

Unraveling the Exosome-miR-133a Axis: Targeting TGF- β Signaling via WJ-MSC-Derived Exosomes for Anti-Fibrotic Therapy in Liver Fibrosis

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ABSTRACT

Background: One of the primary drivers of liver fibrosis is the excessive accumulation of ECM, primarily caused by the over-proliferation of HSCs. The activation of HSCs by TGF- β has a critical role in initiating fibrosis. Recent studies have suggested that miRNA-133a significantly regulates the fibrogenesis process, which its downregulation is associated with the fibrosis progression. Understanding the role of miRNA-133a provides potential therapeutic insights for targeting TGF- β signaling and mitigating liver fibrosis. We investigated whether exosomes could attenuate liver fibrosis by enhancing the antifibrotic effects of miR-133a.

Methods: The LX-2 cell line was treated with TGF- β for 24 hours, followed by an additional 24 hours of treatment with exosomes. After this treatment period, we assessed the mRNA expression levels of α -SMA, collagen 1, and miR-133a, as well as the protein levels of p-Smad3.

Results: TGF- β exposure significantly increased the expression level of α -SMA and collagen 1 genes and elevated the levels of p-Smad3 protein. Additionally, it resulted in a significant downregulation of miR-133a compared to the control group. Exosome administration effectively reduced the TGF- β -induced upregulation of p-Smad3, α -SMA, and collagen 1 genes, but increased miR-133a expression levels.

Conclusion: Our findings indicate that by partially mitigating the downregulation of miR-133a, exosomes can effectively inhibit the persistent activation of HSCs. Furthermore, in the context of in vitro liver fibrosis, exosomes can suppress the TGF- β /Smad3 pathway, reducing the accumulation of ECM. **DOI: 10.61186/ibj.4357**

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List of Abbreviations:

3' UTR: untranslated region; **cdDNA:** complementary DNA; **COL1A1:** collagen type I; **DLS:** dynamic light scattering; **DMEM:** Dulbecco's modified eagle medium; **ECL:** enhanced chemiluminescence; **ECM:** extracellular matrix; **FBS:** fetal bovine serum; **GAPDH:** glyceraldehyde-3-phosphate dehydrogenase; **HRP:** horseradish peroxidase; **HSC:** hepatic star-shaped cell; **LPS:** lipopolysaccharide; **miR:** microRNA; **MSC:** mesenchymal stem cell; **PBS:** phosphate-buffered saline; **p-Smad3:** phosphorylated Smad3; **PVDF:** polyvinylidene fluoride; **qRT-PCR:** quantitative reverse transcription polymerase chain reaction; **ROS:** Reactive oxygen species; **TBST:** Tris Buffered Saline with Tween 20; **TGF- β :** Transforming growth factor beta; **WJ-MSC:** Wharton's jelly-derived mesenchymal stem cell; **α -SMA:** alpha smooth muscle actin

INTRODUCTION

Liver fibrosis progresses as a result of persistent liver damage and injury. In fibrotic conditions, parenchymal cells undergo apoptosis or necrosis, and the resultant voids are subsequently filled with ECM proteins, such as collagen^[1,2]. Uncontrolled hepatic fibrosis can lead to abnormal tissue remodeling, resulting in the formation of irreversible fibrotic tissue. If left unaddressed, liver fibrosis significantly contributes to the increased mortality rates associated with liver cirrhosis and cancer^[3].

Hepatic fibrosis is primarily driven by HSCs located between hepatocytes and endothelial cells. Reactive oxygen species generated during the apoptosis or necrosis of hepatocytes are identified as key activators of HSCs, believed to induce their trans-differentiation^[4,5]. In a healthy liver, HSCs release ECM components in a regulated manner. However, following significant tissue injury, they differentiate into proliferative, myofibroblast-like cells, and lose their characteristic stellate morphology. These transformed cells secrete excessive amounts of autocrine activators, which further promote the development of fibrosis^[6].

The TGF- β family, which contains over 30 proteins, is a well-established promoter of fibrosis. TGF- β exerts its effects via serine/threonine kinase receptors, primarily T β RI, T β RII, and T β RIII, located on the cell membrane. When TGF- β binds to T β RII, T β RI undergoes phosphorylation, which subsequently activates Smad3 signaling^[7,8]. Smad3C transmits the signal to the nucleus, influencing the expression of numerous genes and regulating various cellular functions, including the activation of HSCs. This activation leads HSCs to secrete increased amounts of ECM proteins, contributing to fibrosis. Consequently, elevated TGF- β levels are associated with the progression and severity of hepatic fibrosis^[9].

The roles of miRNAs are essential to various cellular activities, including apoptosis and cell proliferation^[10]. MiRs typically range from 19 to 22 nucleotides in length and interact with the 3'UTR of newly synthesized mRNA, thereby preventing its translation. In the context of liver fibrosis, miRs play a crucial role in influencing the fibrotic response by modulating the expression of genes associated with both the progression and resolution of fibrosis^[11]. MiRs exhibit a dual function in liver fibrosis; some are pro-fibrotic, facilitating the progression of fibrosis, while others are anti-fibrotic, reducing its development. One example is miR-133a, which acts as an anti-fibrotic agent by inhibiting the expression of the TGF- β gene, reducing the activation of HSCs^[12,13].

Recently, there has been considerable interest in investigating the therapeutic potential of MSCs for the treatment of liver diseases. MSCs are a versatile group of pluripotent progenitor cells recognized as a promising resource for tissue regeneration and repair^[14]. Their capability to differentiate into various cell types has garnered attention as a potentially effective strategy for managing hepatic fibrosis. This remarkable ability to differentiate makes MSCs a valuable option for addressing and potentially reversing fibrotic damage in the liver^[15]. However, challenges such as high production and preservation costs, along with relatively long preparation time, created significant barriers to the application of MSCs. Research has demonstrated that MSCs secrete extracellular vesicles, including microvesicles and exosomes, which deliver paracrine factors to target cells. These vesicles play a vital role in MSC function, thereby facilitating tissue regeneration and modulating immune responses^[16].

