Research Article

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Regenerative Medicine

Human mesenchymal stromal cell lysates as a novel strategy to recover liver function

Aim: It is unknown if the beneficial effects of mesenchymal stromal cells (MSC) transplantation into the liver are dependent on their anchorage and differentiation into hepatocytes or rather the result of the release of stem cell intracellular content with hepatoprotector properties. **Materials & methods:** The effects of intact MSC transplantation were compared with the infusion of MSC lysates in an experimental rat model of acute liver failure. **Results:** A more powerful hepatoprotective and antiapoptotic effect was obtained after infusion of MSC lysates than intact MSC. Changes in IL-6 levels and miRNAs might explain the beneficial effects of MSC lysates. **Conclusion:** Infusion of MSC lysates show a better hepatoprotective effect than the transplantation of intact MSC.

Keywords: acute liver failure • apoptosis • cell lysates • cell therapy • mesenchymal stromal cells

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Morbidity and mortality of patients with hepatic pathologies have increased notably during the last decades and the number of liver transplants has increased progressively [1]. Currently, for some hepatic pathologies, such as acute liver injury or advanced cirrhosis, orthotopic liver transplantation is the only therapeutic option. However, its clinical use is limited due to poor long-term graft survival, donor organ shortage, high costs associated with the procedure and the requirement for lifelong regimen of immunosuppressors [2,3]. Cell therapies are an appealing alternative to repair and regenerate injured livers and over the last decade the information derived from stem cells research has increased notably. Part of this interest is due to the potential use of these cells as beneficial treatment contributing to regeneration of injured livers [4,5]. A large body of evidence demonstrates that several types of stem cells (embrionary, induced pluripotent stem or adults stem cells) can be differentiated *in vitro* [6–8] or *in vivo* [9,10] into hepatocytes.

In this regard, several questions remain unsolved, including what is the best source of stem cells for an effective therapy in hepatology or the mechanisms whereby these cells exert hepatoprotective effects. Notably, it is unknown whether the therapeutic effects of stem cells are due to an anchorage into the injured liver and their subsequent differentiation to functional cells or on the contrary the paracrine effects of these cells are responsible of these beneficial effects.

Bone marrow mesenchymal stromal cells (MSC) are considered highly suitable for stem cell therapies due to their relatively easy obtention process and their wide potential of differentiation toward many cell types. Besides, MSC may be obtained from the same patient and without genetic modifications [11] and they can be largely expanded until an appropriate number of cells is achieved. However, although several preclinical studies have reported the use of MSC to improve hepatic function [12–14], the underlying mechanisms for hepatoprotective effect remain unclear.

This study was aimed to compare the therapeutic potential of the transplant of intact human MSC or MSC cell lysates to

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restore hepatic function of rats with thioacetamide (TAA)-induced acute liver failure. We obtained data on molecular mechanisms underlying the hepatoprotective effects, including activation of antiapoptotic signaling, cell proliferation and miRNA content.

Materials & methods

Ethic statement

All animals care and experimental procedures were approved by Research and Ethics Committees of IMIBIC/Reina Sofia University Hospital in accordance with Directive 2010/63/EU of the European Parliament and with institutional guidelines for the care and use of laboratory animals and the Declaration of Helsinki. All human samples were collected after obtention of the corresponding signed informed consent.

Human MSC isolation

All research samples used in this study represented excess bone marrow collected at diagnosis or during routine follow-up of patients in the hematology service of the Hospital Universitario Reina Sofía. Bone marrow obtained by aspiration from the iliac crest of healthy donors was cultured in flasks (Falcon™; Becton and Dickinson Pharmigen, NJ, USA) seeding at 10 µl of total bone marrow cells/cm^2 with α-minimum essential medium (Sigma-Aldrich) supplemented with 2 mM L-glutamine (BioWhittaker, Switzerland), 15% fetal bovine serum (BioWhittaker), 100 U/ml penicillin (Penilevel laboratories ERN, Barcelona, Spain), 0.1 mg/ml streptomycin (Normon laboratories, Seville, Spain) and 1 ng/ml of b-FGF (Peprotech EC, London, UK). Cells were allowed to adhere for 48 h and nonadherent cells were washed out with 100 mM phosphate buffered saline (PBS) pH 7.4 (Sigma-Aldrich). After 48 h, α-minimum essential medium supplemented with 10% fetal bovine serum and 1 ng/ml b-FGF was added twice weekly. All cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. When adherent cells reached 90% confluence, they were detached with 0.25% trypsin-EDTA (BioWhittaker), washed twice with PBS and recovered by centrifugation at 1800 rpm for 5 min at 4°C. In addition, MSC cell lysates were also obtained from 3 × 10^6 intact MSC which were broken by sonication (at intensity medium 10 cycles of 30 s of sonication [Diagenode, Liege, Belgium] plus an incubation of 30 s in ice). The total absence of cellular viability was checked by the trypan blue test (Sigma-Aldrich) and 0.3 ml of cell lysate was prepared to be infused by portal vein as described below.

To reduce variability, therapy with intact MSC or lysate was used from a pool of four different male individuals. MSC were used between passages 2 and 4.

Immunophenotype analysis of MSC

According to the International Society of Cell Therapy, MSC have a characteristic immunophenotype expressing surface markers such as CD90, CD105 or CD73 and lacking expression of CD34 or CD45 [15]. In this study, the immunophenotype of the MSC was evaluated by incubation with human monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE). Anti-CD90-PE (Sigma-Aldrich), anti-CD34-FITC (Miltenyi Biotec, Germany), anti-CD45-PE (Sigma-Aldrich), anti-CD73-PE and anti-CD90-PE (BD Pharmigen, NJ, USA), anti-CD13-FITC (Beckman Coulter, Inc., CA, USA) and anti-CD105-FITC (R&D Systems, MN, USA) were used following manufacturer's instructions. Briefly, 5 × 10^4 cells were acquired by flow cytometry (FACSCalibur; Becton and Dickinson) excluding cellular debris in a side scatter/forward scatter dot plot and analyzed using CellQuest software. The percentage of positive cells was calculated after subtraction of background fluorescence as measured with the appropriate isotype control mouse PE-IgG2a and mouse FITC-IgG1 (Miltenyi Biotec).

