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Therapeutic potentials of Mesenchymal Stromal Cells-Derived Extracellular Vesicles in Liver Failure and Marginal Liver Graft Rehabilitation: A Scoping Review

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Abstract:

Liver failure includes distinct subgroups of diseases: Acute liver failure (ALF) without preexisting cirrhosis, acute-on-chronic liver failure (ACLF) (severe form of cirrhosis associated with organ failures and excess mortality), and liver fibrosis (LF). Inflammation plays a key role in ALF, LF, and more specifically in ACLF for which we have currently no treatment other than liver transplantation (LT). The increasing incidence of marginal liver grafts and the shortage of liver grafts require us to consider strategies to increase the quantity and quality of available liver grafts. Mesenchymal stromal cells (MSCs) have shown beneficial pleiotropic properties with limited translational potential due to the pitfalls associated with their cellular nature. MSC-derived extracellular vesicles (MSC-EVs) are innovative cell-free therapeutic for immunomodulation and regenerative purposes. MSC-EVs encompass further advantages: pleiotropic effects, low immunogenicity, storage stability, good safety profile, and possibility of bioengineering. Currently, no human studies explored the impact of MSC-EVs on liver disease but several preclinical studies highlighted their beneficial effects. In ALF and ACLF, data showed that MSC-EVs attenuate hepatic stellate cells activation, exert antioxidant, anti-inflammatory, anti-apoptosis, anti-ferroptosis properties, and also promote regeneration of the liver, autophagy, and improve metabolism through mitochondrial function recovery. In LF, MSC-EVs demonstrated anti-fibrotic properties associated with liver tissue regeneration. Normothermic-machine perfusion (NMP) combined with MSC-EVs represents an attractive therapy to improve liver regeneration before LT. Our review suggests a growing interest in MSC-EVs in liver failure, and gives an appealing insight into their development to rehabilitate marginal liver grafts through NMP.

Keywords: Mesenchymal stromal cells-derived extracellular vesicles, Liver transplantation, Acute liver failure, Liver fibrosis, Acute-on-chronic liver failure, Normothermic-machine perfusion

Abbreviations

EVs	Extracellular vesicles
ALF	Acute liver failure
APAP	Acetaminophen
BMSCs	Bone marrow-derived mesenchymal stromal cells
CCl₄	Carbon Tetrachloride
CD	Cluster of differentiation
D-GalN	D-galactosamine
DC	Decompensate cirrhosis
HSCs	Hepatic stellate cells
UCMSCs	Umbilical cord-derived mesenchymal stromal cells
IL-1β/6/8	Interleukin-1b β /6/8/10
IRI	Ischemia-reperfusion injury
LPS	Lipopolysaccharide
MELD	Model for end-stage liver disease
miRNA	Micro ribonucleid acid
mRNA	Messenger ribonucleid acid
MSC-EVs	Mesenchymal stromal cells-derived extracellular vesicles
MSCs	Mesenchymal stromal cells
NMP	Normothermic machine perfusion
LT	Liver transplantation
OS	Oxidative stress
SIRS	Systemic inflammatory response syndrome

Declarations

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Authors' contributions: Conceived and designed the study: AS, AM; collected the data: AS, AM; analyzed the study: AS, AM, PRD; drafted the manuscript: AS, AM, PRD, CG, OS, CT, FC, LA, JG. All authors have read and approved the manuscript.