Exosomes are small extracellular vesicles, ranging from 30 to 100 nm in diameter, and carry a diverse range of proteins and other molecules. MSCs are among the cell types that produce these vesicles^[17,18]. The use of exosomes for therapeutic purposes offers several advantages over traditional cell therapy, potentially mitigating issues such as the risk of transplant rejection and supporting a shift toward this innovative approach^[19]. In traditional cell treatment, direct injection of cells can lead to unintended consequences that are generally absent in exosome-based therapies. Moreover, exosomes are easy to produce and store due to their small size and simplicity, making them more viable for therapeutic applications^[20,21].

LPS, primarily derived from bacteria, is well-known for its ability to trigger pro-inflammatory responses. It activates immune cells and stimulates the release of inflammatory cytokines, including TNF- α and IL-6. LPS enhances the release of pro-inflammatory factors during inflammatory processes, which subsequently increases the production of exosomes^[20,21]. Additionally, studies have shown that preconditioned MSCs exhibit more pronounced paracrine effects by releasing a greater number of exosomes^[22]. Consequently, researchers are investigating both LPS-treated and untreated exosomes to assess their potential impact on hepatic fibrosis by activating exosomes and MSCs. The goal of this study was to determine whether exosome treatment can improve the condition by examining its effect on anti-fibrotic miRNA levels, specifically miR-133a, in liver-based LX2 cells.

MATERIALS AND METHODS

Culturing and treating HSCs

The entire process of experimental design, exosome isolation, characterization, and their application in the study is schematically illustrated in Figure 1. The immortalized hepatic stellate cell line, LX-2, was generously provided by Professor S. Friedman. The cells were cultured in six-well plates using DMEM supplemented with 10% FBS. Following the starvation period, the cells were exposed to TGF- β (5 ng/mL)^[23] for 24 hours. Subsequently, exosomes derived from WJ-MSCs were diluted in DMEM and used to replace the existing medium, followed by an additional 24-hour incubation. The experimental design included three groups: a control group that received no treatment, a group that received TGF- β treatment, and a group that received both TGF- β and WJ-MSC exosomes. Before extraction, the cells were washed twice with PBS in preparation for subsequent studies.

WJ-MSC isolation and culture

Fresh umbilical cords were obtained from a single woman who had undergone a full-term cesarean section. After washing the cord with PBS to remove contaminants, Wharton's jelly was carefully processed. It was cut into small fragments, approximately 1 cm in

size, and transferred to DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. These fragments were then incubated in a 37 °C incubator with 5% CO₂ for 72 hours to allow the cells to adhere to the surface of the culture plates.

Differentiation assays for WJ-MSCs

In this study, WJ-MSCs from passages 3 to 5 were utilized. The cells were cultured in BN media (0012.4-BN0012.5; Biomedical Engineering, Iran), specifically formulated for both adipogenic and osteogenic differentiation. To induce osteogenic differentiation, WJ-MSCs were seeded in six-well plates at a density of 20,000 cells/mL and treated with osteogenic media twice a week for 21 days. Following this period, the cells were fixed in 10% formalin for 10 minutes and stained with Alizarin Red (Sigma-Aldrich, USA) at room temperature for 45 minutes to assess calcification. In parallel, adipogenic differentiation was induced using adipogenic media, and the cells were stained with Oil Red O to evaluate lipid accumulation.

Identification of WJ-MSCs surface markers

To identify the surface markers of WJ-MSCs, we utilized conjugated monoclonal antibodies according to the manufacturer's protocol, after rinsing the cells with PBS. The employed antibodies were FITC-conjugated

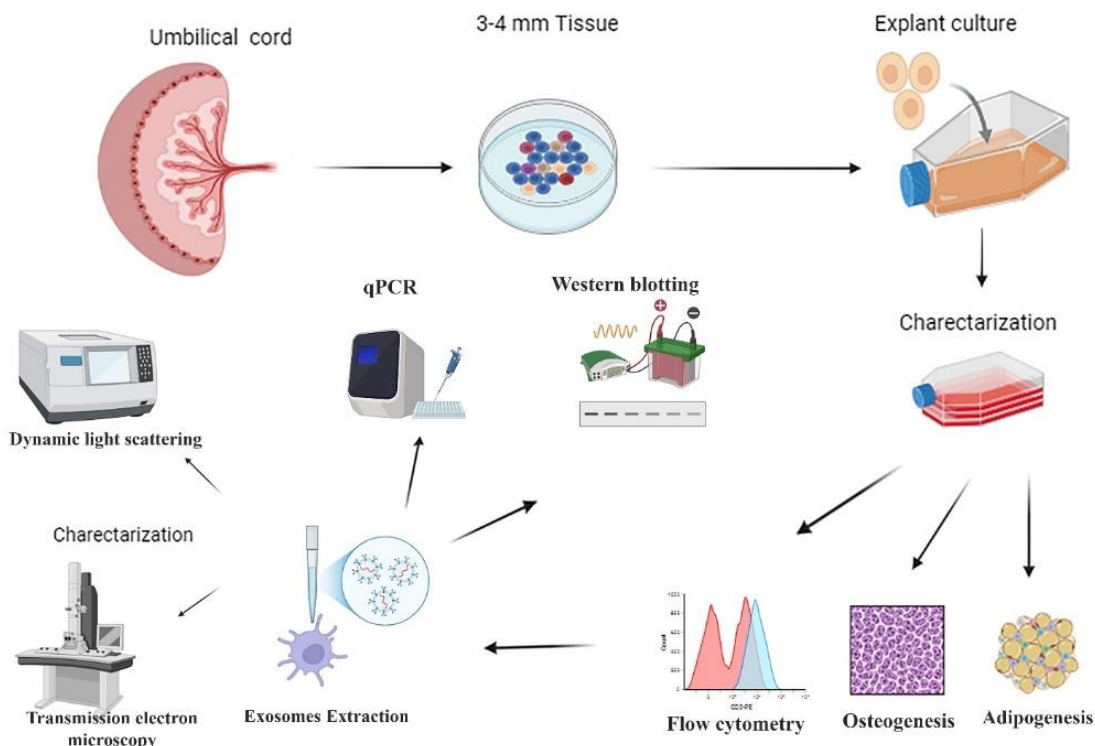


Fig. 1. Schematic diagram of the experimental procedure to study the potential effects of exosomes derived from WJ-MSCs on activated HSCs in vitro.

mouse anti-human CD45, PE-conjugated mouse anti-human CD44, FITC-conjugated mouse anti-human CD105, and PE-conjugated mouse anti-human CD34. All antibodies were obtained from Sigma-Aldrich.