Multipotent analysis of human MSC

Adipogenic and osteogenic differentiation of MSC were performed according to the described protocols by Casado-Diaz *et al.* [16]. Briefly, adipogenesis was performed by culturing human MSC for 10 days with an adipogenic medium (0.5 µM dexamethasone, 0.5 mM isobutylmethylxanthine, and 50 µM indomethacin; Sigma-Aldrich). Oil red staining was performed to detect fat accumulation in human MSC induced to adipocytes. After adipocyte differentiation, cells were fixed, washed with water and subsequently stained for 15–20 min with a mixture of 8.2 ml of 0.3% (w/v in isopropanol) of oil red plus 6.8 ml of distilled water. Cells were then washed with water and visualized under a light microscope (all chemicals from Sigma-Aldrich). Osteogenesis of human MSC was induced by treatment with 1 µM dexamethasone, 0.2 mM ascorbic acid and 10 mM β-glycerophosphate for 21 days. Alizarin red staining was carried out in order to detect mineralization and calcium deposits. Briefly, after 21 days, cells were washed with PBS, followed by fixation in 4% formaldehyde and 2% sucrose for 15 min. Fixation solution was removed and cells were rinsed with water and stained with 40 mM Alizarin-red (pH 4.2) for 20 min at room temperature. Stained cells were further processed by five rinses with water pH 7, followed by a wash in PBS with rotation. Stained

cultures were photographed. Representative images from three independent experiments are shown.

Experimental design

Female Wistar rats weighing 200–250 g (Animal Breeding Facility of the Reina Sofia University Hospital, Cordoba, Spain) were kept under controlled conditions of light (12 h light:12 h dark cycle) and temperature (22°C), and provided with food (Purina, Barcelona, Spain) and water *ad libitum*.

Acute liver failure in rats was induced through a single dose intraperitoneally (400 mg/kg weight) of TAA (Sigma-Aldrich), a hepatotoxic molecule that induces apoptosis and necrosis. To check the hepatotoxic effects of TAA, blood and hepatic samples were collected at 0, 12, 48 h and 4 days. Five rats per group were used in each time frame. Hematoxylin and eosin staining of liver sections and serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were evaluated. The hepatotoxic effect of TAA was observed 12 h later of TAA administration being maximal at 48 h. Four days later, a spontaneous reversion of hepatic injury was observed with a decrease of transaminases and changes in hepatic histology (see results section, Figure 1). Therefore, TAA-treated rats were used to compare the effects of MSC-based therapies using intact MSC or MSC cell lysates at 48 h of TAA administration. Both therapies started after 12 h