Introduction

Acute liver failure (ALF) associates a severe liver dysfunction without pre-existing liver disease. ALF is a rare disease (with an incidence of 10 cases per million per year worldwide) with a heterogeneous presentation, multiple etiology (e.g. acetaminophen (APAP), hepatitis viruses, hepatic ischemia), and in several cases, a lethal outcome¹. Pathophysiology of ALF relies on injury induced by specific cause of ALF (e.g., APAP-induced liver injury) and on dysregulated inflammation associated with multiple organ failure². With more than 160 million people suffering from cirrhosis, and approximately 800 000 people dying each year from cirrhosis worldwide, cirrhosis can be considered a major cause of the global burden on health³. Pathophysiology associates chronic liver inflammation, remodeling of the healthy liver replaced by liver fibrosis and regenerative nodules. After an initial asymptomatic phase, cirrhosis evolved from compensated cirrhosis to decompensated cirrhosis (DC). In the case of a precipitating event, DC can evolve towards acute on chronic liver failure (ACLF)⁴ combining acute DC with at least 2 organ failures and significant excess mortality within 28 days of hospitalization^{5,6}. ACLF occurs in up to 40% of DC and carries high mortality ranging from 30% to 100% at 1 year, depending on severity⁷. Sepsis, alcoholic hepatitis, and digestive hemorrhage represent more than 90% of the precipitating event leading to ACLF⁸. Inflammatory signature in ACLF is characterized by a maladaptive systemic inflammatory response syndrome (SIRS) combining intense systemic inflammation and strong reactive oxygen species (ROS)-induced injury with a close relationship between the intensity of SIRS, the number of organ failures, and mortality on day 28⁹. SIRS appears therefore as a major therapeutic target of interest to limit the evolution of ACLF and improve patient survival⁴. There is currently no specific treatment for ACLF, and only organ support strategies might allow bringing selected patients to liver transplantation (LT), the only curative option for improving the survival of ACLF patients¹⁰. However, access to LT remains restricted by a persistent graft shortage and a demographic shift in donor profile (obesity), increasing the incidence of marginal graft. This has led countries to use extended criteria for liver donors (e.g., donation after circulatory death, elderly donors, and high-extended rate of steatosis) inducing an excess risk of ischemia-reperfusion injury (IRI) and primary graft dysfunction, both responsible for altered outcomes¹¹, and increased mortality¹². Aside from oxidative stress (OS), cell death, and metabolic disruptions, inflammation also plays a crucial role in IRI-induced liver damage by inducing immune activation with innate-adaptive intense immune crosstalk¹³. For those patients, mitigating inflammation and OS might improve liver

function and overall survival. Mesenchymal stromal cells (MSCs) are among the innovative immunomodulatory emerging therapies currently being explored. MSCs exert pleiotropic effects including anti-inflammatory, immunomodulatory, anti-fibrotic, pro-metabolomic, anti-apoptotic, and anti-oxidant properties representing a therapeutic option in liver injury¹⁴. Although a recent meta-analysis summarizing clinical studies using MSCs in liver diseases suggested that they were safe to use in this clinical setting¹⁵, pitfalls remain concerning the use of cell-based therapy (*e.g.* uncontrolled differentiation or vascular occlusion), which limits its development and clinical use¹⁶. Thus, MSC-derived extracellular vesicles (MSC-EVs) are opening up new opportunities¹⁷ as these entities naturally tend to mirror their parental cell properties in liver injury while circumventing the pitfalls inherent to the use of MSC. *In vitro* and *in vivo* studies demonstrated that MSC-EVs recapitulate the beneficial effects of MSC in reducing cell death and OS, improving hepatocyte function by enhancing autophagy, and mitigating cytokine storm¹⁸. This scoping review will describe the potential therapeutic effects of MSCs and MSC-EVs in ALF, liver fibrosis, ACLF, and rehabilitation of marginal liver grafts.

Material and methods

Eligibility criteria

We performed a scoping review by selecting the most relevant articles by combining "Mesenchymal stromal cells", "extracellular vesicles", "exosomes", "acute liver failure", "liver fibrosis", "acute-on-chronic liver failure", "liver transplantation", "liver graft rehabilitation". We have not taken any restrictions regarding the date or location of the publications. The final search occurred on December 12, 2022. We reviewed our findings to identify work relevant to MSC-EVs in liver injury.

Information sources

Our research is conducted in several databases such as PubMed, Web of Science, Cross Ref, and Google scholar.