LPS-induced activation of WJ-MSCs

MSCs were exposed to 1 µg/mL concentration of LPS, which was first diluted in DMEM in order to investigate the effects of LPS preconditioning on MSC differentiation^[23]. Subsequently, a serum-free medium was used to replace the LPS-containing medium for the differentiation assays.

Exosomes extraction

The cells were first cultured in DMEM enriched with 15% FBS prior to exosome extraction. The concentration of FBS was subsequently reduced to zero percent before extraction. The collected medium was then centrifuged at 300 ×g for 10 minutes. Exosome extraction was performed using an EXOCIB extraction kit from CIB Biotech Co. (Iran), according to the protocols provided by the manufacturer. The isolated exosomes were resuspended in 50-200 L of PBS and stored at -80 °C. BCA assay kit (Parstous, Iran) was used to measure the protein concentration of the exosomes.

Exosome characterization

Exosomes were characterized using TEM and DLS. For TEM, exosomes were fixed with 4% paraformaldehyde, placed on carbon-coated grids, and stained with 1% phosphotungstic acid. Their morphology was observed using a Zeiss EM10C microscope (100 KV; Germany), confirming their characteristic cup-shaped structure. DLS analysis was performed using a Zetasizer Nano ZS system (Malvern Instruments, UK) to assess size distribution. Exosomes were solubilized in PBS with 0.05% Tween-20, diluted to 1 µg/mL, and measured at 23 °C. The size distribution

ranged between 30 and 150 nm and was consistent with typical exosome sizes. The protein concentration of the exosomes was determined using BCA protein assay kit. Exosome identification was conducted by Western blotting analysis, employing standard exosome markers, CD9 (1:300, Santa Cruz, USA) and CD81 (1:100, Santa Cruz). Briefly, 20 µg of protein from each sample was separated on a 10% SDS-PAGE, transferred to a PVDF membrane, and blocked with 5% non-fat milk at 37 °C for one hour. The membrane was then incubated at 4°C overnight with primary antibodies against CD9 and CD81. Following three 10-minute washing in TBST, the membranes were incubated for two hours at 37 °C with mouse anti-rabbit IgG-HRP (1:1000, Santa Cruz), washed again, and treated with chemiluminescent substrates. Blots were visualized using an ECL system.

qRT-PCR analysis

To generate cDNA templates, we employed a cDNA synthesis kit following the extraction of total RNA using the RNA Isolation Kit; both kits were obtained from Yekta Tajhiz, Iran. The genes studied included α -SMA, collagen 1, and miR-133a. As per the manufacturer's guidelines, qRT-PCR was performed using the QuantStudio™ 3 System (ABI, USA) and SYBR Green master mix (Ampliqon, Denmark). The primer sequences for these genes were designed by Sinaclon Company, Tehran, Iran (Table 1).

Western blotting

HSCs were lysed using RIPA buffer supplemented with protease and phosphatase inhibitors. The BCA assay was employed to precisely quantify the protein concentration. Following this process, the protein samples were resolved using SDS-PAGE (30 µg per lane) and transferred to PVDF membranes. After three washes with TBST, the membranes were incubated at 4-5 °C overnight with a primary antibody specific for p-Smad3, diluted at 1:1500. Subsequently, a secondary

Table 1. Primer sequence for RT-PCR

Gene	Primer sequence	PCR product size (bp)
<i>COL1A1</i>	F: 5'-TGAAGGGACACAGAGGTTCA-3' R: 5'-ACGATCAACTCCAGGAGAA-3'	188
α -SMA	F: 5'-CAAGTCCTCCAGCGTTCTGA-3' R: 5'-GCTTCACAGGATTCCCGTCTT-3'	196
<i>Hsa-miR-133a</i>	F: 5'-CTTGGTCCCCTTCAACCAAG-3'	149
<i>U6</i>	F: 5'-GCAGCACATATACTAAATTGG-3'	168
<i>GAPDH</i>	F: 5'-TCGGAGTCAACGGATTTGGT-3' R: 5'-TTCCCGTTCTCAGCCTTTGAC-3'	181

