ to ensure significant liver damage and development of fibrosis.

Cytokine Assay

To evaluate the immunomodulatory effects of treatments, an in-depth analysis of the inflammatory microenvironment was conducted. At the end of the study, blood samples were collected from both treated and control animals. The collected blood was permitted to clot at room temperature for 30 minutes, after which it was centrifuged at 3000 rpm for 10 minutes to isolate the serum. The serum samples obtained were then stored at –80 °C until they were analyzed.

A range of pro-inflammatory and anti-inflammatory mediators—including tumor necrosis factor- α (TNF- α), interferon- γ (IFN- γ), interleukin (IL)6, IL-4, and IL-10—were simultaneously quantified using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, USA). All assays were performed following the manufacturer's protocols, and to ensure greater accuracy, each sample was tested in triplicate.

RNA Extraction and Real-time PCR

Following the manufacturer's instructions, TRIzol reagent was used to separate RNA from hUCMSC-derived EVs. Using the MiRcute miRNA First-Strand cDNA Synthesis Kit, 1 μ g of the collected RNA was reverse-transcribed into complementary DNA (cDNA) in order to examine miR-10a expression. The MiRcute miRNA qPCR Detection Kit was used to quantify the amounts of miR-10a on an ABI 7500 Real-Time PCR instrument, and the $2^{-\Delta\Delta C_t}$ method was used to determine the relative expression.

Liver tissue samples were collected from mice in different experimental groups, and total RNA was extracted using the RNeasy Mini Kit (Bio FACT, South Korea) according to the manufacturer's protocol. Briefly, the tissues were homogenized in RLT buffer, followed by the addition of 70% ethanol to promote RNA binding to the silica membrane in the spin column. After multiple wash steps to remove contaminants, RNA was eluted in RNase-free water and quantified with a NanoDrop spectrophotometer. For cytokine expression analysis, 1 μ g of total RNA was reverse-transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Bio FACT, South Korea). Primers for cytokines including TNF- α , IFN- γ , IL-6, IL-4, and IL-10 were used in real-time polymerase chain reaction (PCR). 10 μ L of SYBR Green Master Mix (Bio FACT, South Korea), 1 μ L of cDNA, and 0.5 μ M of each primer were included in each 20 μ L PCR reaction. The thermal cycling procedure started with a 10-minute initial denaturation at 95°C, then 40 cycles of annealing and extension at 95°C for 15 seconds and 60°C for 1 minute. Using the $2^{-\Delta\Delta C_t}$ method, cytokine expression levels were measured after being standardized against the housekeeping gene *GAPDH*.

Statistical Analysis

Data normality was assessed using the Shapiro-Wilk test to determine whether the data followed a normal distribution. To compare groups with a normal

distribution, a one-way analysis of variance (ANOVA) was performed, followed by Tukey's post hoc test for multiple comparisons. For groups that did not follow a normal distribution, the Kruskal–Wallis test was used. Data were expressed as mean values with standard deviation, and a p value < 0.05 was considered statistically significant. Graphs were created using GraphPad Prism 8, and statistical analyses were conducted with IBM SPSS Statistics software.

RESULTS

Characterization and differentiation Potential of hUCMSCs

The analysis of hUCMSCs revealed a distinctive immunophenotype and potential for multilineage differentiation, confirming their classification as MSCs. Flow cytometric evaluation highlighted a distinctive pattern of cell surface antigens, showing positive expression for CD90 and CD105, and negative for CD34 and CD45, supporting their identification as MSCs. The hUCMSCs were also shown to differentiate into both adipogenic and osteogenic lineages, underscoring their multipotency. Adipogenic differentiation was evidenced by the detection of lipid droplets using Oil Red O staining after 21 days of culture. Similarly, osteogenic differentiation was confirmed through Alizarin Red staining, which revealed calcium deposits, indicating osteogenic activity after the same incubation period (Figure 1). Collectively, these findings confirm that the isolated hUCMSCs exhibit the characteristic immunophenotype and differentiation potential of MSCs.