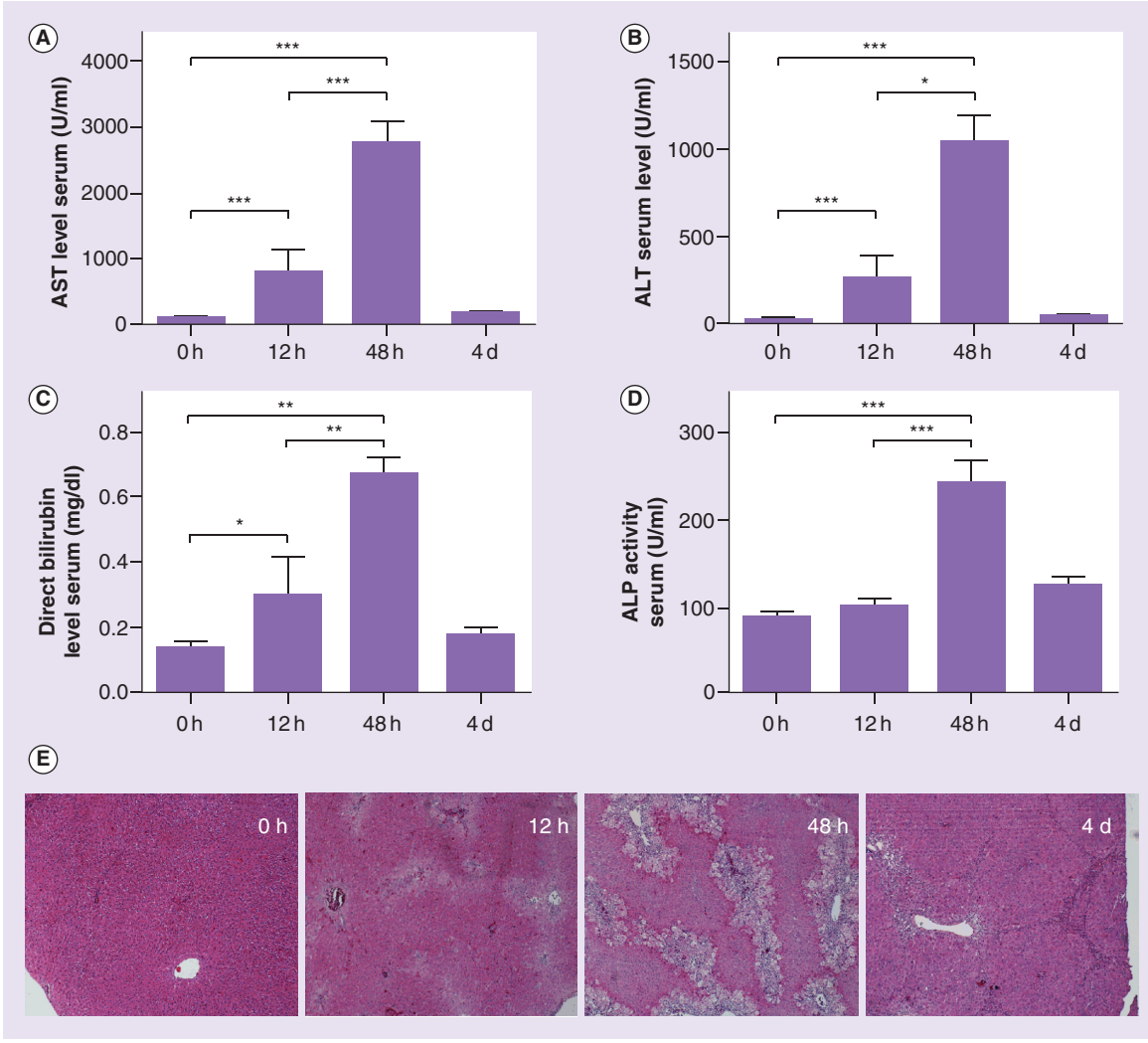

Figure 1. Thioacetamide administration induces hepatic injury in rats. A single dose of TAA 400 mg/kg body weight intraperitoneally increased the serum levels of **(A)** AST, **(B)** ALT, **(C)** direct bilirubin, **(D)** ALP and **(E)** liver hematoxylin and eosin staining showed necrotic areas after 12 h, revealing a bigger hepatotoxic effect after 48 h of TAA administration. Data represent the mean ± standard error.
***p < 0.001; **p < 0.01; *p < 0.05.
ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; d: Days; TAA: Thioacetamide.

of TAA injection. Figure 2 summarizes the followed protocol.

Surgical procedures

Rats were anesthetized with 3% sevofluorane and the surgical procedure was initiated by a midline ventral incision with exposure of the portal vein. Saline solution (TAA group), intact MSC (TAA+MSC group) or MSC cell lysates (TAA+Lys group) were infused into portal vein (n = 7 rats per group) in a final volume of 0.3 ml with a needle of 26G. In TAA+MSC group, 3×10^6 intact MSC were transplanted into portal vein of TAA-treated rats. In TAA+Lys group, cell lysate obtained from sonication of 3×10^6 MSC was infused. After infusion, a smooth pressure on portal vein was applied for 15–30 s to avoid bleeding. Prolene 6/0 was used for abdominal and skin closure. Fentanil (Ratiopharm, Madrid, Spain) (0.4 mg/kg weight) was used as analgesic agent.

In order to assess whether the effects of MSC were specific, human mononuclear cells (MNC) from peripheral blood were also transplanted into portal vein. Ficoll gradient was carried by centrifugation at 3000 rpm for 20 min at 4°C (without brake) and 3×10^6 MNC were collected and subsequently transplanted into portal vein of TAA-treated rats (TAA+MNC). No signs of possible rejection or complications such as pulmonary thrombosis were evidenced in any of the experimental groups.

Finally, 48 h after TAA administration, all animals were anesthetized with pentobarbital (100 mg/kg body weight) and sacrificed by abdominal aortic puncture. Blood was collected and the plasma and serum obtained were frozen at -20°C until the measurement of biochemical parameters. The liver was quickly removed, washed with cold saline solution and liver samples were stored at -80°C or fixed in formaldehyde 4% for biochemical or histological examination, respectively.

Parameters of hepatic function

Serum concentrations of AST, ALT, total bilirubin and alkaline phosphatase (ALP) were measured as indicators of hepatic injury according to standard diagnostic kits (Biosystems SA, Barcelona, Spain).

Western blot analysis

Blots were immunolabeled using specific antibodies and horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.) and revealed using the ECL Advance western blotting detection system (Amersham Biosciences, Uppsala, Sweden).

Real-time quantitative PCR analysis

Total RNA was extracted from liver samples with Trizol reagent (Sigma-Aldrich) and quantified by spectrophotometry (ND-1000; Nanodrop Technologies, DE, USA). A total of 1 µg RNA was treated with DNAse (DNAse kit; Sigma-Aldrich) and QuantiTect real-time PCR kit (Qiagen, Hilden, Germany) was used for cDNA synthesis. Real time-quantitative PCR was performed in a Light cycler 480 thermal system (LC480; Roche Diagnostic, Basel, Switzerland). The expression of rat *cyclin D1* was assessed with specific primers (sense 5′ GCACAACGCACTTTCTTTCC 3′, and antisense 5′TCTTCCTCCACTTCCCCTTC 3′), and the expression of the ribosomal *18S* gene (sense 5′ GTA ACC CGT TGA ACC CCA TT 3′, and antisense 5′CCA TCC AAT CGG TAG TAG CG 3′) was used as a housekeeping control. PCR primers were designed with the free Oligo 7 software. Quantification of relative gene expression was calculated by the $2^{(-\Delta ct)}$ method.

miRNA analysis

Comparative expression of miRNA-124a, miRNA-125b and miRNA-155 was carried out in MSC, MSC cell lysates and MNC from peripheral blood of healthy donor. Total RNA was extracted with TriZol reagent

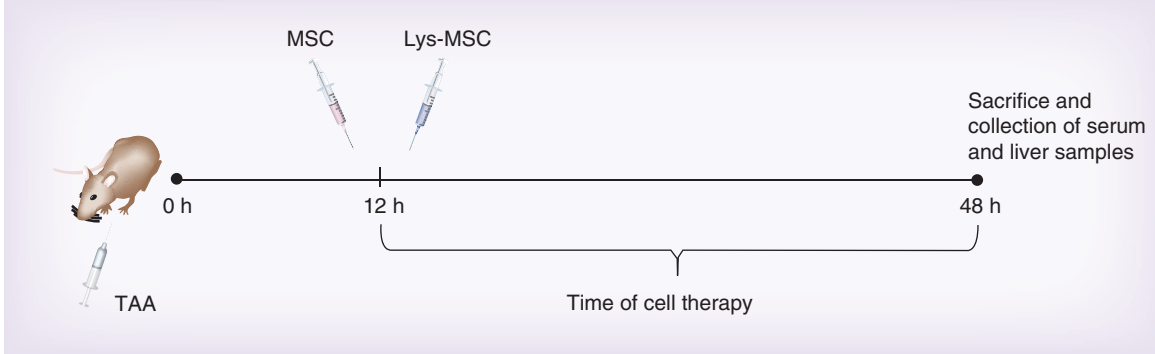

Figure 2. Experimental design. A total of 12 h later of TAA administration intact or Lys-MSC were infused by portal vein. Hepatic and serum samples were collected 36 h later of stem cell therapy.
Lys: Lysates; MSC: Mesenchymal stromal cells; TAA: Thioacetamide.