F: forward; R: reverse

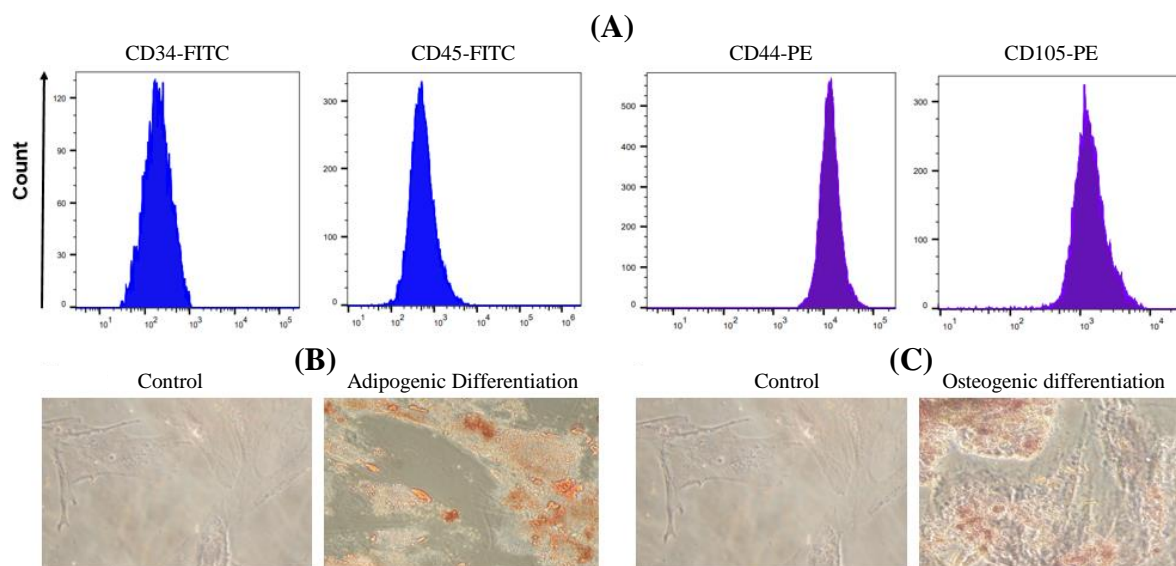


Fig. 2. Immunophenotyping and differentiation abilities of WJ-MSCs. (A) Flow cytometry analysis showing the expression of CD44 and CD105 and the reduced levels of CD34 and CD45 on the surface of WJ-MSCs. (B) Oil red O staining demonstrating clear red intracellular lipid deposits in WJ-MSC adipocytes after 21 days. (C) Alizarin Red S staining indicating significant orange-red calcium deposits in WJ-MSC osteocytes at day 21.

antibody was applied at a dilution of 1:10,000. An ECL reagent (Bio-Rad, USA) was used to detect the antibodies.

Statistical analysis

The experiments were conducted in triplicate. Statistical comparisons were performed using GraphPad Prism 9.0 software, employing ANOVA, Tukey's HSD test, and presenting the data as mean \pm SEM for the data. Statistical significance was determined with a *p* value of less than 0.05.

RESULTS

WJ-MSCs characterization

Surface marker expression and in vitro cell differentiation capabilities were assessed in this section. By the third passage, WJ-MSCs had developed into spindle-shaped monolayers, with fibroblast-like cells firmly adhering to the flasks. The MSCs were found to be positive for the stem cell markers CD34 and CD45, as determined by flow cytometry analysis. However, the MSCs did not express CD44 or CD105, which excludes populations derived from endothelial or hematopoietic cells, confirming the isolation of MSCs (Fig. 2A). After 21 days of culture, Oil Red O staining revealed an abundance of lipid droplets, indicating that WJ-MSCs can differentiate into adipocytes (Fig. 2B). In addition, the induction of differentiation into osteoblasts, followed by Alizarin Red staining, revealed a significant calcium deposit, which is characteristic of osteogenic

differentiation and confirms the process of osteogenic differentiation (Fig. 2C).

Exosome characterization

Exosomes exhibited a spherical shape, as measured by TEM, with diameters ranging from 50 to 200 nm (Fig. 3A). Further analysis of the size distribution of the isolated exosomes, conducted using a zeta sizer, revealed that approximately 70-80% of the exosomes had diameters between 75 and 80 nm (Fig. 3B). The expression levels of protein markers CD9 and CD81 were significantly higher in the exosomes than WJ-MSCs, as determined by Western blotting analysis (Fig. 3C).

Effect of TGF- β and exosome on liver fibrosis markers

The expression levels of the fibrosis markers α -SMA and *collagen 1* were significantly elevated in the presence of TGF- β (5 ng/mL) compared to the control group (Fig. 4). This increase in the mRNA levels of the two markers correlates with TGF- β -induced liver fibrosis. However, treatment with WJ-MSC-derived exosomes at a concentration of 50 μ g/mL resulted in reduced expression levels of *collagen 1* and α -SMA by 1.76 and 3.26 folds, respectively (Fig. 4A and 4B). Although there was a substantial decrease in α -SMA expression level in the LPS + exosome-treated group compared to the control group, no significant changes were observed in *collagen 1* expression in this treatment group.

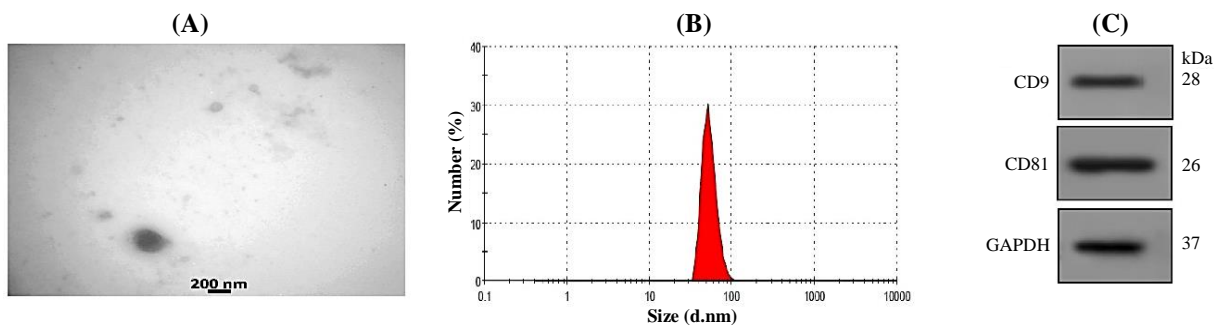


Fig. 3. Exosome characterization. (A) Visualization of the shape and size of the vesicles by TEM. (B) Size analysis by the Malvern Zetasizer, showing that approximately 85% of the exosomes are between 70 and 75 nm. (C) Western blotting analysis for CD9 and CD81 protein markers in WJ-MSC-exosome.

Effect of TGF- β and exosome on miR-133a expression

The real-time PCR results indicated that the administration of TGF- β (5 ng/mL) decreased the expression level of *miR-133a* in LX2 cells (Fig. 4). In contrast, treatment with LPS + exosome (50 μ g/mL) resulted in a 1.08-fold increase in the *miR-133a* expression level (Fig. 4C).

Effect of TGF- β and exosome on Smad3 level

In LX2-HSC cells, we investigated the effect of exosomes on TGF- β signaling. Our results indicated that the level of p-Smad3 significantly increased by 5.87-fold compared to the control group, following TGF- β treatment (Fig. 5). However, when WJ-MSC-derived exosomes were administered, the quantity of p-Smad3 decreased significantly to 2.86-fold relative to the control (Fig. 5A). Furthermore, p-Smad3 level was notably lower in the cells treated with LPS + exosome (50 μ g/mL) compared to those treated with exosome alone, resulting in a reduction of 1.94-fold.