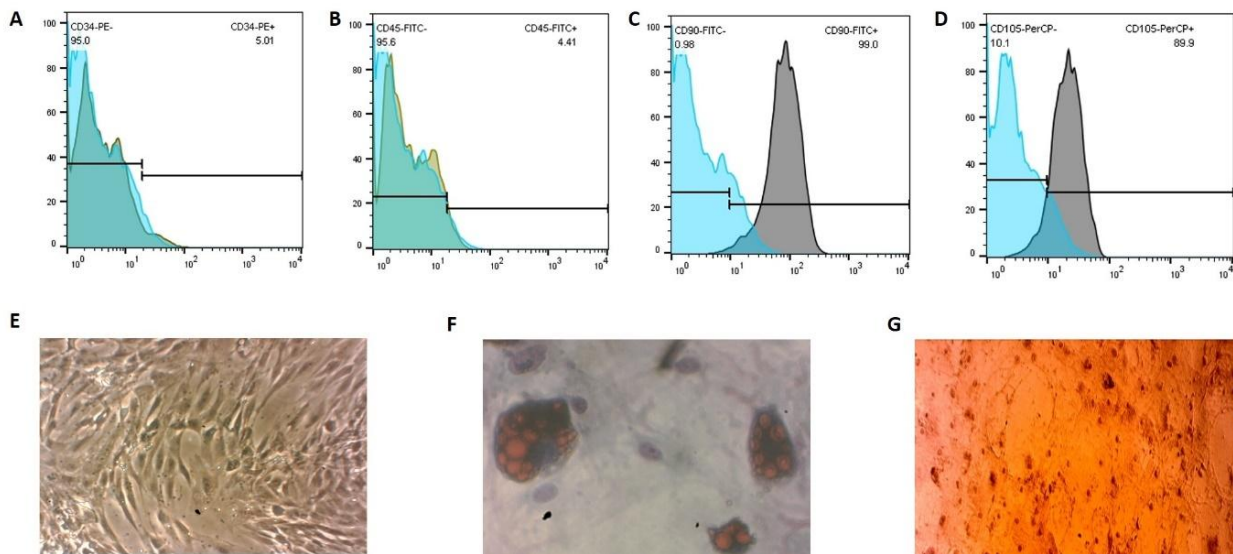


Figure 1. Immunophenotypic characterization and differentiation potential of hUCMSCs. Flow cytometry results show lack of expression of CD34 (A) and CD45 (B), with positive expression of CD90 (C) and CD105 (D), confirming their classification as MSCs. Additionally, the figure presents a fibroblastic morphology with adherence growth (E) and evidence of adipogenic differentiation, indicated by lipid droplets stained with Oil Red O after 21 days (F), and osteogenic differentiation, demonstrated by calcium deposits visualized through Alizarin Red staining under the same culture conditions (G). hUCMSC: human umbilical cord mesenchymal stem cell; MSC: mesenchymal stem cell.

Characterization of EVs Derived from miR-10a-Transfected hUCMSCs

The analysis of EVs derived from hUCMSCs transfected with miR-10a demonstrated distinctive exosomal characteristics. hUCMSCs were transfected with miR-10a mimics and cultured for 72 hours before EV isolation from the cell culture supernatants. Flow cytometry confirmed the presence of the exosomal marker CD63 in the EV sample, verifying both the successful isolation and exosomal identity of the particles (Figure 2A).

miR-10a Enrichment in hUCMSC-derived EVs

Quantitative real-time polymerase chain reaction (RT-PCR) analysis demonstrated the successful incorporation of miR-10a into EVs derived from transfected hUCMSCs. EVs isolated from hUCMSCs transfected with the miR-10a mimic showed significantly higher levels of miR-10a compared to those from control-transfected cells, confirming the efficient encapsulation of the miR ($p < 0.0001$) (Figure 2B).

