**RESEARCH ARTICLE**

# Mesenchymal stem cells versus their extracellular vesicles in treatment of liver fibrosis: Is it possible to compare?

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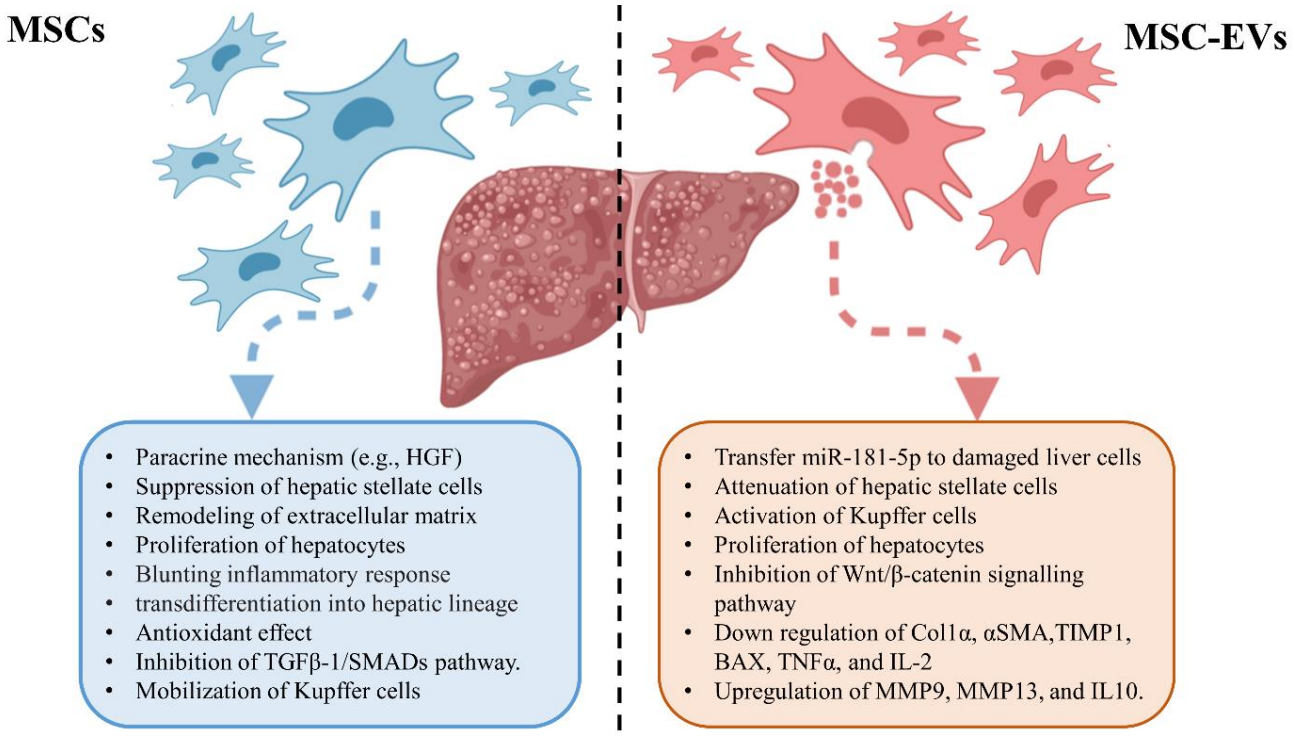
**Abstract**

Liver fibrosis (LF) is a worldwide health problem that is associated with a range of complications and high mortality. Due to the scarcity of liver donors, mesenchymal stem cell (MSC) therapy emerged as an alternative therapeutic strategy. However, it is widely accepted that most of the transplanted MSCs exhibit their therapeutic impact mainly via a bystander paracrine (medicinal) capacity. In addition to their secretory proteins, MSCs also produce various types of extracellular vesicles (EVs) that are classified into three main subtypes: microvesicles, exosomes and apoptotic bodies. Thanks to their peculiar cargo composition (e.g., proteins, lipids, and nucleic acids), EVs serve as an advantageous candidate for cell-free therapy. Recently, MSC-derived EVs (MSC-EVs) have gained the podium due to their regenerative and immunomodulatory effect. In mitigation/treatment of LF, a plethora of recent studies have shown the anti-inflammatory, anti-fibrotic and cytoprotective effects of both MSCs and MSC-EVs in various in vitro and in vivo models of LF. However, despite the limited evidence, we sought in this mini review to sort out the established data and formulate several challenging questions that must be answered to pave the way for further clinical applications. One of the major questions to ask is "Which is the best therapeutic approach, MSCs or MSC-EVs?" We tried to highlight how difficult it might be to compare the two approaches while our understanding of both candidates is still deficient. Among the major obstacles against such comparison is the inaccurate equivalent dose determination, the unknown in vivo behavior, and the undetermined lifespan/fate of each. Currently, the fields of MSCs and MSC-EVs seem to be rich in ideas but lacking in appropriate technologies to test these ideas. Nevertheless, continuous efforts are likely to help resolve some of the challenges listed here.