Mesenchymal stromal cells in liver injury

General properties of mesenchymal stromal cells

MSCs are spindle-shaped cell adherent to plastic, showing multipotent differentiation capacities *in vitro*, and are characterized by a strong immunomodulation capacity towards key immune cells driving the pathogenesis of SIRS¹⁹. Main source tissues of MSCs are adipose tissue, bone marrow, placenta, or umbilical-cord tissues¹⁹. MSCs are well characterized *in vitro* by the expression of cluster of differentiation (CD) 73, CD90, and CD105, the lack of expression of non-mesenchymal cells (e.g., hematopoietic and endothelial cells) CD11 β , CD14, CD19, CD34, CD45, CD79 α , the weak expression of CD106, detectable of human leukocyte antigen-DR isotype. MSCs harbor multi-lineage differentiation potential into osteoblasts, adipocytes, or chondroblasts¹⁹. MSCs general properties are numerous with among others anti-inflammatory, immunomodulatory, anti-ROS, anti-apoptotic, pro-metabolomic, anti-fibrogenesis, and pro-regenerative properties²⁰. Immunomodulatory effects of MSCs are mainly paracrine, mediated by the secretion of soluble mediators, organelles, and extracellular vesicles (EVs) into their environment.¹⁴ Considering their pleiotropic properties (**Figure 1**), MSCs appear to be a therapeutic candidate of interest for inflammatory diseases and may have several interests for treating liver ALF, LF or ACLF but also in marginal liver grafts rehabilitation.

Mesenchymal stromal cells and acute liver failure

MSCs have been tested in 3 different types of animal models of ALF: surgical (i.e., partial hepatectomy), pharmacological (i.e., APAP, D-galactosamine (D-GalN)), or immune-mediated (i.e., Concanavalin A , viral hepatitis)²¹. In such preclinical models of ALF, MSCs improved survival, decreased hepatic cytolysis, icterus and inflammation²². MSCs exert antioxidant properties by enhancing super oxidase dismutase and antioxidant response²³. Altogether, MSCs showed huge potential to alleviate liver injury and improved the regeneration of liver tissues in ALF.

Mesenchymal stromal cells and liver fibrosis

MSCs have been evaluated in following LF models: i) surgical model (e.g., bile duct ligation); ii) chemical model (e.g., Carbon tetrachloride (CCL₄), thioacetamide); iii) immune-mediated damage (e.g., Concanavalin A); iv) alcohol-induced liver damage; v) diet-induced nonalcoholic steatohepatitis; vi) transgenic model; vii) and organoid liver fibrosis²⁴. In such preclinical

models of cirrhosis, MSCs attenuated the progression of fibrosis due to their immunomodulatory properties and could safely improve liver function²⁵.

Mesenchymal stromal cells and acute-on-chronic liver failure

Preclinical ACLF models typically include the first hit of chronic injury followed by a second hit of acute liver injury. For example, some authors described the ACLF model using CCl₄-induced fibrosis followed by multiple injections of lipopolysaccharide (LPS)/D-GalN to trigger acute liver injury or even the combination of bile duct ligation for fibrosis with LPS to trigger acute liver injury²⁶. In serum porcine, LPS/D-GalN mice ACLF models, intravenous administration of human umbilical cord-derived MSCs (UCMSCs) promoted liver repair, fibrosis resolution, and improved liver function²⁷. In a similar ACLF model, Liao *et al.* showed a higher 48-hour survival rate in the UCMSCs-treated group associated with reduced liver cytolysis, and improved liver histology²⁸.