DISCUSSION

HSCs play a central role in mediating fibrosis-promoting lesions resulting from liver damage. These essential mesenchymal cells, located in the space of Disse within the liver, significantly contribute to fibrogenesis. The progression of liver fibrosis is accelerated by pro-fibrogenic cytokines, which stimulate the proliferation and differentiation of HSCs into myofibroblast-like cells^[24]. Due to its function as an inhibitor of liver fibrosis, miR-146a has recently received attention in hepatic research^[13,23]. Our understanding of the functional role of miR-133a during HSC activation remains limited, despite its increasing significance. Numerous studies have indicated that multiple pro-fibrogenic pathways can activate HSCs^[25,26]. In this context, it has been shown that TGF- β is a key regulator that stimulates the synthesis of ECM proteins by HSCs. Additionally, the involvement of miRNAs in the regulation of TGF- β signaling in HSCs has previously been established^[27]. It has been

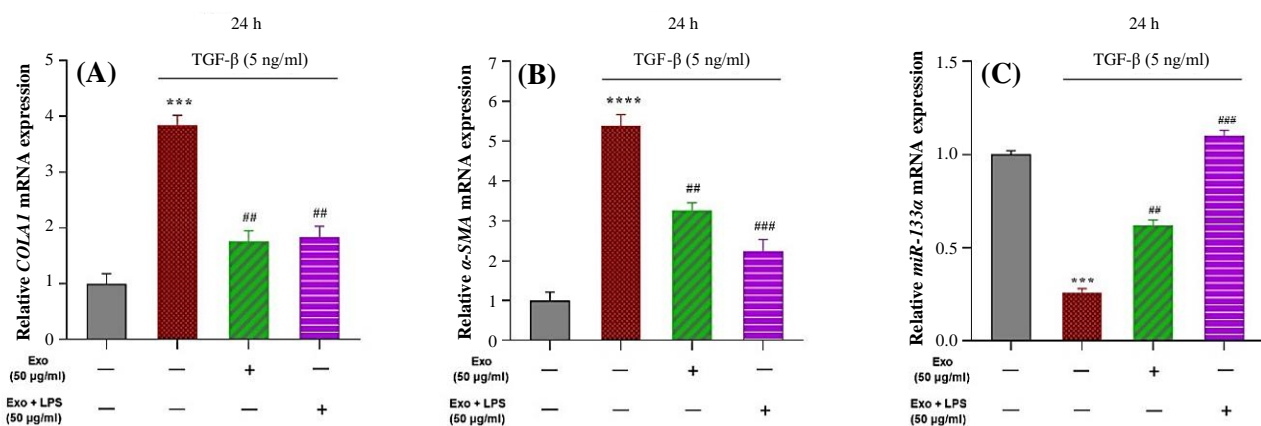


Fig. 4. Evaluation of the mRNA expression levels of *COL1A1*, *α -SMA*, and *miR-133a* in the LX2 cell line in the presence of TGF- β and exosomes. Data from three replicates (mean \pm SEM) were compared to the control. GAPDH was used as a reference gene (** p < 0.001, **** p < 0.0001, ## p < 0.01, ### p < 0.001).

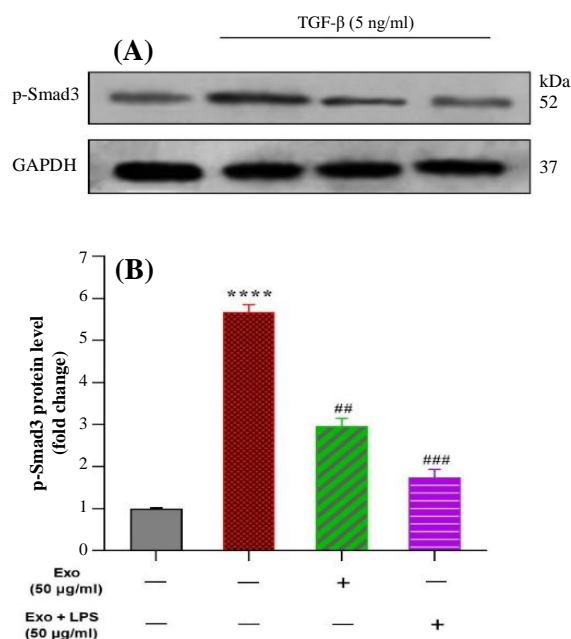


Fig. 5. Effect of one hour exosome treatment on TGF- β -induced Smad3 phosphorylation in the LX2 cell line. The results from three replicates (mean \pm SEM) were analyzed with a significance threshold of $p < 0.05$. GAPDH protein was used as an internal control (**** $p < 0.001$, ## $p < 0.01$, and ### $p < 0.001$).

demonstrated that increased level of miR-101 inhibits TGF- β signaling in HSCs by targeting and reducing the expression of TRI and KLF6^[28], two essential transcriptional activators in the TGF- β pathway. Similarly, miR-19b inhibits the expression of *Smad3* and *TGF-RII*, preventing TGF- β signaling. Moreover, TGF- β -treated HSCs exhibit lower levels of α -SMA and collagen 1 because of targeting TGF- β 2 by miR-378a. Suppressing pro-fibrotic miRNAs may represent a promising therapeutic strategy for treating liver fibrosis^[29]. He et al. have demonstrated that the substantial inhibition of miR-21 in mice with hepatic schistosomiasis markedly reduces liver fibrosis^[30].

In the field of liver fibrosis research, characterizing WJ-MSCs and their exosomes is a crucial step toward understanding the therapeutic potential of these cells. Our study meticulously adhered to the guidelines established by the International Society for Stem Cell Research for WJ-MSC characterization, which included evaluating surface marker expression and assessing differentiation potential. The confirmation of CD44 and CD105 expression, along with the absence of CD34 and CD45, is consistent with the identification of MSCs and is a commonly observed finding across various studies^[31-33]. This consistency in surface marker expression underscores the reliability of these markers for MSC identification. The WJ-MSC ability to differentiate into adipocytes and osteoblasts, as

demonstrated by Oil Red O and Alizarin Red staining, is a hallmark of MSC plasticity. This multilineage differentiation capacity is a key characteristic of MSCs and has widely been reported in the literature^[34]. The capability of WJ-MSCs to differentiate into these lineages emphasizes their potential for tissue regeneration and repair, corroborating findings from a previous study that underline the therapeutic potential of MSCs in various diseases^[35].