**Keywords:** Mesenchymal stem cells; Extracellular vesicles; Exosomes; Cirrhosis; Cell-free therapy

**Graphical abstract**




**List of abbreviations**

| | |
|---|---|
| AD | Adipose-derived |
| AFP | Alpha-fetoprotein |
| ALB | Albumin |
| ASCs | Adipose derived stromal cells |
| BA | Biliary atresia |
| BDL | Bile duct ligation |
| BM | Bone marrow |
| CBDL | Common bile duct ligation |
| $CCl_4$ | Carbon tetrachloride |
| CFU-F | Colony forming unit fibroblast |
| CK18 | Cytokeratin 18 |
| CM-DiI | Chloromethyl-DiI |
| c-Met | Mesenchymal epithelial transition factor–phosphorylated type |
| DLS | Dynamic light scattering |
| DMN | Dimethyl nitrosamine |
| ECM | Extracellular matrix |
| EGFP | Enhanced green fluorescence protein |
| EMT | Epithelial mesenchymal transition |
| EVs | Extracellular vesicles |
| Ex | Exosomes |
| GSH | Glutathione |
| h | Human |
| hAm-MSCs | human amnion-derived mesenchymal stem cells |
| Hep Par 1 | Human Hepatocyte Paraffin 1 |
| HGF | Hepatic growth factor |
| HNF-4 | Hepatocyte Nuclear Factor 4 |
| HSCs | Hepatic stellate cells |
| hUC | human umbilical cord |
| hUCPVCs | Human umbilical cord perivascular cells |
| IH | Intrahepatic |
| IHC | Immunohistochemistry |
| IP | Intraperitoneal |
| IS | Intrasplenic |
| IV | Intravenous |
| KC | Kupffer cell |
| LPS | Lipopolysaccharide |
| M1 | Macrophages 1 |
| MDA | Malondialdehyde |

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|---|---|
| miR | Micro RNA |
| MMP | Matrix metalloproteinases |
| MSCs | Mesenchymal stem cells |
| MVs | Microvesicles |
| NTA | Nanoparticle Tracking Analysis |
| PKH dye | Paul Karl Horan dye |
| SC | Subcutaneous |
| SMA | Smooth muscle actin |
| SOD | Superoxide dismutase |
| SPIO | Superparamagnetic iron oxide |
| SRY | Sex-determining region Y |
| TAA | Thioacetamide |
| TGF-β | Transforming growth factor-β |
| TIMP | Tissue inhibitor of metalloproteinases |
| TS | Trans-splenic |
| UC | Umbilical cord |
| WB | Western blot |

## 1. Background

Significant liver fibrosis (LF) is the eventual fate of all chronic liver injuries, mainly impacting younger patients who usually perish due to the sequelae of liver failure, portal hypertension, and cirrhosis-induced hepatocellular carcinoma. Among other etiologies, LF is impeded by hepatitis B and C, iron overload, biliary obstruction, and autoimmune liver disease.

Worldwide, liver cirrhosis and liver cancer represent the 11$^{th}$ and the 16$^{th}$ most common cause of death, respectively. Together, they account for 3.5% of all worldwide demises [1]. Thus far, liver transplantation seems to be the only effective remedy for patients with decompensated liver disease. Nevertheless, the scarcity of donors in many countries has highlighted the need for alternative therapies. Stem cells have shown great potential in liver injury repair and disease treatment, despite various pressing issues to be resolved before the clinical application.

Among the various of stem cell types, MSCs have become a popular research topic thanks to their ethical and safety profile, easy isolation, large-scale expansion, and paracrine activity. MSCs have been isolated from various tissue types, for instance; bone marrow (BM),[2] adipose tissue,[3] amniotic fluid,[4] amniotic membrane,[5] dental tissues,[6] endometrium,[7] menstrual blood,[8] peripheral blood,[9] placenta,[2] fetal membrane,[10] salivary glands,[11] skin,[12] sub-amniotic umbilical cord lining membrane,[3] synovial fluid and Wharton's jelly. Therefore, MSCs are considered to be a promising candidate for regenerative medicine. Many studies displayed the effect of MSC-based therapy in a variety of disorders such as, acute myocardial infarction,[13] stroke,[14] liver cirrhosis,[15] amyotrophic lateral sclerosis,[16] graft-versus-host disease,[17] solid organ transplant rejection,[18] chronic wound healing [19], bone repair [20], and autoimmune disorders.

MSCs are well known of their ability to differentiate into various mesodermal lineage cells (e.g., osteoblasts, chondroblasts, adipocytes, myocytes, and tenocytes), they can probably generate cells of other lineages, endodermal (e.g., hepatocytes, enterocytes, and islet cells), and ectodermal (e.g., epithelial, glial, and neural cells) [21].