Mesenchymal stromal cells and marginal liver grafts rehabilitation

Facing liver graft shortage and an increase in marginal liver grafts, researchers proposed several approaches to rehabilitate marginal grafts to improve patients' prognosis before and after LT. Recent studies based on animal models highlighted the potential role of normothermic machine perfusion (NMP) in shortening cold ischemia time, increasing organ preservation time, and optimizing organ rehabilitation and regeneration²⁹. Among therapies tested during NMP, MSCs showed pleiotropic properties to attenuate most of the liver graft IRI in which inflammation plays a key role³⁰. Recent studies explored the applications of MSCs during NMP, and four of them focused on liver rehabilitation during NMP (rat liver³¹⁻³³ or porcine liver³⁴). The administration of MSCs during NMP improved bile production, decreased hepatic cytolysis, improved liver regeneration, attenuated the level of mitochondrial damage, and enhanced the protective effect of NMP³⁵. Further evidence demonstrated the superiority of NMP plus MSCs to improve the liver graft quality, especially marginal liver grafts³². **Figure 2** highlights the therapeutic capabilities proposed by NMP combined with MSCs.

Clinical evaluation of mesenchymal stromal cells in human liver diseases

In ALF, clinical trials showed that MSCs administration was possible and safe. However, despite encouraging results, interpretation remains cautious due to the lack of patients and

the relative heterogeneity in their design³⁶. **In LF**, more than ten clinical studies evaluated the impact of MSC-based therapies in liver fibrosis with good safety³⁷. Infusion of bone marrow-derived mesenchymal stromal cells (BMSCs) in 9 cirrhotic patients in a randomized placebo-controlled phase I-II single-center study found a significant improvement in the model for end-stage liver disease (MELD) score³⁸. In two studies assessing the impact of BMSCs on alcoholic liver cirrhosis, results showed a good safety profile, an improvement in liver function, and a clear amelioration in liver fibrosis^{39,40}. UCMSCs improved liver function documented with enhanced serum albumin levels and reduced total bilirubin level⁴¹. **In ACLF**, a phase I/II trial evaluated the safety and efficacy of human UCMSCs involving 43 ACLF patients with hepatitis B infection. No significant side effects were observed in UCMSCs group, and UCMSCs increased survival rate, alleviated liver damage, and reduced MELD score⁴². In another trial, BMSCs infusion was safe in patients with ACLF (n=4)⁴³. Lin *et al.* showed that mortality was higher in the control group (n=54) than in the BMSCs group (n=56) in hepatitis B virus-related ACLF (37.0% versus 17.9%, p=0.02)⁴⁴. Moreover, a recent meta-analysis of 12 clinical studies showed a beneficial effect of MSCs on liver function in both LF and ACLF (including the MELD score, albumin levels, and coagulation function)⁴⁵. All these data show that the administration of MSCs in the clinical setting of patients with ALF, cirrhosis, or ACLF is feasible and safe. Several promising signals suggesting beneficial effects are emerging, but the number of well-conducted RCTs is still too limited to conclude that MSCs have a therapeutic effect in these indications.

Actual limitations of Mesenchymal stromal cell-based therapy

Despite a solid rationale, and these promising preclinical and clinical data, the translation and clinical use of MSC-based therapies remain problematic in several ways. **First**, chromosomal instability and senescence have been demonstrated, and there is a potential tumorigenic effect of MSC-based therapy that has raised concerns⁴⁶. However, no MSC-mediated cancers have been reported in human clinical studies³². **Second**, MSCs can also be infected by viruses and thus be the vectors of viral dissemination, although this risk has not been shown in clinical studies to date. **Third**, MSCs are often significantly expanded *in vitro* to obtain sufficient cells for infusion, which could reduce replicative potential, induce telomere attrition, reduce DNA polymerase and repair efficiency and induce genetic instability which could promote cell senescence and functional alterations⁴⁷. For clinical trials, cell culture usually lasts to the third or fourth passage to limit genetic instability⁴⁸.