Anti-inflammatory effects of miR-10a-enriched EVs on serum cytokine profiles

To assess the impact of miR-10a-enriched EVs on serum cytokine levels, mice were separated into three groups: one receiving PBS as a control, another treated with miR-control EVs, and a third administered miR-10a-EVs. Serum samples were collected and analyzed for key inflammatory markers using ELISA. Treatment with miR-10a-EVs led to a significant decrease in pro-inflammatory cytokines, TNF- α , IFN- γ , and IL-6 compared to both the PBS control ($p < 0.0001$, $p = 0.0002$, $p < 0.0001$, respectively) and miR-control EV group ($p = 0.0128$, $p = 0.0341$, $p = 0.0344$, respectively). These results indicate a strong anti-inflammatory effect of miR-10a-enriched EVs. Interestingly, the anti-inflammatory cytokine profile was enhanced in the miR-10a-EV treated group. IL-10 levels were significantly higher compared to both control and miR-control groups ($p < 0.0001$, $p = 0.0123$), suggesting an improved anti-inflammatory response. IL-4 showed an increase compared to PBS control ($p < 0.0001$). However, no significant result was obtained when compared with the miR-control group ($p = 0.9263$) (Figure 3).

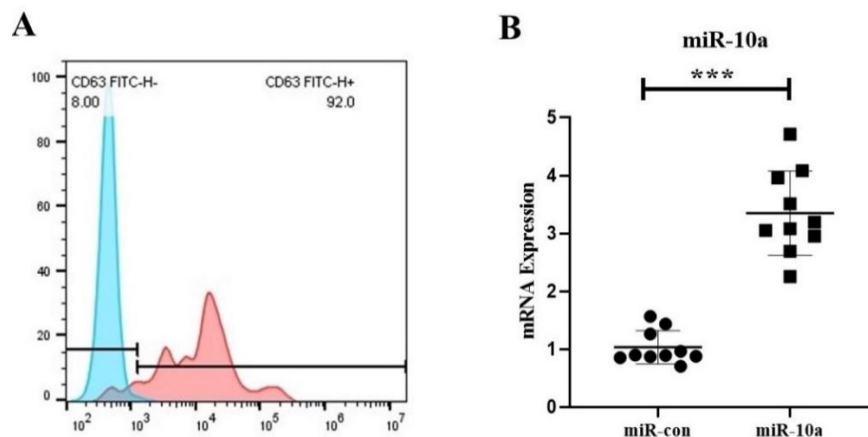


Figure 2. Characterization and miR encapsulation in EVs derived from miR-10a-transfected hUCMSCs. Flow cytometry analysis confirmed the presence of the exosomal marker CD63 in EVs isolated from hUCMSCs transfected with miR-10a mimics (A). Quantitative RT-PCR analysis demonstrating the incorporation of miR-10a into EVs (B). The data is presented as the mean \pm SD. Statistical significance is indicated as *** p <0.001. EV: extracellular vesicle; Humsc: human umbilical cord mesenchymal stem cell.