For decades, MSC-based therapies were believed to boost the structure and function of injured/diseased tissues by means of direct cell replacement. Nevertheless, it soon became clear that a relatively limited number of MSCs were eventually retained at the sites of injury, suggesting their bystander paracrine capacity. [22] Consequently, a conceptual shift has confirmed that MSCs enhance tissue repair mainly thru their paracrine factors, cell-to-cell communication or various types of extracellular vesicles (EVs). [23]

 

However, a major question remains to be answered: Is it possible to determine which approach is more effective for MSC therapy, cell-based or cell-free? In other words, in terms of LF therapeutics, which way to go, MSCs or their EVs (MSC-EVs)?

In this review, we will discuss both choices in light of recent publications and try to see if it is possible to answer such pressing question.

## 2. MSC-based cell therapy for LF

Many studies investigated the therapeutic effects of MSCs on LF (as seen in table 1), albeit the underlying mechanisms are not fully understood. Many studies showed that MSCs act mainly through paracrine mechanisms rather than trans-differentiation. The paracrine mechanism is the secretion of soluble factors and the transmission of microvesicles containing proteins, mRNAs, genes and miRNAs. [23] One of the secreted factors is hepatic growth factor (HGF), which displays antiapoptotic activity in hepatocytes and acts as an important part in the regeneration of the liver. [24-26] In addition, the antifibrotic effects of MSCs can be a result of a combined set of mechanisms including modulation of immune system, inhibition of transforming growth factor-β (TGF-β) mediated differentiation of hepatic stellate cells (HSCs) into myofibroblasts, [25,27,28] oxidative stress inhibition, and matrix remodeling. [29,30]

Various studies suggested that MSCs depict an antifibrotic effect through up-regulation of matrix metalloproteinase (MMP-13) and down-regulation of tissue inhibitor metalloproteinase (TIMP-1). [31,32] It was also stated that the increased levels of hepatic glutathione (GSH) and superoxide dismutase (SOD) and decrease of malondialdehyde (MDA) following MSCs injection suggested their possible therapeutic role involving an antioxidant effect. [33,34]

In addition, MSC therapy has an immunosuppressive effect, thru up-regulating anti-inflammatory T regulatory cells and reducing pro-inflammatory T-helper (1 and 17) cells. It was noted that the gene expression levels of IL-1β, IL-6, and INF-γ were reduced. [35-38]

One of the suggested effects of MSCs therapy in LF is their transdifferentiation into hepatic progenitor cells or hepatocytes. This was confirmed by loss of CK90 expression, increased hepatic HGF, MMP-2 mRNA and CK19 mRNA.[33,39] A possibility of increased binding between HGF and its receptor; mesenchymal epithelial transition factor–phosphorylated type (c-Met) in the liver after MSC transplantation was also reported, leading to liver cell proliferation. [40] On the other side, it has been displayed that MSCs could promote the conversion of Kupffer cells (KCs) from the (M1) macrophage phenotype to the M2. The M2 macrophages are known to secrete IL-10 leading to apoptosis of M1 macrophages and subsequently recovery of liver insult. [41]

Interestingly, MSCs are capable of homing to sites of injury and inflammation. However, the dynamics and molecular mechanisms of MSCs trafficking to sites of injury are not fully understood which makes a barrier to clinical translation of MSC-based therapies. [42] It could be of utmost importance to trace the transplanted MSCs in vivo and determine their short-term as well as long-term fate. Many studies have used the Sex-determining region Y (SRY) gene as a method for in vivo cell tracking. [24,28,29,31,32,41] Other studies detected human hepatocyte-like cells [human Hepatocyte Paraffin 1 (Hep Par 1), alpha-fetoprotein (AFP), cytokeratin 18 (CK18),

 

CK19, CK7, and albumin (ALB)] in non-human species. [25,27]

In addition, Transgenic mice expressing enhanced green fluorescence protein (EGFP) have been successfully used for MSCs tracking. [28] Fluorescence microscopy have been used to confirm the liver-specific homing of fluorescent MSCs labelling dyes. [35,38,39] The tracking of MSCs was also detected by double staining of anti-human specific nuclear antigen and anti-human albumin. [40] Another tracking methods was the use of superparamagnetic iron oxide (SPIO) nanoparticles-labelled MSCs, followed by their visualization in liver tissue by Prussian blue staining. Labelling with SPIO nanoparticles was considered to be a nontoxic and noninvasive method for MSCs tracking after injection. [29]

Concerning the administration route, the most used mode of MSC transplantation to date was the intravenous (IV) route. This mode of administration has revealed clinical effectiveness in different studies. [23] However, some studies reported that most of the intravenously administered MSCs settle in the lung microvasculature even in the absence of lung injury. In addition to the IV route, local infusion of MSCs into the portal vein comprises a convenient way for MSCs transplantation. This delivers a bigger number of cells to the injured liver, thus increasing their functional potential. Yet, numerous studies postulated that the transportal infusion of MSCs could lead to a temporary interruption to the hepatic blood flow. [43] In addition, the trans-splenic (TS) route has been used as a potential method of MSC administration. Unfortunately, only 1% of the transplanted cells were retained in the liver parenchyma. [29,39] Thus, all of the aforementioned transplantation avenues are still far from being considered ideal.