Fourth, although MSCs are immune privileged due to a low immunogenicity profile, they are not protected from the risk of graft rejection and immune-related clearance⁴⁹. **Fifth**, the manufacturing process of MSCs remains not standardized and there is a myriad of steps introducing heterogeneity like donors characteristics (ages, comorbidity), tissue sources, cell isolation techniques, culture and preservation conditions, which could impact the quality and translational potential of MSC-based therapy¹⁹. **Sixth**, pharmacokinetics and pharmacodynamics remain unknown due to MSCs-related factors (e.g., donor age, number of culture passages), and non-MSCs-related factors (e.g., MSCs labeling, host cytotoxic response)⁵⁰. **Seventh**, since the manufacturing process is not yet standardized, it is still quite unrealistic to imagine such therapeutics outside of an expert center for cell-based therapies. Indeed, after expansion of the cells *in vitro*, it is necessary to cryopreserve the cells (frequently in liquid nitrogen) until needed to maintain their potency by slowing down the metabolic process and then thawing the cells at the patient's bedside before administration⁵¹. **Eighth**, during intravenous injection of MSCs, a large proportion of MSCs are trapped in the lung and could never reach the peripheral organs, notably the liver⁵², which explains in part the numerous studies evaluating direct intra-hepatic or intra-splenic injections^{53,54}.

For those all reasons, special attention has been paid to MSC-EVs as promising therapeutics agents mimicking the pleiotropic effects of MSCs and opening up new opportunities as a cell-free therapeutic approach in liver diseases¹⁷.

Mesenchymal stromal cells-derived extracellular vesicles in liver injury

Interest in the mesenchymal stromal cells-derived extracellular vesicles

Exosomes were first described in 1983⁵⁵ but were initially considered as cellular waste products. However, in the early 2000s, several studies emerged showing that these nanoparticles harbored a wide variety of bioactive molecules, such as micro ribonucleic acid (miRNA), messenger RNA (mRNA), cytokines, growth factors as well as lipid mediators and other proteins/peptides⁵⁶. EVs are phospholipid bilayer bound nanoscale vesicles (30-1000 nm with small EVs <200 nm and medium/large EVs >200 nm), released by a wide variety of cell types and include exosomes (30–150 nm), and microvesicles (100-1000 nm). Note that apoptotic bodies (500-5000 nm) and oncosomes (1-10 μ m) are other types of vesicles, related to EVs but not strictly belonging to the EVs size category⁵⁷. Together with soluble molecules and organelles, MSC-EVs compose the MSCs secretome. *In vitro* and *in vivo* studies demonstrated that MSC-EVs recapitulate the beneficial effects of MSCs in liver disease by reducing cell death and OS and ameliorating hepatocyte function by enhancing autophagy and mitigating cytokine¹⁸. MSC-EVs recapitulate the pleiotropic effects of MSCs by transferring their intra-cytoplasmic bioactive cargo (e.g., proteins, lipids, miRNA, mRNA) into target cells¹⁷ with a good immunocompatibility^{58,59}. This large body of data shows that MSC-EVs represent a crucial vectoring element of the paracrine therapeutic effect of MSCs. Thus, MSC-EVs could be an innovative therapeutic approach for both immunomodulation and regenerative purposes in liver diseases while circumventing the pitfalls inherent in the use of MSCs⁶⁰.

Compared to MSCs, MSC-EVs have several substantial advantages. **First** of all, they are not composed of nuclei (“cell-free-based” therapy) leading to a lack of self-replicate capability preventing an excess risk of cancer⁴⁶. **Secondly**, MSC-EVs are nanovesicles allowing to limit the morphological side effects of MSCs such as pulmonary trapping during intravenous injection with a potential risk of pulmonary embolism and right ventricle overload⁶¹. **Thirdly**, MSC-EVs are stable in long-term storage and easily usable in clinical practice, as an off-the-shelf medicinal products. Optimizing the lyophilization process of MSC-EVs could contribute

to generating long-term stable MSC-EVs formulations at 4°C, and improve MSC-EVs translation into the clinics⁶². MSC-EVs have been demonstrated stable during long-term storage at –80°C up to 6 months⁶³ and during short-term storage at 0-4°C (up to 8 days)⁶⁴. MSC-EVs are also more resistant than MSCs to damage caused by freeze-thaw cycles⁶⁵. **Fourthly**, MSC-EVs demonstrated a good safety profile in several clinical trials⁶⁶. For example, authors showed a good safety and feasibility of the administration of either nebulized or intravenous MSC-EVs in COVID-19 patients^{67,68}. **Finally**, MSC-EVs can be modified through bioengineering to be uploaded in specific molecules, carrying specific cargo⁶⁹ or focusing on selected molecular target⁷⁰.