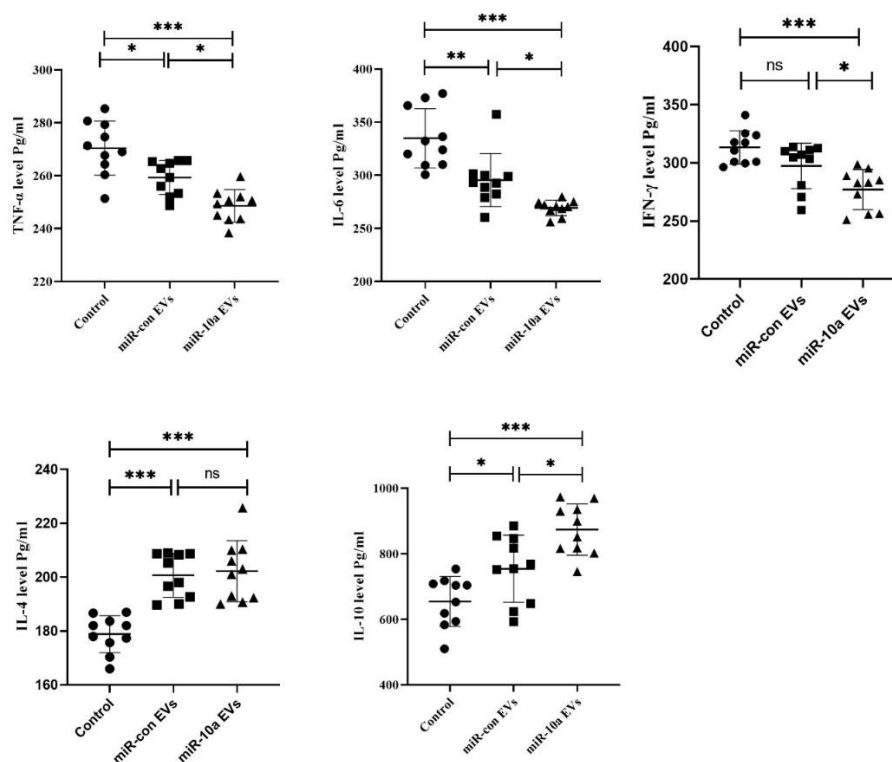


Figure 3. Impact of miR-10a-enriched EVs on serum cytokine profile. Treatment with miR-10a-EVs resulted in a significant reduction of pro-inflammatory cytokines, including TNF- α , IL-6, and IFN- γ , compared to control groups. Additionally, the anti-inflammatory cytokines IL-4, and IL-10 were significantly elevated in the miR-10a-EV group, indicating an enhanced anti-inflammatory response. The data is presented as the mean \pm SD. Statistical significance is indicated as *** p <0.001, ** p <0.01, and * p <0.05. N=10 in each group. EV: extracellular vesicle; IFN: interferon; IL: interleukin; TNF: tumor necrosis factor.

hUCMSC-EVs with Elevated miR-10a Levels Modulate Cytokine Expression in the Liver Tissue

Critical cytokine expression was assessed by measuring levels of IL-4, IL-10, TNF- α , IFN- γ , and IL-6 in a CCl₄-induced liver fibrosis model after treatment with miR-10a-enriched EVs. In comparison to EVs containing the miR-control (miR-con), the results showed that miR-10a EVs significantly decreased the levels of pro-inflammatory cytokines TNF- α and IL-6 in liver tissue ($p=0.0386$ and $p=0.0217$, respectively). They also decreased TNF- α , IFN- γ , and IL-6 in comparison to PBS control ($p<0.0001$, $p=0.001$, $p<0.0001$, respectively). Conversely, the expression of anti-inflammatory cytokines IL-4 and IL-10 was notably elevated in the miR-10a EV group compared to

both control ($p<0.0001$ and $p<0.0001$, respectively) and miR-control groups ($p=0.0364$ and $p<0.0001$, respectively).

Furthermore, comparing the cytokine profiles between the miR-control EV group and untreated CCl₄-induced fibrosis control mice revealed significant differences. The miR-control EVs caused a moderate decrease in pro-inflammatory cytokines TNF- α , IFN- γ , and IL-6 ($p=0.0184$, 0.0209 , and 0.0014 , respectively), although this reduction was less pronounced than that observed in the miR-10a EV-treated group. The anti-inflammatory cytokines IL-4 and IL-10 displayed an increase with miR-control EV treatment compared to untreated controls ($p<0.0001$ and $p<0.0001$, respectively) (Figure 4).