 

**Table (1): Effect of MSCs from different sources on LF**.

| Cell source | LF model | | Cell dose | | | Cell characterization (in vitro) | Cell tracking (in vivo) | Main results | Mechanism (s) of action | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|
| | Species | LF Induction | Cell Number | Injection Frequency | Injection route | | | | | |
| Rat BM-MSCs | Rats | IP $CCl_4$ (1 ul/g BW twice/week for 9 weeks) | 3 x $10^6$ | Single dose at week 9 | IV | • Positive for CD90<br>• Negative for CD45<br>• CFU-F assays | No tracking | • Regression of fibrous tissue deposition<br>• Attenuation of hepatocellular damage, structurally and functionally | • Paracrine mechanisms | [23] |
| hUC-MSCs | Hamsters | IP $CCl_4$ 100 µl/animal twice/week for 8 weeks | 3 x $10^6$ | Single dose at week 8 | IH | • Multi-differentiation potential<br>• Positive for CD44, CD90, CD73, CD105<br>• Dual expression of CD105/90<br>• Negative for HLA-DR, CD34 and<br>• CD45 | IHC for human hepatocyte-like cells | • Regression of fibrous tissue deposition<br>• Improved liver functions | • Paracrine mechanisms<br>• Suppression of hepatic stellate cells. | [25] |
| hUC-MSCs | Rats | SC $CCl_4$ 2 ml/kg (twice/ week for 7 weeks) | 5 x $10^6$ | Single dose at week 3 | IV | • Positive for CD90, CD105 and CD73<br>• Negative for CD34, CD19, CD11b, HLA-DR and CD45 | Immunohistochemically for human hepatocyte-like cells | Improved biochemical and histopathologic changes | • Suppression of hepatic stellate cells.<br>• Decreased collagen deposition<br>• Enhanced extracellular matrix remodeling *via* the up-regulation of MMP-13 and down-regulation of TIMP-1. | [27] |

 

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| Rat BM-MSCs | Rats | IP $CCl_4$ (1 ml/kg BW twice/week for 6 weeks) | 3 x $10^6$ | Single dose at week 6 | IV | • Positive for CD29 | SRY gene | Improved histopathological examination, biochemical and molecular findings. | • Up-regulation of MMP-1<br>• Down-regulation of TIMP-1 | 32 |
| Mice BM-MSCs | Mice | IP $CCl_4$ (7 ml/kg body weight twice/week for 12 weeks) | 1 x $10^6$ | Single dose at week 8 | IV | • Positive for CD90, CD29 and CD105<br>• Negative for CD45, CD34 and CD80 | • SRY<br>• EGFP transgenic mice | Regression of fibrous tissue deposition | • Paracrine mechanisms<br>• Immunomodulatory properties on hepatic stellate cells and native hepatocytes. | 28 |
| hUC-MSCs | Rats | Oral $CCl_4$ via gavage tube (first at a dose of 0.5 ml /kg of BW and then at 1 ml/kg each time twice/week for 8 weeks) | 5 x $10^5$ | Single dose at week 4 | IH | • Positive for CD44 and CD105, CD29, CD51, SH2 and SH3 | Double Staining of anti-human specific nuclear antigen and anti-human albumin | • Improved biochemical and histopathological parameters<br>• Reduced hepatic inflammation | Increased binding between HGF and its receptor (c-Met) leading to liver cell proliferation | 40 |
| Mice BM-MSCs | Mice | BA by rhesus rotavirus in neonatal mice (20 µl of 1.2x$10^5$ pfu/ml of RRV within 24 h of birth) | 1 x $10^6$ | Single dose on day 7 | IP | • Positive for CD5<br>• Negative for CD45, CD11b, | No tracking | Reduced MDA levels and increased GSH and SOD levels | Antioxidant effect | 34 |
| Rat BM-MSCs | Rats | SC $CCl_4$ (0.2 ml/100 g BW twice/ week for 6 weeks) | 1 x $10^7$ | Single dose at week 6 | IV | • Positive for CD29 and CD44 | GFP-labeled BM-MSCS | Enhancement of liver function, confirmed by histopathology | Blunted inflammatory response | 35 |
| Rat BM-MSCs | Rats | SC $CCl_4$ (0.2 ml/100 g BW | 3 x $10^6$ | Single dose at week 6 | IV | • Positive for CD29 | SRY | • Decrease in liver collagen gene expression | Paracrine mechanisms such as secretion of HGF, which shows | 24 |

 