We summarized in this review several aspects of MSC-EVs in ALF, liver fibrosis, ACLF, and rehabilitation of marginal liver grafts.

Mesenchymal stromal cells-derived extracellular vesicles and acute liver failure

In animal models of ALF, MSC-EVs improved liver function⁷¹ with a strong anti-inflammatory effect⁷². In this context, MSC-EVs have been shown to reduce hepatic cytolysis⁷³ and limit hepatocyte necrosis⁷⁴. These effects led to increasing survival in treated rodents in ALF⁷⁵. Concerning the underlying mechanisms mediating these effects, authors showed that MSC-EVs reduced apoptosis⁷⁶ by promoting autophagy⁷⁷ and limiting OS-induced liver damage⁷⁸. Pro-regenerative effects of MSC-EVs in ALF have also been highlighted⁷⁹. Recent studies highlighted the importance of restoring mitochondrial metabolic function in ALF⁷⁸. The use of MSC-EVs can inhibit ferroptosis and promote cell regeneration factors^{80,81}. All studies evaluating the effect of MSC-EVs in liver injury are summarized in **table 1**.

Mesenchymal stromal cells-derived extracellular vesicles and liver fibrosis

In several preclinical models of liver fibrosis, MSC-EVs have been shown to improve survival⁸², alleviate liver fibrosis, reduce liver inflammation, enhance liver regeneration, induce autophagy, and reduce collagen maturation⁸³. There is a clear decrease in liver cytolysis⁸⁴, a frequent decrease in proinflammatory cytokines (e.g., Tumor necrosis factor- α , IL-6, IL-1 β , Transforming growth factor- β)⁸⁵, and strong repression in pro-fibrotic genes⁸⁶. The modulation of innate immunity by blocking the activation of hepatic stellate cells (HSCs) and by decreasing the quantity of Kupffer cells appear to be a cornerstone of the mechanism of action of MSC-EVs⁸⁷. Authors showed that MSC-EVs inhibited HSCs and induced a

transition from the activated to the quiescent form of HSCs through the activation of several signaling pathways (i.e., Wnt/ β -catenin pathway⁸⁴). Although the underlying mechanisms remain still not fully understood, one hypothesis expressed by Bruno *et al.* is that MSC-EVs can alleviate liver fibrosis by restoring a physiological expression of miRNA in the liver down-regulating HSCs⁸³. **Table 2** summarized preclinical studies involving MSC-EVs use in liver fibrosis.

Mesenchymal stromal cells-derived extracellular vesicles and acute on chronic liver failure

There is currently only one study focusing on the impact of MSC-EVs in ACLF. Hepatic exosomal miRNAs exploration during MSC-EVs treatment revealed an elevation of several miRNAs (e.g., miR-20a-5p) involved in a strong reduction of inflammation (decreases IL-8 serum levels)⁸⁸. Our work has shown that this axis of research is currently underdeveloped and undergoing evaluation. It is a crucial innovative area to develop in the next few years to determine whether it is worth translating into the clinic.