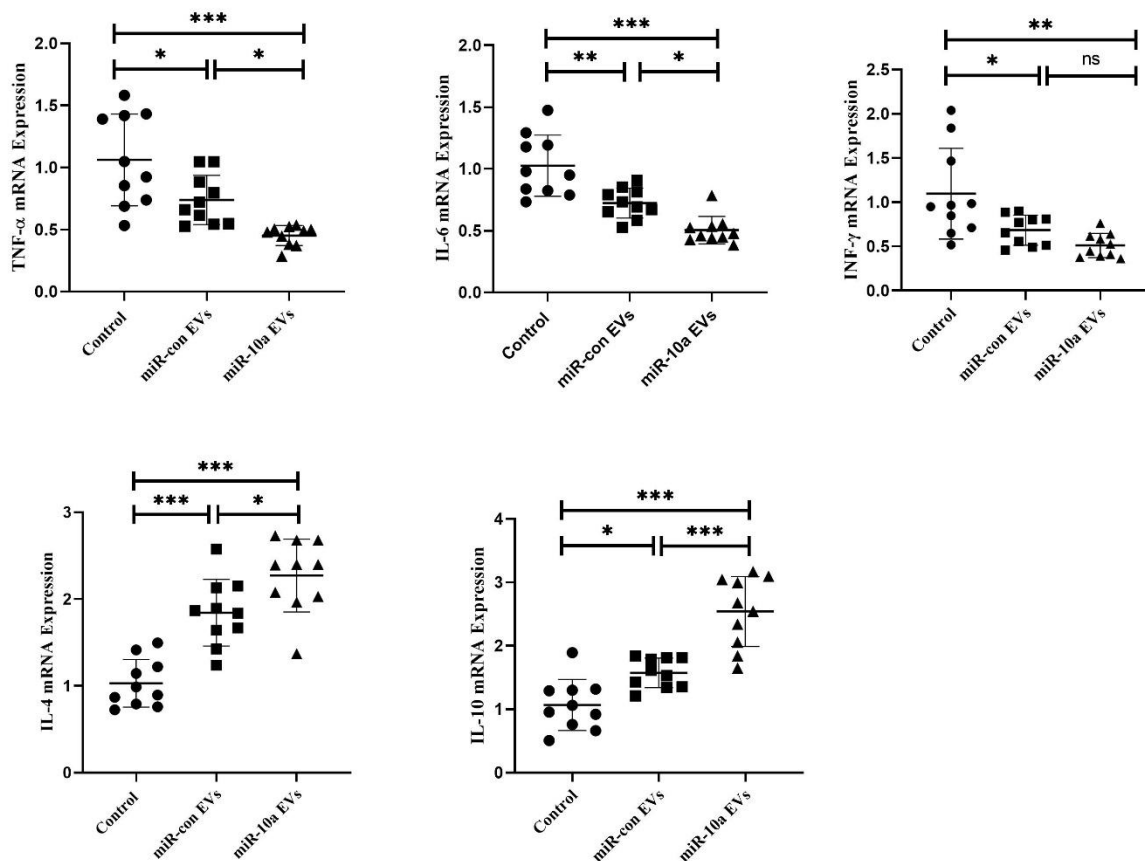


Figure 4. mRNA expression of the anti- and pro-inflammatory markers in liver tissue. Treatment with miR-10a-EVs resulted in a significant reduction of pro-inflammatory gene expression, including TNF- α , IL-6, and IFN- γ , compared to control groups. Additionally, the anti-inflammatory genes IL-4 and IL-10 were significantly elevated in the miR-10a-EV group. The data is presented as the mean \pm SD. Statistical significance is indicated as *** $p<0.001$, ** $p<0.01$, and * $p<0.05$. N=10 in each group. EV: extracellular vesicle; IFN: interferon; IL, interleukin; TNF: tumor necrosis factor.

These results indicate that miR-10a-enriched EVs exhibit a stronger anti-inflammatory potential in CCl₄-induced liver fibrosis compared to control EVs, underscoring the potential therapeutic value of miR-10a for liver fibrosis treatment.

DISCUSSION

The study's findings provide valuable information about the therapeutic potential of miR-10a in EVs derived from MSCs for the treatment of liver fibrosis. The results demonstrated that miR-10a-enriched EVs can change the inflammatory environment in a CCl₄-induced liver fibrosis model, effectively promoting an anti-inflammatory state, as evidenced by increased levels of the anti-inflammatory cytokines IL-4 and IL-10 and a concurrent decrease in pro-inflammatory markers such as TNF- α , IL-6, and IFN- γ . These findings are consistent with other studies that focus on the function of miRs in regulating inflammation and fibrogenesis during liver fibrosis.^{14,15}

A key feature of this study is the encapsulation of miR-10a within EVs derived from MSCs, which provides a stable delivery system that protects the miR from rapid degradation in vivo.^{16,17} The encapsulation method likely facilitated sustained release and improved bioavailability, thereby enhancing the therapeutic impact. The observed increases in IL-10 and IL-4 levels after treatment suggest that miR-10a is instrumental in creating an anti-inflammatory environment, which is vital for reducing fibrosis and promoting tissue repair.