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| | | twice/week for 6 weeks) | | | | | | • Decrease in hydroxyproline content | antiapoptotic activity in hepatocytes | |
| Rat BM-MSCs | Rats | 0.04% TAA in drinking water for 8 weeks | 2 x $10^6$ | Single dose at week 8 | TS | • Positive for CD90 | Cell Stalker-CSR | • Repair of damaged hepatocytes<br>• Intracellular glycogen restoration<br>• Regression of fibrous tissue deposition | In vivo transdifferentiation from MSCs to hepatic progenitor cells | [39] |
| Rat ASCs | Rats | SC $CCl_4$ (1.5 ml/kg twice/week for 12 weeks) | 5 x $10^6$ | Double dose at weeks 10 &12 | Portal vein | • Positive for CD73 and CD90<br>• Negative for CD45 | No tracking | histological regression of fibrous tissue deposition | Paracrine mechanisms such as HGF secretion | [26] |
| Rat BM-MSCs | Rats | BDL | 3 x $10^6$ | Single dose at week 4 | IV | • Positive for CD105 and CD90 and CD73<br>• Multi-differentiation potential | PKH-26 fluorescent dye | Restored liver functions and histological structure | • An antioxidant effect.<br>• Transdifferentiation of MSCs into hepatic lineage | [33] |
| Rat BM-MSCs | Rats | CBDL | 1 x $10^6$ | Single dose at week 2 | IV | • Positive for CD29, CD90, and CD106<br>• Negative for CD3, CD4 and CD25<br>• Multi-differentiation potential | GFP-labeled BM-MSCs | Decreased fibrosis scores | Blunted inflammatory response | [38] |
| Mice BM-MSCs | Mice | IP $CCl_4$ (1 ml/kg BW twice/week for 12 weeks) | 1 x $10^6$ | Single dose at week 12 | IV | • Cell morphology: spindle-shaped | No tracking | Regression of fibrous tissue deposition | Inhibition of TGFβ-1/SMADs pathway. | [30] |
| Mice BM-MSCs | Mice | IP $CCl_4$ (1 µl/g BW twice/week for 6 weeks) | 1 x $10^6$ | Twice/week for three weeks | IV | • Positive for CD29 and CD44 | No tracking | Regression of fibrous tissue deposition | Immunosuppressive and anti-inflammatory effects | [37] |

 

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| | | | | starting at week 6 | | • Negative for CD45 CD31 CD11b CD86 and CD135 and CD31 | | and inflammatory response | | |
| Rat BM-MSCs | Rats | IP $CCl_4$ (0.5 ml/kg BW twice/week for 4 weeks) | 3 x $10^6$ | Single dose at week 4 | IV | • Positive for CD29, CD73 AND CD105<br>• Negative for CD34, CD45 and MHC-II | SRY | Regression of fibrous tissue deposition in terms of liver functions and histological features. | Immunosuppressive and antioxidant effects | 31 |
| hUC-MSCs | Rats | IP DMN (10 ml/kg for three consecutive days/ week for 3 weeks) | 5 x $10^6$ | Single dose on day 7 | IV | • Positive for CD44, CD73, CD90 and CD105<br>• Negative for CD11b, CD19, CD34,<br>• CD45 and HLA-DR<br>• Multi differentiation potential | SRY | Alleviated liver fibrosis | Mobilization of KCs | 41 |
| Rat BM-MSCs | Rats | IP $CCl_4$ (1 ml/kg BW twice/week for 8 weeks) | 1 x $10^6$ | Single dose at week 8 | Portal vein | • Positive for CD90+, CD44+,<br>• Negative for CD34<br>• CFU-F assays | Prussian blue staining for SPIO labeled BM-MSCs | Regression of fibrous tissue deposition on histological and molecular levels. | • Inhibition of TGFβ-mediated cell differentiation into myofibroblasts<br>• ECM Remodeling | 29 |

 

## 3. Limitations of MSC-based cell therapy

Despite the plethora of studies presenting MSCs as heroic cells in treatment of LF, several critical concerns to be resolved before the establishment of MSC therapy, for instance: [1] The MSC source. Despite the numerous tissues from which MSCs are obtained, understanding what population is optimal for liver regeneration is still lacking. [2] The MSC dose (cell number, route and frequency of injection). A critical step in the development of any novel therapeutic agent is the optimal dosing. Some studies reported that the degree of tissue repair was proportionate to the dose of MSCs. Still, further studies are needed to establish an optimal therapeutic dose and route of injection. [3] The MSC fate in vivo. Once MSCs are transplanted, it is still arguable how many cells manage to engraft successfully into the fibrosed liver. Furthermore, MSC life span and performance (i.e., differentiation, proliferation, cell fusion, or paracrine secretion) in vivo are still questions without definitive answers. [4] The MSC tumorigenicity. Another limitation of MSC-based cell therapy was the debatable theories regarding their role in tumor pathogenesis. However, these effects were explained by many as a result of contamination of MSCs culture or the use of immune-deficient animal models. [29] On the other side, accumulating evidence revealed that MSC therapy can be a safe and effective strategy for LF.

## 4. MSC-EVs-based cell-free therapy for LF

EVs are broadly defined as lipid bilayer enclosed cargo of biomolecules released by cells into their surrounding microenvironment, and include particles described as ectosomes, exosomes, microvesicles, and apoptotic bodies, among others. The size of EVs vary widely from <50 nm to several micrometers in diameter. As well, their chemical compositions, and suggested functions depend mainly on their cell of origin and their process of production. [44] MSC-EVs derived from different MSCs origins, have been shown to ameliorate chronic LF in different animal models (as seen in table 2), mainly through the reduction of collagen deposition modulating the inflammatory response, and hepatic cell proliferation/survival.