Mesenchymal stromal cells-derived extracellular vesicles and rehabilitation of marginal liver graft

Several teams have successfully performed therapeutic interventions directly on human or animal liver grafts perfused on NMP⁸⁹ like small interfering RNA (siRNA) uptakes⁹⁰, defatting cocktails⁹¹, enkephalin⁹², or MSCs⁹³, as previously discussed. A recent study by Rigo *et al.* used human liver stromal cells (HLSC)-derived extracellular vesicles (HLSC-EVs) in an *ex vivo* NMP rat liver model⁹⁴. Findings showed that HLSC-EVs attenuated IRI with a decrease in hepatic cytolysis. They also elegantly demonstrated that MSC-EVs were efficiently internalized into hepatocytes⁹⁴. Moreover, human HLSC-EVs-based therapy during NMP in rats liver transplantation from donors after circulatory death reduced liver injury, enhanced liver regeneration, and limited necrosis extension⁹⁵. These two studies highlighted the efficiency and feasibility of EVs infusion during NMP procedure, although no study has yet evaluated the impact of human MSC-EVs in an *ex vivo* perfused large animal or human liver NMP model. We still do not know whether MSC-EVs can rehabilitate marginal grafts, allowing some grafts to be recovered and reconditioned to be eligible for LT and/or to improve the function of other marginal grafts to improve recipients' outcomes⁹⁶. This is a field of research that deserves to be explored in the coming years.

MSC-EVs in human studies

Regarding the clinical use of MSC-EVs, a dozen studies have been published and another dozen are in the process of including patients⁴⁹. Findings demonstrate the feasibility and safety of administering clinical-grade MSC-EVs to patients. None of these studies have focused on liver disease. Whether in ALF, cirrhosis, or ACLF, no clinical study testing MSC-EVs has yet been conducted to the best of our knowledge. The good safety records of clinical studies using MSCs and the compelling preclinical data concerning MSC-EVs in liver injury led us to expand the investigation of MSC-EVs in liver injury and to consider MSC-EVs as a promising future therapy. This is an innovative avenue of research, with a solid rationale that has yet to be consolidated. Several questions remain to be addressed before we can advance to the first phases of clinical development. **Figure 3**, summarizes the potential impact of MSC-EVs on liver injury

Discussion

When considering the clinical transfer of MSC-EV, several heterogeneities emerge at multiple levels that are discussed here, including the tissue of origin (bone marrow, adipose tissue, human umbilical cord for example), the culture conditions (hypoxia cell, stimulation by growth factors for example), the therapeutic dose, and the optimal mode of administration of MSC-EVs⁹⁷.

Manufacturing challenge. MSCs need to be expanded multiple times to generate enough cells to reach therapeutic doses of MSC-EVs. Scaling-up cell production involves costly large batch culture devices limiting its availability. Several bioreactors (e.g., three or two-dimensional bioreactors) are currently developed to allow large scale manufacturing of MSC-EVs⁹⁸.

MSC-EVs production. There is also considerable heterogeneity in the techniques used at each stage of MSC-EVs production, and the optimal techniques for expansion (e.g., 2D versus 3D, with or without microcarriers) and MSCs stress (e.g., hypoxia versus serum starvation), extraction, and purification of MSC-EVs (e.g., ultracentrifugation versus tangential flow filtration) have yet to be determined⁹⁹.

Best parental cells. The selection of the best tissue of origin and parental stromal cells as well as the qualitative and quantitative screening of MSC-EVs remains to be determined and should be standardized. Selection of the donors remains a question of importance due to the potential risk of spreading viruses and the poor quality of MSCs derived from specific patients³⁶.

Standardizing characterization. Although the International Society for Cell and Gene Therapy[®] recommendations have made progress towards standardization of the techniques used to characterize EVs, there is still some heterogeneity in the combination of characterization panels, including with different levels of priority, nanotracking analyzer counting, protein, and mRNA quantification, electron microscopy imaging, westernblotting, flow cytometry detected marker expression panel⁵⁷. This heterogeneity induces a potentially important variability in the therapeutic effects of the EVs used, rendering the comparison between studies challenging. The best-standardized potency assays also remain to be established to guarantee a certain level of clinical therapeutic effect.