The decrease in pro-inflammatory cytokines TNF- α , IL-6, and IFN- γ observed in treated mice further highlights the effectiveness of miR-10a-EVs in suppressing the fibrotic response. TNF- α and IL-6 play central roles in the inflammatory response linked to chronic liver injury by stimulating hepatic stellate cell (HSC) activation and supporting the continuous buildup of extracellular matrix (ECM).¹⁸⁻²⁰ The capacity of miR-10a-EVs to reduce these cytokines suggests that miR-10a effectively disrupts this harmful cycle, thereby diminishing fibrogenesis.

IFN- γ has a complex role in liver injury, typically associated with pro-inflammatory and pro-fibrotic functions.²¹ The reduction of IFN- γ observed in this study indicates that miR-10a-EVs not only target direct fibrotic factors but also influence immune signaling pathways that lead to tissue damage. This broad effect

on both direct and indirect mechanisms of fibrosis emphasizes the potential of miR-10a-EVs as a therapeutic strategy for liver fibrosis.

Another important finding from this study is the performance of the miR-control EVs, which showed moderate anti-inflammatory effects compared to the untreated control group. This suggests that EVs derived from MSCs have some inherent anti-inflammatory properties due to their natural cargo of bioactive molecules, as shown in previous research.²² However, the greater efficacy seen with miR-10a-loaded EVs emphasizes the significance of targeted miR loading to enhance specific therapeutic effects.

By encapsulating miR-10a within EVs derived from MSCs, this study presents an innovative method that ensures targeted delivery while leveraging the natural biocompatibility of EVs to reduce potential side effects. This approach not only improves the specificity of the treatment but also potentially enhances its safety profile, making it a promising avenue for future liver fibrosis therapies.

Despite these encouraging results, certain limitations should be acknowledged. The specific mechanisms behind the observed changes in cytokine levels are not fully understood. Future research should aim to clarify the particular signaling pathways (e.g., nuclear factor κ B (NF- κ B), Janus kinase-signal transducers and activators of transcription (JAK/STAT), transforming Growth Factor-beta 1 (TGF- β 1)/Smads) activated by miR-10a in hepatic cells and HSCs. Additionally, while this study emphasized cytokine expression as a key marker of anti-inflammatory and anti-fibrotic activity, more thorough histopathological analyses would further validate the degree of tissue remodeling and reversal of fibrosis.

In summary, this research underscores the promise of EVs derived from MSCs and loaded with miR-10a as a potential treatment for liver fibrosis. The miR-10a-EVs demonstrate an ability to modulate the balance between pro- and anti-inflammatory cytokines, promoting an anti-inflammatory environment that facilitates tissue repair and slows fibrotic progression. These results open new avenues for exploring EV-based miR therapies, potentially offering an innovative alternative to existing treatment approaches for liver fibrosis.

Future studies should clarify the molecular mechanisms pertaining to miR-10a, especially concerning hepatic stellate cells and TGF- β 1/Smads

signaling, with the addition of histopathological evaluations to determine fibrosis reversal. For clinical translation, optimizing scalable EV production and miR-10a loading is critical, followed by preclinical safety studies to evaluate biodistribution and immunogenicity. Early-phase clinical trials should then assess safety and efficacy in liver fibrosis patients. These steps position miR-10a-EVs as a transformative therapy, addressing the urgent need for effective anti-fibrotic treatments.

STATEMENT OF ETHICS

This study was approved by the Zhinanzhen Biology Ethics Committee (ID: A2024000517).

FUNDING

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

Not applicable.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

AI ASSISTANCE DISCLOSURE

Not applicable.

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