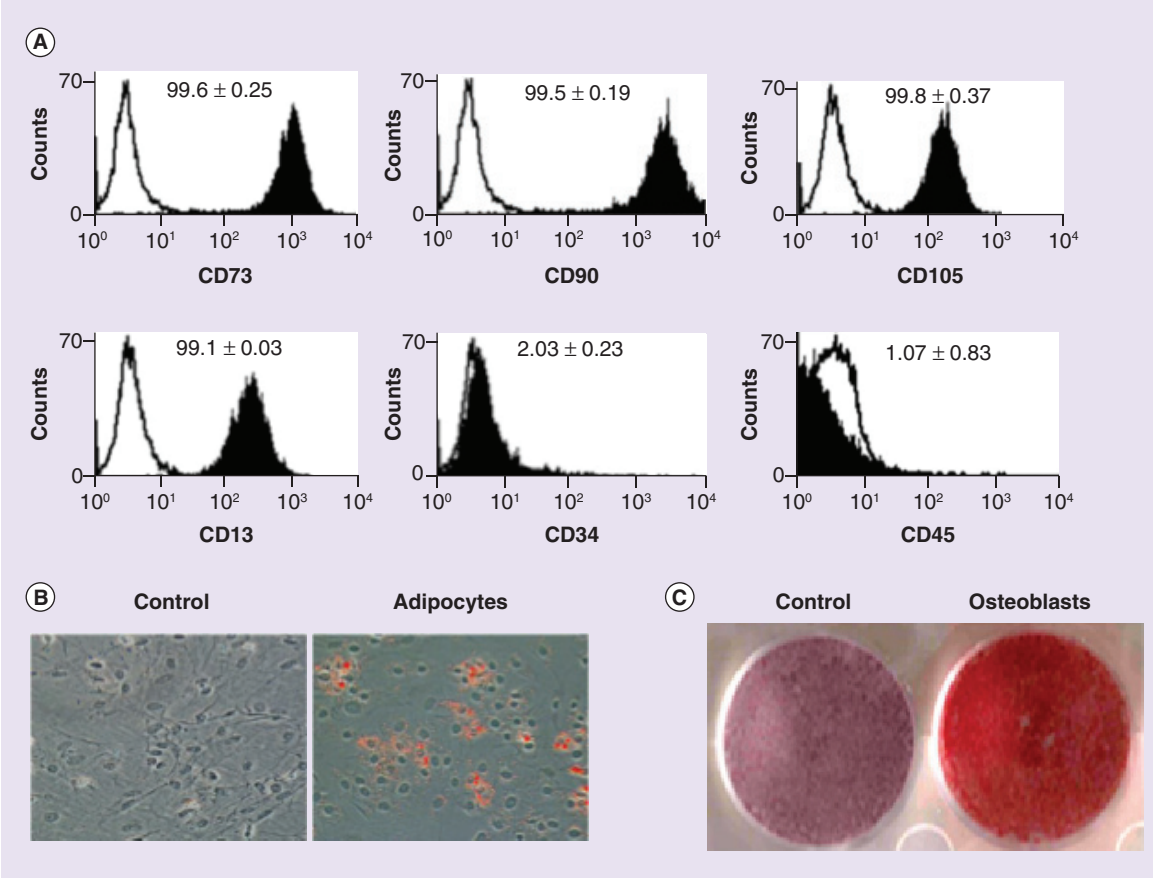

Figure 3. Immunophenotype and pluripotency analysis of mesenchymal stromal cells. (A) Immunophenotype of transplanted mesenchymal stromal cells was analyzed by flow cytometry. Image is representative of three independent experiments. **(B)** Human mesenchymal stromal cells were differentiated into osteoblasts. Alizarin red staining indicating mineralization and calcium deposits was carried out after 21 days with osteogenic stimulus and **(C)** adipocytes differentiation was observed after 14 days with adipogenic stimulus (lipid drops, red color). Please see color figure at www.futuremedicine.com/doi/full/10.2217/rme.14.59.

and miRNA expression was quantified by real-time quantitative PCR using Taqman probes (Qiagen) and a LC480 instrument. Data were analyzed comparatively using the $2^{(-\Delta ct)}$ method. U6 small nuclear RNA was used as housekeeping control for the normalization of miRNAs expression data. Results are shown as fold-changes with respect to MSC.

Immunohistochemical analysis

Liver biopsies were fixed in 4% formaldehyde, processed in graded alcohol, xylene and embedded in paraffin. These chemicals were purchased from Merck Sharp & Dohme, Madrid, Spain). Tissue sections (4 µm) were cut and hematoxylin and eosin, Masson's trichromic staining and immunohistochemistry for CD90, proliferating cell nuclear antigen (PCNA) and β-catenin were performed according to standard procedures. Immunohistochemistry analyses were carried out with a DakoCytomation staining kit (Glostrup, Denmark). Liver sections were dewaxed in xylene, rehydrated in ethanol and incubated at 100°C in ChemMate™ Target Retrieval Solution pH 6.0 (Dako, Barcelona, Spain) for 20 min. After washing in PBS, slides were incubated for 10 min in 3% hydrogen peroxide to block endogenous peroxidase. Then, slides were incubated with anti-human CD90 (Sigma-Aldrich), anti-rat PCNA (Santa Cruz Biotechnology, Inc.) and anti-rat β-catenin (Cell Signaling, MA, USA) antibodies for 30 min. Subsequently, slides were washed 5 min in PBS and incubated 30 min with a horseradish peroxidase-labeled polymer. Diaminobenzidine for 5 min was used to develop the brown color of positive areas. Finally, the slides were counterstained with hematoxylin and mounted in Eukitt mounting medium (Labobam, Navarra, Spain).