The research community has consistently promoted the importance of RNA contents in EVs. For instance, EVs, exosomes in particular, derived from hUC-MSCs were able to reduce hepatic inflammation and collagen deposition in carbon tetrachloride-($CCl_4$-) induced LF. At a molecular level, the expression of collagen types I and III, and TGF-β transcripts was reduced by the administration of EVs. In Addition, EVs reduced epithelial-to-mesenchymal transition (EMT)-pathway by reducing Smad2 phosphorylation.[45]

The administration of EVs derived from murine adipose stem cells (ASCs) over-expressing miR-181-5p was found to reverse the $CCl_4$-induced liver injury and down-regulate fibrosis-promoting transcripts, such as collagen I, vimentin, alpha-smooth muscle actin (α-SMA), and fibronectin. In addition, in vitro miR-181-5p down-regulated signal transducer and activator of transcription (STAT)-3 and Bcl-2, thus suppressing HSCs' activation, and induced autophagy through the up-regulation of Beclin-1. [46]

Furthermore, EVs from amnion-derived MSCs (Am-MSC) attenuated fibrosis, KC number, and HSC activation. Am-MSC-EVs were shown to reduce the expression level of pro-inflammatory molecules, in vitro. TNF-α, IL-1-beta, and MCP-1, in KCs stimulated

 

with LPS were significantly reduced. In addition, the reduction of nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) transcriptional activity induced by LPS was demonstrated, basically through the inhibition of the phosphorylation of IkB-α and p65 by suppressing the earlier steps of the LPS/TLR4 signaling pathway.[47]

In another rat model of $CCl_4$-induced LF, EVs derived from hBM-MSCs have been shown to reduce HSC activation.[48] This latter effect has been linked to the inhibition of several genes in the Wnt signaling pathway, such as peroxisome proliferator-activated receptor (PPAR)-gamma, beta-catenin, WNT3a, and WNT10b. Moreover, the EV-treated animals exhibited reduced mRNA expression levels of inflammatory cytokines (e.g., IL-1, IL-2, IL-6, IL-8, IL-10, and TNF-α).[48] Similar findings were also demonstrated using murine BM-MSC-EVs on both histopathological and biochemical levels by down-regulating the expression of α-SMA.[23,49] Moreover, decrease in level of quantitative gene expression of TGF-β, collagen-1α, IL-1β was reported.[50]

In a thioacetamide (TAA)-induced chronic LF model in mice, antifibrotic effects of EVs obtained from hESC-derived MSCs has been reported. Further molecular analyses showed a concomitant up-regulation of matrix metalloproteinase (MMP) 9 and 13, anti-apoptotic genes, and anti-inflammatory cytokines (e.g., IL-10 and TGF-β), accompanied by down-regulation of collagen, α-SMA, and TIMP-1 transcripts, and of pro-apoptotic and pro-inflammatory genes (e.g., TNF-α and IL-2). [50]

One of the main drawbacks in the use of EV treatments is their rapid clearance from target organs and hence may reduce their efficiency. For this reason, Mardpour et al. [51] tested EVs encapsulated in polyethylene glycol macromeres (gel-EVs) in the treatment of TAA induced LF. Authors claimed that the gel-EVs accumulated in the liver and gradually released their content over a period of 1 month, as indicated by in vivo tracking experiments. The authors concluded that on both histological and molecular levels, gel –EVs had a superior anti-fibrotic, anti-apoptotic, and anti-inflammatory effect when compared with the free-EVs.[51]

EVs derived from human cord perivascular cells (hUCPVCs), an alternative source of MSCs, were also tested in a TAA-chronic liver injury model. In comparison to naïve hUCPVC-EVs, the hUCPVC-EVs over-expressing insulin growth factor 1 (IGF-1) showed a stronger anti-fibrotic effect and were the only group capable of reducing the activation of HSCs. [52]

 