Route of administration. While MSC-EVs are rapidly eliminated in the blood with a short plasma half-life¹⁰⁰, they are still detectable in the liver 24 hours after infusion¹⁰¹. Thus, the

best administration routes to expect conclusive results in human studies still need to be investigated (i.e., intravenous versus intrasplenic, portal, or intra-arterial injection)⁵⁷.

Standardizing the therapeutic dose. To achieve a similar therapeutic effect, the dose of MSC-EVs used in published studies is commonly higher than the equivalent dose of MSCs¹⁰². However, there is a broad range of therapeutic doses used in animal studies, spanning 0.1 to 250 µg of protein or 2.0×10^8 to 5.0×10^{11} particles per dose, assuming that 1 µg of protein equates to around 2.0×10^9 MSC-EVs¹⁰³. Optimal treatment regimen (single versus multiple injections) remains also to be defined.

Bioengineering of MSC-EVs. Further studies explored the impact of either EVs released from upstream pre-treated MSCs or downstream MSC-EVs modified after MSCs release. Indeed, due to their ability to carry many active molecules, MSCs-EVs can be exploited as drug delivery systems and can be modified (e.g., uploaded) to deliver specific therapeutic agents. MSCs-EVs can be viewed as cargo vectors with high stability in the bloodstream, with specific targeting capabilities, and the ability to cross physiological barriers by pre-treatment and/or gene modification¹⁰⁴. Studies using modified MSC-EVs in ALF, LF and, ACLF are summarized in the **Table 3**.

Conclusion and perspectives

Finally, EVs derived from several MSCs sources have confirmed beneficial properties such as anti-inflammatory, immunomodulatory, pro-regenerative, anti-apoptotic, antioxidant, anti-fibrotic, and pro-metabolomic effects in rodent models of ALF, ACLF, and LF. While circumventing critical pitfalls inherent to their parent cells, MSC-EVs offer a cell-free-based therapeutic approach for liver diseases by recapitulating the effects of MSCs. Evidence regarding the effects of MSC-EV in ACLF and marginal liver graft rehabilitation remains to be expanded. These are areas that have not yet been fully explored. Although good safety records of clinical trials testing MSC-EVs in other contexts than liver diseases have been published, the clinical impact of MSC-EVs on liver disease remains unknown and needs to be investigated. Some questions still need to be addressed such as the optimal tissue of origin of MSCs, MSC-EVs characterization, route of administration, therapeutic dose, cost of production, and mechanisms of action. The prospect of using bioengineered MSC-EVs as a carrier for targeted therapy is also attractive. Finally, there is still a long way before using MSC-EVs in daily life at the bedside and to be able to define a clear framework for using MSC-EVs in liver injury.

Figure Legends

Figure 1: Classical sources of mesenchymal stromal cells, general properties and perspective in liver diseases

Figure 2: Overview of exosome of mesenchymal stromal cells expected effects in liver diseases

Figure 3: Perspectives in graft rehabilitation

Table Legends

Table 1: Preclinical data of mesenchymal stromal cells-derived extracellular vesicles on acute liver failure, acute-on-chronic liver failure and ischemia-reperfusion liver injury.

Table 2: Preclinical data of mesenchymal stromal cells-derived extracellular vesicles on liver fibrosis.

Table 3: Bioengineering applications of mesenchymal stromal cells-derived extracellular vesicles in liver injury.

” Take-home message”:

Mesenchymal stromal cells-derived extracellular vesicles are a future innovative approach in acute liver failure, cirrhosis, acute-on-chronic liver failure, and rehabilitation of marginal liver graft due to their pleiotropic actions (e.g., anti-inflammatory, immunomodulatory, pro-regenerative, anti-apoptotic, antioxidant, anti-fibrotic, pro-metabolomic effects). Expanding preclinical findings and developing clinically relevant models are needed to confirm promising emerging results, and before advancing one step further their clinical translation into first clinical phases trials.

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