Apoptotic cell death assay

Apoptosis was measured using the cell death detection enzyme-linked immunosorbent assay plus (Roche Diagnostic, Barcelona, Spain) according to the manufacturer's instruction. Briefly, liver homogenates were incubated with both antihistone antibody labeled with

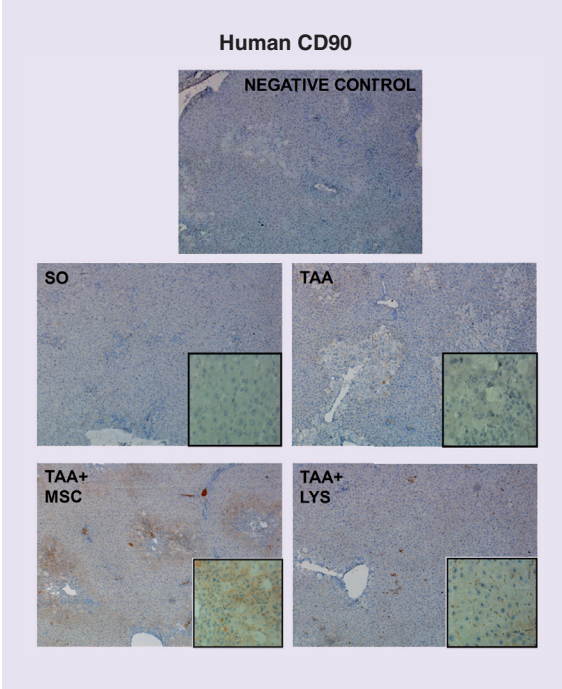

Figure 4. Presence of human CD90⁺ cells into the liver parenchyma of thioacetamide-treated rats.
Immunohistochemical analysis for the liver expression of human CD90 (a marker of human MSC) was carried out in the liver of rats after 48 h of TAA injection. Image is representative of the following groups: SO, TAA, TAA+MSC infusion, TAA+LYS (TAA plus cellular LYS of MSC) or only secondary antibody. Magnification: 5× and 40×.
LYS: Lysates; MSC: Mesenchymal stromal cells; SO: Sham-operated; TAA: Thioacetamide.

biotin and anti-DNA antibody conjugated with peroxidase in streptavidin-coated microplates, for 2 h. Microplates were washed and incubated with substrate for colorimetric measurement at wavelength 405 nm with reference at 490 nm.

Intracellular measurement of IL-6 levels

Intracellular levels of IL-6 were measured into cell lysates of MSC or MNC. IL-6 kit from BenderMedsystems (Vienna, Austria) was used according to manufacturer's instructions.

Statistical analysis

Results are expressed as means ± standard error. Comparisons were made using analysis of variance test. Statistical significance was set at $p \leq 0.05$. SPSS software was used for statistical analysis.

Results

TAA administration induces hepatic injury in rats

Twelve hours after TAA administration, liver injury serum markers such as AST, ALT, ALP and direct bilirubin were increased (Figure 1A–D). Histological changes revealed the presence of apoptotic cells and necrotic areas around central vein and in the hepatic parenchyma (Figure 1E). The greatest hepatotoxic effect of TAA was detected 48 h after TAA administration, with highest levels of transaminases, ALP and total bilirubin in serum samples, as well as with pronounced necrotic and apoptotic areas around central vein. Four days after TAA administration, the values of transaminases and the histology denoted a recovery of liver function. Therefore, the effect of cell therapy was evaluated 48 h after TAA administration when the greatest hepatic damage was found.

Immunophenotype & pluripotency of transplanted cells

Immunophenotype of transplanted cells into the portal vein of TAA-treated rats was analyzed by flow cytometry. Characteristic immunophenotype of human MSC was observed in these cells, positive for specific markers such as CD13⁺, CD73⁺, CD90⁺ and CD105⁺ and negative for CD45 and CD34 (Figure 3A). The pluripotentiality of MSC to differentiate into other cell types such as osteoblasts or adipocytes was confirmed through the presence of osteoblasts and matrix mineralization (Figure 3C) and adipocytes with specific lipid drops (Figure 3B).

Human MSC transplanted into rat portal vein localized into the liver

A total of 48 h after TAA administration, it was assessed if cells infused through portal vein engrafted into liver parenchyma. Expression of human CD90 antigen, a major marker of human MSC, was detected into the liver parenchyma of rats with human MSC infusion (TAA+MSC). Importantly, human CD90⁺ cells were localized around those necrotic areas induced by TAA (Figure 4). In TAA+Lys-treated rats, the human MSC marker was also detected with less intensity into hepatic parenchyma and sinusoids. Boxed areas in the right panel are shown magnified CD90 staining. CD90-positive staining detected after stem cell transplantation (MSC or Lys) was exclusively due to the presence of this human antigen (Figure 4). No positive staining for human CD90 was found in healthy or TAA-treated rats , confirming the human specificity of this antibody.

Transplantation of human MSC or infusion of MSC cell lysates improves hepatic function of TAA-treated rats

To assess the effects of both cell therapies (MSC or MSC cell lysates) on the liver function of TAA-treated rats, several parameters related to hepatic function were evaluated. A total of 48 h after TAA administration,

significant changes in ALT, AST, ALP and direct bilirubin were detected (Figure 5). Transplantation of intact MSC (TAA+MSC) significantly reduced the serum levels of hepatic injury markers. Comparatively, the infusion of MSC cell lysates decreased more efficiently transaminase levels than intact MSC (Figure 5). Serial sections for histological studies (hematoxylin and eosin, Masson's trichrome and β-catenin staining) corroborated the transaminase data. The images presented in Figure 6 clearly show that human MSC transplantation reduces the necrotic areas induced by TAA. As compared with intact MSC, the administration of MSC cell lysates (TAA+ Lys) was more efficient in the repairing of the hepatic architecture with less necrotic and apoptotic foci (Figure 6).