**Table (2): Effect of MSCs –EVs from different sources on LF**.

| MSCs-EVs source | LF model | | MSCs-EVs dose | | | MSCs-EVs characterization (in vitro) | MSCs-EVs tracking (in vivo) | Main results | Mechanism(s) of action | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|
| | Species | LF induction | EVs concentration | Injection timing and Frequency | Injection route | | | | | |
| hUC-MSCs-EX | Mice | IP $CCl_4$ (0.6 mL/kg BW, twice within 1 week) | 250 mg hUCMSC-Ex in 330 mL PBS | Single dose at week 6 | IH | • BCA protein quantification<br>• Morphology: TEM<br>• WB: CD9, CD81 | cross linkable membrane dye, CM-Dil *In- vivo* imaging system | • Reduced collagen deposition (Masson's Trichrome)<br>• Inhibition of collagen mRNA<br>• Recovery of serum AST | Inhibition of EMT and reduction of TGF- β phosphorylation through inactivating TGF- β1/Smad signalling pathway Smad2 expression. | [45] |
| miR-181-5p modified murine ASCs-Ex | Mice | IP $CCl_4$ (0.05 ml/kg BW twice/week for 8 weeks.) | 0.4 µg/µl, 100 µl | Twice a week for 8 weeks concomitantly with $CCl_4$ | IS | • BCA protein quantification<br>• Morphology: TEM<br>• WB: CD63, CD81 | No tracking | • Down-regulation of collagen I, vimentin, α-SMA and fibronectin in liver<br>• Improvement of liver function | • Selective transfer of miR-181-5p to damaged liver cells<br>• In vitro activated autophagy and down regulated expression of fibrotic genes by inhibiting the STAT3/Bcl-2/Beclin 1 pathway. | [46] |

 

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| hAm-MSCs-EVs | Rat | IP $CCl_4$ (2 mL/kg BW twice/week for six weeks) | 20 µg/kg BW | Single dose at week 3 | IV | • Qubit protein assay<br>• Size distribution: qNano system<br>• Morphology: SEM<br>• WB: CD 81 | No tracking | • Decreased collagen deposition (Masson's trichrome)<br>• Decreased HSCs activation (α-SMA)<br>• Reduced inflammation (decrease number of CD $68^+$ KCs)<br>• Decreased levels of pro inflammotory and fibrotic cytokines (TNF-α, IL 1β and Il-6, and TGF- β. | Attenuating HSC and KC activation. | 47 |
| hBM-MSCs-Ex | Rat | IP $CCL_4$ (3 ml/kg BW twice/ week for 8 weeks) | 250 mg Ex | Single dose at week 8 | IV | • BCA protein quantification<br>• Morphology: TEM<br>• WB: CD9, CD63, CD81, TSG101, and Alix<br>• NTA concentration and size distribution | No tracking | • Reduced collagen deposition (Masson's trichrome and Sirus red)<br>• Reduced hepatic hydroxyproline<br>• Reduced oxidative stress (MDA)<br>• Reduced HSCs activation (α-SMA)<br>• Decreased levels of pro- inflammatory cytokines IL-1,2,6,8,10 & TNF-α)<br>• Increased proliferation (Ki-67 and HNF-4) | Inhibits Wnt/β-catenin signalling pathway:<br>• Stimulating hepatocyte regeneration<br>• Inhibiting HSCs | 48 |

 

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| hES-MSCs-EVs | Rat | IP TAA (200 mg/kg BW twice/ week for 16 weeks) | 350 µg EVs | Single dose at week 16 | IS | • BCA protein quantification<br>• Size and relative intensity: DLS<br>• Morphology: SEM<br>• WB: CD81, CD63, and TSG101 | PkH-26 red florescence linker membrane lipophilic dye | • Rreduced collagen deposition and density (Masson's trichrome)<br>• Decreased apoptosis (caspase density). | • Down-regulation of major contributors to fibrosis: *Col1α*, *α-SMA*, and *TIMP1*<br>• Up-regulation of key factors in degradation of ECM *MMP9*, *MMP13*<br>• Up-regulation of anti-apoptotic gene (*BCL-2*) and anti-inflammatory cytokines (*TGF-β1* and IL-10)<br>• Down-regulation of pro-apoptotic gene (*BAX*) and pro-inflammatory cytokines (TNFα and *IL-2*) | 50 |
| hES-MSCs –EVs | Rats | IP TAA (200 mg/kg BW twice/ week for 16 weeks) | 350 µg protein content | Single dose at week 16 (Free-EVs and hydrogel-laden EVs) | IP | • BCA protein quantification<br>• Size and relative intensity: DLS<br>• Morphology: SEM and TEM<br>• WB: TSG101, CD81, CD63, and CD9 | PKH-26 red florescence linker membrane lipophilic dye | • Reduced fibrosis (Masson'strichrome and IHC anti collagen)<br>• Reduced HSCs activation (α-SMA)<br>• Reduced inflammation and necrosis<br>• Reduced apoptosis (Caspase 3)<br>• Improved serum AL | • Inactivation of HSCs α-SMA on gene and protein levels<br>• Reduction in TIMPI increases degradation of ECM *MMP9* and *MMP13*<br>• Reduction of TNF-α and IL2 as pro-inflammatory cytokines and elevated levels of anti-inflammatory cytokine IL10<br>• Hydrogel has better results due to | 51 |

 