Exosome characterization is a pivotal aspect of our study, as these extracellular vesicles are increasingly recognized for their role in cell-to-cell communication and potential therapeutic applications. The spherical shape and size distribution of the exosomes, as observed using TEM and DLS, are typical features of exosomes and align with the findings from other studies^[36,37]. The predominance of exosomes with diameters ranging from 70 to 75 nm is noteworthy, as this size falls within the expected range for exosomes. This finding further validates the isolation and characterization of exosomes in our study.

The impact of TGF- β on liver fibrosis marker genes, such as α -SMA and *collagen 1a*, has been well-documented in the literature^[38]. TGF- β is a master regulator of fibrosis, and its ability to induce the expression of these markers is a central mechanism in the fibrotic process^[39,40]. Our findings that TGF- β significantly upregulates these markers are in agreement with previous studies highlighting the consistent profibrotic effects of TGF- β ^[39,40]. The reduction in *collagen 1a* and α -SMA gene expression following treatment with WJ-MSCs exosomes suggests an anti-fibrotic effect of these exosomes. This is a remarkable result that is consistent with the previous findings^[41,42], as it demonstrates the potential of MSC-derived exosomes to counteract TGF- β -induced fibrosis.

The mechanism by which exosomes exert the anti-fibrotic effect may be due to the transfer of anti-fibrotic molecules, such as miRNAs, to target cells, a hypothesis that warrants further investigation. When our results are compared to those of the study that used models of cholesterol-induced liver fibrosis, it is evident that TGF- β consistently and significantly influences the upregulation of liver fibrosis markers, specifically collagen1 and α -SMA. This consistency of results from different experimental paradigms highlights the central role of TGF- β in the pathogenesis of liver fibrosis. The observed increase in these markers indicates the activation of HSCs and the promotion of a fibrotic environment, which is a hallmark of the progression of liver fibrosis. This outcome suggests that the mechanism of action of WJ-MSC derived exosomes may be independent of the initial trigger of fibrosis, emphasizing their broad applicability as a therapeutic

agent. The reduction in α -SMA and collagen 1 expression after exosome treatment implies the modulation of profibrotic signaling pathways, possibly through the inhibition of TGF- β signaling. These mechanistic insights create opportunities for further research into the specific molecular cargo of WJ-MSCs exosomes, which could be used to combat liver fibrosis.

The modulation of miR-133a expression by TGF- β and exosome treatment is another significant finding of the present study. The downregulation of miR-133a by TGF- β is consistent with its role as an anti-fibrotic miRNA, since its suppression would facilitate fibrogenesis^[43]. The increased expression level of miR-133a in exosomes derived from LPS-preconditioned MSCs is a distinctive finding that highlights our study. This observation indicates that LPS preconditioning could enhance the anti-fibrotic properties of exosomes, likely by modifying their composition. Exosomes, enriched with elevated levels of miR-133a or other beneficial molecules, may play a crucial role in modulating fibrotic pathways and mitigating liver fibrosis. A comparison between our findings and other studies examining the impact of miR-193a-5p and miR-381-3p on liver fibrosis involving 42 patients with liver fibrosis reveals the complex interplay of various miRNAs in the pathophysiology of fibrosis^[43]. Our results showed that TGF- β administration reduced miR-133a expression, suggesting an antifibrotic function for this miRNA. Conversely, the overexpression of miR-193a-5p and miR-381-3p in the Wang et al. study (involving 42 patients) promotes fibrogenesis, as evidenced by increased HSC proliferation and migration, along with a decrease in apoptosis^[43]. The diverse effects of these miRNAs underscore the complex regulatory networks that control liver fibrosis and the potential for targeting specific miRNAs as a therapeutic approach. The elevated expression level of miR-133a following exosome treatment in our study suggests that exosomes may contain factors involved in the fibrotic effects of TGF- β , likely through the upregulation of miR-133a.

The effects of exosomes on Smad3 phosphorylation provide a mechanistic insight into their anti-fibrotic action. Smad3 is a key mediator of TGF- β signaling, and its phosphorylation is essential for the transduction of fibrotic signals^[41,44]. Decrease in *p-Smad3* expression levels observed after exosome treatment, especially in the LPS + exosome group, suggests that exosomes can disrupt TGF- β signaling, a key pathway in liver fibrosis. This finding is in line with previous studies^[45,46] in which the impact of exosomes on Smad3 phosphorylation was investigated and emphasized the novelty of our research. Conversely, treatment with WJ-MSCs exosomes significantly reduced the p-Smad3 level, indicating that these exosomes contain factors

capable of inhibiting the TGF- β /Smad3 pathway. The additional reduction in p-Smad3 level observed in the LPS + exosome treatment group, suggests a potential synergistic effect between LPS and exosomes in inhibiting Smad3 phosphorylation. These results highlight the therapeutic potential of exosomes in alleviating liver fibrosis by modulating key fibrogenic signaling pathways.

CONCLUSION

The present study enhances the understanding of liver fibrosis by emphasizing the pivotal roles of HSCs and the therapeutic potential of WJ-MSCs and their derived exosomes. The unique properties of these exosomes show promise in reducing key fibrotic markers, such as collagen 1 α and α -SMA, suggesting a novel approach to mitigating fibrogenesis. Our findings illustrate how exosome-mediated alterations in miR-133a and the dephosphorylation of Smad3 can disrupt TGF- β signaling. Notably, the increased anti-fibrotic effects observed with LPS-preconditioned exosomes highlight their potential for enhancing therapeutic outcomes through the strategic manipulation of exosomal content. This research positions MSC-derived exosomes as targeted therapeutic agents, warranting further investigation into their mechanisms and clinical applications in the treatment of hepatic fibrosis.

DECLARATIONS

Acknowledgments

No artificial intelligence was used in this study.

Ethical approval

This study was conducted with the approval of the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (ethical code: IR.AJUMS.REC.1400.461).

Consent to participate

Not applicable.

Consent for publication

All authors reviewed the results and approved the final version of the manuscript.