Under normal conditions, the presence of β-catenin is essential to maintain cell–cell junction in hepatic tissue. As shown in boxed areas, lower part of the panel, an evident loss of β-catenin in the plasmatic membrane of hepatocytes was observed in those necrotic and apoptotic areas induced by TAA. Notably, MSC transplantation (TAA+MSC) abolished this TAA-induced loss of β-catenin expression. Moreover, infusion of MSC cell lysates (TAA+Lys) was able to completely recover β-catenin expression in hepatocyte membranes, restoring the normal architecture of the hepatic liver parenchyma in liver-injured rats (Figure 6).

Transplantation of human MSC & the infusion of cellular lysates activates antiapoptotic pathways & decreases cell death

As expected, TAA administration clearly increased apoptosis compared with control rats (SO) (Figure 7A). This TAA-induced cell death was reduced after human

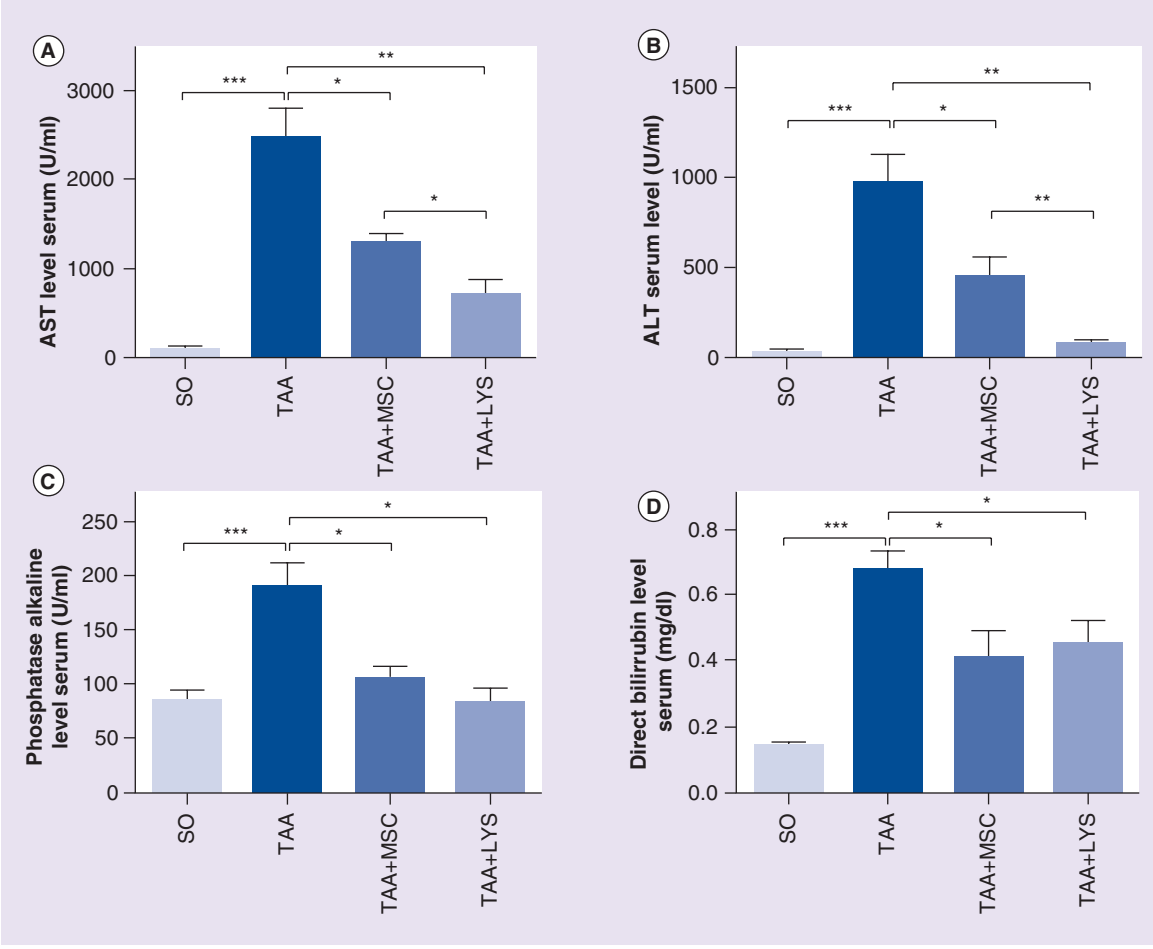

Figure 5. Transplantation of human mesenchymal stromal cells or the infusion of mesenchymal stromal cell lysates of mesenchymal stromal cells improves hepatic function of thioacetamide-treated rats. After 48 h of a single injection of TAA serum levels of **(A)** AST, **(B)** ALT, **(C)** direct bilirubin and **(D)** ALP were analyzed using colorimetric kits. Data represent the mean ± standard error.
***p < 0.001; **p < 0.01; *p < 0.05.
ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; LYS: Lysates; MSC: Mesenchymal stromal cells; SO: Sham-operated; TAA: Thioacetamide.