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| | | | | | | | | | sustained systemic delivery for up to 1 month | |
| hUCPVCs - EVs | Mice | IP TAA (0.2 mg/g BW, 3 times/week, for 8 weeks). | 15 µg EVs | 3 doses (one /5 days) at week 6 | IV | • Size and relative intensity: DLS<br>• Morphology: TEM<br>• Flow cytometry: CD63 and CD81 | No tracking | • Reduced collagen deposition (Sirus red COL1A2)<br>• Reduced HSCs activation (α-SMA)<br>• Reduced pro-fibrogenic cytokine TGF-β1 | • hUCPVCs overexpressing hIGF-I reduced gene expression of fibrogenic-related molecules<br>• Triggering hepatic macrophages to switch their phenotype towards anti-inflammatory phagocytes | 52 |
| MSCs-MV | Rat | SC $CCL_4$ (0.2ml/100 g BW twice/week for 6 weeks) | (4 µg/ml PBS) | Twice a week for 4 weeks at week 6 | IV | • Bradford protein quantification<br>• Morphology: TEM<br>• Size and relative intensity: DLS<br>• WB: CD63, CD81 and CD83 | No tracking | • Reduced collagen deposition and inflammation<br>• Increased serum albumin, VEGF<br>• Decreased serum ALT<br>• Decreased gene expression of TGF-β, collagen-1α, IL-1β. | • Anti-fibrotic<br>• Anti-inflammatory<br>• Pro-angiogenic properties | 49 |
| Rat BMSCs-EVs | Rat | IP $CCL_4$ (1 µl/g BW twice/week for 9 weeks) | 80 µg protein content | Single dose at week 9 | IV | • Lowry protein quantification<br>• Morphology: TEM<br>• Size and relative intensity: DLS | No tracking | • Decreased collagen deposition (Masson's trichrome)<br>• Decreased HSCs activation (α SMA)<br>• Decreased inflammation Improved liver regeneration<br>• Improved serum AST and ALT | • Anti-fibrotic effect induced by attenuating HSC activation<br>• Anti-inflammatory properties though paracrine action. | 23 |

 

## 5. Limitations of MSC-EVs-based cell-free therapy

Although MSC-EVs-based therapeutics for the treatment of LF seem to have a great potential, numerous challenges must be addressed before moving from benchtop research to bedside medicine. [1] First of which is defining the best cell source to obtain the EVs. Since EVs can be obtained from various stem cell sources, maintained in different culture conditions (e.g., hypoxia, growth factors), all of which may alter the EVs contents and thus influence their effects in tissue regeneration. Therefore, a direct comparison of the effect of EVs from different cell sources on LF is still required. [2] Developing an optimized method for EVs isolation that is highly reproducible and efficient with high yield of purified EVs represents another major challenge for the clinical application of EVs. [3] Establishing a better understanding of how EVs work and defining their active components is also required to identify which components are beneficial and which might be harmful. [4] A database of absorption, distribution, metabolism, and excretion of EVs should also be established in order to reach the maximum therapeutic potential and well-established dosage. [5] Most researchers address the beneficial effects of EVs, but the unknown negative effects have to be also clarified. [6] Moreover, better targeting mechanisms of EVs should be developed to decrease off-target effects. Obviously, further research is still necessary for methodology standardization and large-scale production to ensure continuous supply of high quality MSC-EVs with predictable and reproducible therapeutic effects.

## 6. Conclusion remarks

Due to the true unmet need for an optimal therapeutic avenue to mitigate the progression and manifestation of LF, a plethora of studies suggest that MSC-based cell therapy should be highly considered. In addition, one of the most impressive aspects of MSCs therapy for LF is the wide variety of their cell-free products that could be considered as promising candidates in pre-clinical and clinical research. More specifically, MSC-EVs are considered the next-generation cell-free therapeutics for uncurable diseases. Nonetheless, we are still far from a concrete understanding of the exact mechanism(s) behind any discerned improvement. Establishing real comprehension of their therapeutic benefits will enable the medical community to make more informed decisions as to whether MSCs or their EVs (MSC-EVs) could be a worthwhile choice for treatment of LF. In our recent study, [23] we tried to establish a provisional comparison between the two modalities in order to determine which can be a better option when it comes to LF. However, we were challenged by a huge number of unknowns that significantly hindered our goal. At the beginning, we had to set up an equivalent dose for both MSCs and MSC-EVs. Nevertheless, the ambiguity of the fate of both, in vivo, made it almost impossible to determine how many cells would be equivalent to a precise quantity of EVs. Moreover, one of the most adopted EVs quantification method is their "protein content equivalent", which is far from giving accurate and reliable results. Recently, thanks to the introduction of Nanoparticle Tracking Analysis (NTA), it is possible to make an estimate of the particle concentration and size distribution even in a polydisperse sample. However, being a relatively new technique, NTA is still undergoing active standardization which is a prerequisite for reproducibility and data interpretation Clearly, efficient commercialization of MSCs/MSC-EVs would offer an entirely new therapeutic paradigm in health care. Yet, unfortunately, and despite the encouraging

 

data obtained by either modality, a competent comparison is still out of reach.

## Declaration of Competing Interest

The authors have no commercial, proprietary, or financial interest

## Author Contributions

Conception and design of the study: N.A. Acquisition of data: N.A., D.M.R., Y.H.K, M.M. Drafting or revising the manuscript: N.A., M.M. All authors have approved the final article.

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