Authors' contributions

SS: performed all assays; SM: contributed to interpreting the results, designed the study and revised the manuscript; FGH: analyzed the data; GHO: wrote the first draft; SSB: analyzed the data; AKH: designed

the study; MH: designed the study and wrote the first draft.

Data availability

All relevant data can be found within the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Supplementary information

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REFERENCES

- Afarin R, Babaahmadi Rezaei H, Yaghoobi H, Mohammadtaghvaei N. Fibroblast growth factor 21 reduces cholesterol-induced hepatic fibrogenesis by inhibiting TGF- β /Smad3C signaling pathway in LX2 cells. *Hepat Mon.* 2021;21(4): e113321. <https://doi.org/10.5812/hepatmon.113321>.
- Mohammadzadeh G, Afarin R, Salehipour Bavarsad S, Aslani F, Asadzadeh S, Shakerian E. Comparison of the effects of cholesterol, palmitic acid, and glucose on activation of human hepatic stellate cells to induce liver fibrosis. *J Diabetes Metab Disord.* 2022;21(2):1531-8.
- Roehlen N, Crouchet E, Baumert TF. Liver fibrosis: Mechanistic concepts and therapeutic perspectives. *Cells.* 2020;9(4):875.
- Luangmonkong T, Suriguga S, Mutsaers HAM, Groothuis GMM, Olinga P, Boersema M. Targeting oxidative stress for the treatment of liver fibrosis. *Rev Physiol Biochem Pharmacol.* 2018;175:71-102.
- Liang S, Kisseleva T, Brenner DA. The role of NADPH oxidases (NOXs) in liver fibrosis and the activation of myofibroblasts. *Front Physiol.* 2016;7:17.
- Tacke F, Trautwein C. Mechanisms of liver fibrosis resolution. *J Hepatol.* 2015;63(4):1038-9.
- Fabregat I, Moreno-Càceres J, Sánchez A, Dooley S, Dewidar B, Giannelli G, et al. TGF- β signalling and liver disease. *FEBS J.* 2016;283(12):2219-32.
- Xu F, Liu C, Zhou D, Zhang L. TGF- β /SMAD pathway and its regulation in hepatic fibrosis. *J Histochem Cytochem.* 2016;64(3):157-67.
- Afarin R, Babaahmadi Rezaei H, Yaghouti SH, Mohammad Taghvaei N. The effect of cholesterol on the activation of TGF- β /Smad3C signaling pathway in hepatic stellate cells and its role in the progression of liver fibrogenesis. *J Isfahan Med Sch.* 2021;39(619):212-8.
- Roshani Asl E, Amini M, Najafi S, Mansoori B, Mokhtarzadeh A, Mohammadi A, et al. Interplay between MAPK/ERK signaling pathway and MicroRNAs: A crucial mechanism regulating cancer cell metabolism and tumor progression. *Life Sci.* 2021;278:119499.
- Jiang XP, Ai WB, Wan LY, Zhang YQ, Wu JF. The roles of microRNA families in hepatic fibrosis. *Cell Biosci.* 2017; <https://doi.org/10.1186/s13578-017-0161-7>.
- Wang J, Chu ESH, Chen HY, Man K, Go MYY, Huang XR, et al. MicroRNA-29b prevents liver fibrosis by attenuating hepatic stellate cell activation and inducing apoptosis through targeting PI3K/AKT pathway. *Oncotarget.* 2015;6(9):7325-38.
- Roderburg C, Luedde M, Cardenas DV, Vucur M, Mollnow T, Zimmermann HW, et al. miR-133a mediates TGF- β -dependent derepression of collagen synthesis in hepatic stellate cells during liver fibrosis. *J Hepatol.* 2013;58(4):736-42.
- Zhang S, Yang Y, Fan L, Zhang F, Li L. The clinical application of mesenchymal stem cells in liver disease: the current situation and potential future. *Ann Transl Med.* 2020;8(8):565.
- Tsuchiya A, Takeuchi S, Watanabe T, Yoshida T, Nojiri S, Ogawa M, et al. Mesenchymal stem cell therapies for liver cirrhosis: MSCs as “conducting cells” for improvement of liver fibrosis and regeneration. *Inflamm Regen.* 2019;39(1):1-6.
- Ye JS, Su XS, Stoltz JF, Isla N, Zhang L. Signalling pathways involved in the process of mesenchymal stem cells differentiating into hepatocytes. *Cell Prolif.* 2015;48(2):157-65.
- Lou G, Chen Z, Zheng M, Liu Y. Mesenchymal stem cell-derived exosomes as a new therapeutic strategy for liver diseases. *Exp Mol Med.* 2017;49(6):e346.
- Afarin R, Behdarvand T, Shakerian E, Salehipour Bavarsad S, Rashidi M. Exosomes of Whartons’ jelly mesenchymal stem cell reduce the NOX genes in TGF- β -induced hepatic fibrosis. *Iran J Basic Med Sci.* 2022;25(12):1498-1503.
- Rong X, Liu J, Yao X, Jiang T, Wang Y, Xie F. Human bone marrow mesenchymal stem cells-derived exosomes alleviate liver fibrosis through the Wnt/ β -catenin pathway. *Stem Cell Res Ther.* 2019;10(1):98.
- Hong Y, Lee J, Vu TH, Lee S, Lillehoj HS, Hong YH. Exosomes of lipopolysaccharide-stimulated chicken macrophages modulate immune response through the MyD88/NF- κ B signaling pathway. *Dev Comp Immunol.* 2021;115:103908.
- Afarin R, Aslani F, Asadzade S, Jaberian Asl B, Mohammadi Gahrooie M, Shakerian E, et al. The effect of lipopolysaccharide-stimulated adipose-derived mesenchymal stem cells on NAFLD treatment in high-fat diet-Fed rats. *Iran J Pharm Res.* 2023;22(1):e134807.
- Ti D, Hao H, Tong C, Liu J, Dong L, Zheng J, et al. LPS-preconditioned mesenchymal stromal cells modify macrophage polarization for resolution of chronic inflammation via exosome-shuttled let-7b. *J transl Med.* 2015;13:308.