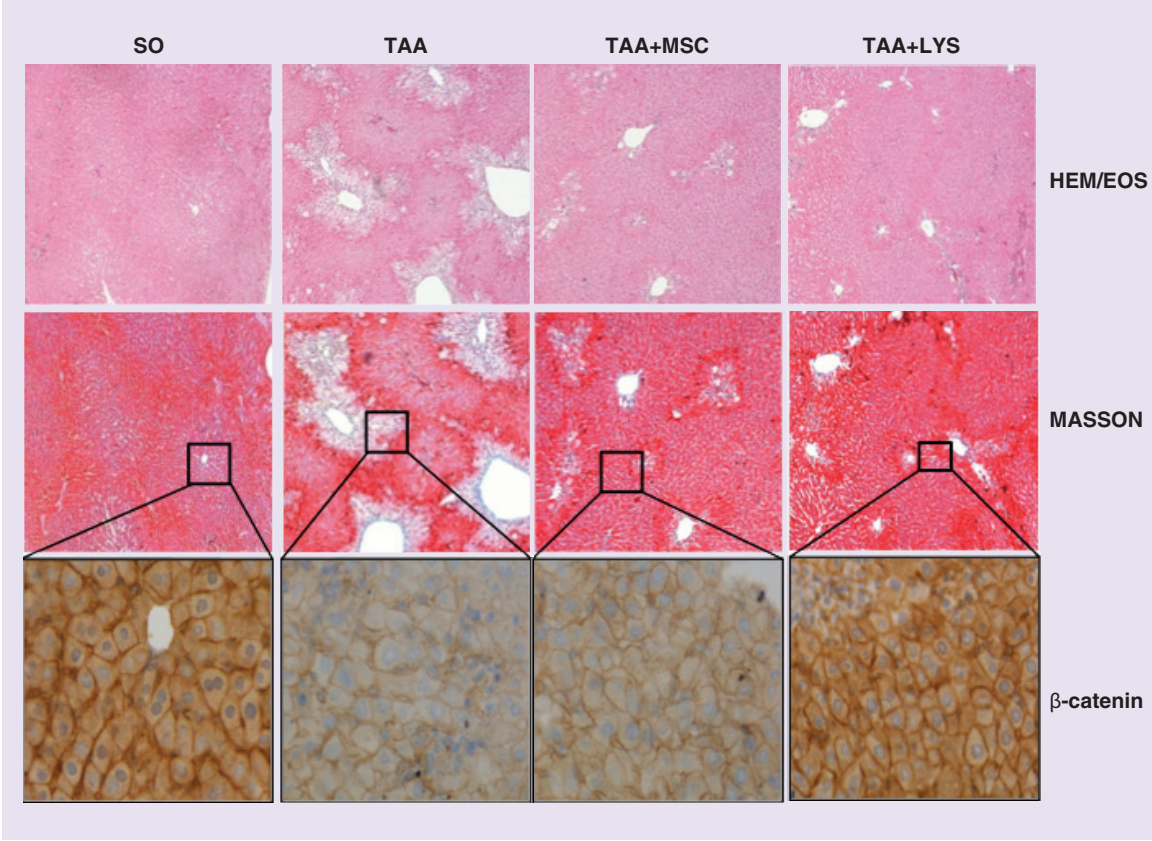

Figure 6. Transplantation of human mesenchymal stromal cells or the infusion of cellular lysates of mesenchymal stromal cells improves hepatic function of thioacetamide-treated rats. Serial histological studies of HEM/EOS, MASSON and immunohistochemical analysis for β-catenin were performed. Boxed areas in lower down panel are shown magnified (20×) of above panels (5×). Images are representative of each group. Magnification of HEM/EOS and MASSON was 5× and 20× for β-catenin.
HEM/EOS: Hematoxylin and eosin; LYS: Lysates; MASSON: Masson's trichromic staining; MSC: Mesenchymal stromal cells; SO: Sham-operated; TAA: Thioacetamide.

MSC transplantation (TAA+MSC) and after MSC cell lysate infusion (TAA+Lys). However, a significant lower apoptosis was found in TAA+Lys compared with TAA+MSC group ($p < 0.05$) (Figure 7A). The levels of NF-κB, a protein involved in cell survival, were reduced in TAA-treated rats. MSC transplantation or MSC cell lysate infusion into the portal vein of TAA-treated rats increased the expression of this antiapoptotic protein (Figure 7B). Moreover, the levels of IκBα protein, which is an endogenous inhibitor of NF-κB, were also decreased after both MSC cell therapies. Additionally, Akt signaling, which is a key antiapoptotic pathway, was activated after cell therapy. Thus, Akt phosphorylation was increased after infusion of intact cells (TAA+MSC) or cellular lysates of MSC (TAA+Lys) (Figure 7B).

Transplantation of human MSC & the infusion of cellular lysates from MSC stimulates hepatic proliferation

Hepatic proliferation was evaluated by quantification of *cyclin D1* mRNA expression. *Cyclin D1* is a specific regulator of G1/S phase during the cellular cycle. TAA administration did not affect *cyclin D1* expression as compared with control group; however, a significant increase in *cyclin D1* mRNA expression was observed after transplantation of human MSC or the infusion of MSC cell lysates (Figure 8A).

To confirm this increase in cell proliferation, we also determined the expression of PCNA, which is a nuclear protein that is only synthesized during cell division. As shown in Figure 8B, control and TAA-treated rats showed similar expression of PCNA; by contrast a higher positive nuclear staining for PCNA was observed after MSC transplantation (TAA+MSC) or infusion of MSC cell lysates (TAA+Lys). These results confirm that both modalities of cell therapy exerted a potent hepatoproliferative effect in TAA-treated rats.

Hepatoprotective effects are specific of human MSC

To verify the specificity of therapeutic benefits of MSC, human MNC were transplanted into rats with hepatic

damage induced by TAA. Human MNC infusion into portal vein was compared with MSC transplantation. MNC did not improve hepatic function as illustrated by the similar values of AST found both in TAA and TAA+MNC rats (Figure 9A). In addition, hematoxylin and eosin staining revealed similar necrotic areas around the central veins in TAA and TAA+MNC groups (Figure 9B).

In order to explain the differential effects of MSC and MNC infusion on the TAA-induced liver damage, the expression of different miRNAs involved in the regulation of apoptosis and NF-κB signaling activation were also analyzed. Results showed that in MSC, miRNA-124a and miRNA-125b were increased significantly as compared with MNC infusion (Figure 10A & B). Moreover, the expression of miRNA-155 in MSC was found to be half than that of MNC (Figure 10C). With respect to MSC, the content of miRNA-124a, miRNA-125b and miRNA-155 in lysates was significantly reduced ($p < 0.001$), although the levels of these miRNAs in lysates were still significantly different with respect to MNC ($p < 0.001$). In addition, the intracellular content of IL-6 was fivefold higher in MSC than in MNC (Figure 10D).