23. Kitano M, Bloomston PM. Hepatic stellate cells and microRNAs in pathogenesis of liver fibrosis. *J Clin Med*. 2016;5(3):38.
24. Shakerian E, Akbari R, Mohammadtaghvaei N, Mohammadi Gahrooie M, Afarin R. Quercetin reduces hepatic fibrogenesis by inhibiting TGF- β /Smad3 signaling pathway in LX-2 cell line. *Jundishapur J Nat Pharm Prod*. 2022;17(1):e113484.
25. Bindal P, Ramasamy TS, Kasim NHA, Gnanasegaran N, Chai WL. Immune responses of human dental pulp stem cells in lipopolysaccharide-induced microenvironment. *Cell Biol Int*. 2018;42(7):832-40.
26. Bansal MB. Hepatic fibrogenesis. Elsevier. 2025;(1):247-72.
27. Marquez RT, Bandyopadhyay S, Wendlandt EB, Keck K, Hoffer BA, Icardi MS, et al. Correlation between microRNA expression levels and clinical parameters associated with chronic hepatitis C viral infection in humans. *Lab Invest*. 2010;90(12):1727-36.
28. Tu X, Zhang H, Zhang J, Zhao S, Zheng X, Zhang Z, et al. MicroRNA-101 suppresses liver fibrosis by targeting the TGF β signalling pathway. *J Pathol*. 2014;234(1):46-59.
29. Lakner AM, Steuerwald NM, Walling TL, Ghosh S, Li T, McKillop IH, et al. Inhibitory effects of microRNA 19b in hepatic stellate cell-mediated fibrogenesis. *Hepatology*. 2012;56(1):300-10.
30. He X, Xie J, Zhang D, Su Q, Sai X, Bai R, et al. Recombinant adeno-associated virus-mediated inhibition of microRNA-21 protects mice against the lethal schistosome infection by repressing both IL-13 and transforming growth factor beta pathways. *Hepatology*. 2015;61(6):2008-17.
31. Isono M, Takeuchi J, Maehara A, Nakagawa Y, Katagiri H, Miyatake K, et al. Effect of CD44 signal axis in the gain of mesenchymal stem cell surface antigens from synovial fibroblasts in vitro. *Heliyon*. 2022;8(10):e10739.
32. L. Ramos T, Sánchez-Abarca LI, Muntión S, Preciado S, Puig N, López-Ruano G, et al. MSC surface markers (CD44, CD73, and CD90) can identify human MSC-derived extracellular vesicles by conventional flow cytometry. *Cell Commun signal*. 2016;14(2):<https://doi.org/10.1186/s12964-015-0124-8>.
33. Walecka I, Gil-Kulik P, Krzyżanowski A, Czop M, Galkowski D, Karwat J, et al. Phenotypic characterization of adherent cells population CD34⁺ CD90⁺ CD105⁺ derived from Wharton's jelly. *Med Sci Monit*. 2017;23:1886-95.
34. Lin K, Yang Y, Cao Y, Liang J, Qian J, Wang X, et al. Combining single-cell transcriptomics and cell tagging to identify differentiation trajectories of human adipose-derived mesenchymal stem cells. *Stem Cell Res Ther*. 2023;14(1):14.
35. Ababneh NA, Al-Kurdi B, Jamali F, Awidi A. A comparative study of the capability of MSCs isolated from different human tissue sources to differentiate into neuronal stem cells and dopaminergic-like cells. *Peer J*. 2022;10:e13003.
36. Wu S, Zhao Y, Zhang Z, Zuo C, Wu H, Liu Y. The Advances and applications of characterization technique for exosomes: from dynamic light scattering to super-resolution imaging technology. *Photonics*. 2024;11(2):101.
37. Dilsiz N. Technology. Exosomes as new generation vehicles for drug delivery systems. *J Drug Deliv Sci Technol*. 2024;95:105562.
38. Huang P, Ma H, Cao Y, Zhan T, Zhang T, Wang X, et al. Activation of primary hepatic stellate cells and liver fibrosis induced by targeting TGF- β 1/Smad signaling in schistosomiasis in mice. *Parasit Vectors*. 2022;15(1):456.
39. Afarin R, Behdarvand T, Shakerian E, Bavarsad SS, Rashidi M. Exosomes of Whartons' jelly mesenchymal stem cell reduce the NOX genes in TGF- β -induced hepatic fibrosis. *Iran J Basic Med Sci*. 2022;25(12):1498-1503.
40. Wang XL, Yang M, Wang Y. Roles of transforming growth factor- β signaling in liver disease. *World J Hepatol*. 2024;16(7):973-9.
41. Jaberian Asl B, Monjezi S, Orak G, Ghaffari F, Salehipour Bavarsad S, Dinarvand N, et al. The up-regulation of miR-146a and miR-29b via exosomes protects against liver fibrosis by inhibiting the TGF- β /Smad3c signaling pathway. *Hepat Mon*. 2024;24(1):e144095.
42. Yu HR, Huang HC, Chen IL, Li SC. Exosomes secreted by Wharton's jelly-derived mesenchymal stem cells promote the ability of cell proliferation and migration for keratinocyte. *Int J Mol Sci*. 2024;25(9):4758.
43. Wang S, Chen Y, Lei G, Ma X, An L, Wang H, et al. Serum exosome-derived microRNA-193a-5p and miR-381-3p regulate AMPK/TGF- β /Smad2/3 signaling pathway and promote fibrogenesis. *Clin Transl Gastroenterol*. 2024;15(2):e00662.
44. Wang R, Shi Y, Lv Y, Xie C, Hu Y. The novel insights of epithelial-derived exosomes in various fibrotic diseases. *Biomed Pharmacother*. 2024;174:116591.
45. Yao Y, Chen R, Wang G, Zhang Y, Liu F. Exosomes derived from mesenchymal stem cells reverse EMT via TGF- β 1/Smad pathway and promote repair of damaged endometrium. *Stem Cell Res Ther*. 2019;10(1):225.
46. Zhu QJ, Zhu M, Xu XX, Meng XM, Wu YG. Exosomes from high glucose-treated macrophages activate glomerular mesangial cells via TGF- β 1/Smad3 pathway in vivo and in vitro. *FASEB J*. 2019;33(8):9279-90.