Discussion

This study was designed to analyze and compare in a rat model of acute liver failure the therapeutical potential of two different methods of delivering MSC. Our results support that both transplantation of intact MSC and the infusion of MSC cell lysates have a positive effect; however, in terms of liver function and antiapoptotic effects, the hepatoprotective impact obtained after the infusion of MSC cell lysates was more efficient than that obtained with transplantation of intact MSC. Compared with intact MSC, MSC cell lysates significantly decreased all parameters associated to hepatic dysfunction. Hematoxylin and eosin staining revealed the presence of large necrotic–apoptotic areas after TAA treatment and this hepatocellular damage affected both hepatocytes and cholangiocytes, suggesting that the high levels of bilirubin and ALP may be due to the destruction of bile ducts. These data agree with other published studies [17,18].

Our study shows for the first time the ability of cell lysate from MSC to induce a hepatoprotective effect, suggesting a potential therapeutic use of MSC cell lysates in the management of acute liver disease.

The beneficial effects of the infusion of cellular lysates of MSC found in our study are in agreement with other studies, suggesting a paracrine mechanism of hepatoprotection induced by MSC. In these studies, MSC-conditioned medium reduces and protects the liver in a model of acute injury induced by D-galactosamine [19,20]. Recently, Du *et al.* have also shown that MSC-conditioned medium reduces liver injury through an increase of hepatocellular proliferation, hepatic regeneration and a reduction of hepatic apoptosis [21]. Our results also show that MSC or MSC cell lysates recovered hepatic function through a decrease of apoptosis and massive hepatocellular death. Our data further support that the activation of NF-κB and Akt signaling pathways are involved in the increase of cell survival by favoring

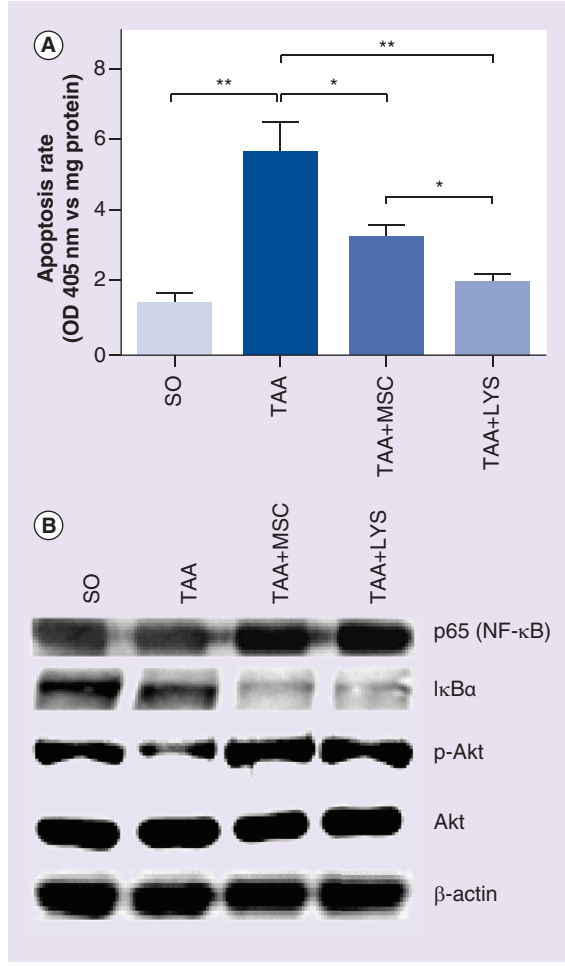

Figure 7. Transplantation of human mesenchymal stromal cells or the infusion of mesenchymal stromal cell lysates activates antiapoptotic pathways and decreases cell death. (A) The levels of apoptosis were measured by enzyme linked immunosorbent assay analysis for DNA fragments associated to cytosolic histones. Data represent the mean ± standard error. **(B)** p65 (from NF-κB), IκBα, phospho-Akt and Akt were analyzed by western blot in liver homogenates from the groups SO, TAA, TAA+MSC and TAA+LYS. β-actin was used as loading control. Image is representative of three independent experiments.
**$p < 0.01$; *$p < 0.05$.
LYS: Lysates; MSC: Mesenchymal stromal cells; OD: Optical density; SO: Sham-operated; TAA: Thioacetamide.

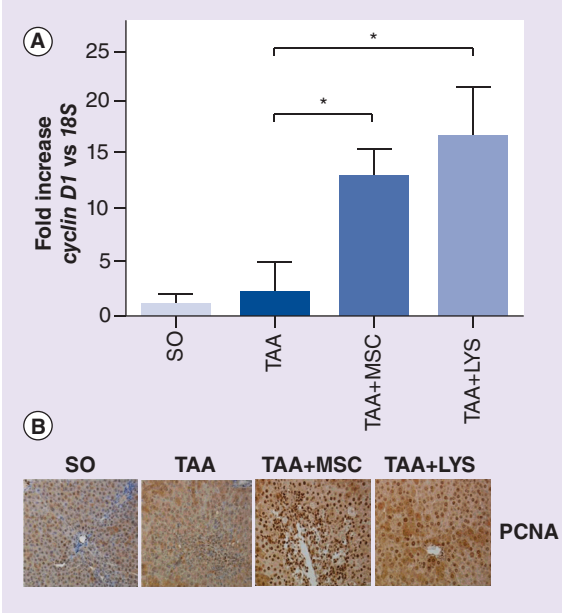

Figure 8. Transplantation of human mesenchymal stromal cells or the infusion of mesenchymal stromal cell lysates stimulates hepatic proliferation. (A) mRNA expression of rat *cyclin D1*. Data represent the mean ± standard error (*p < 0.05 vs TAA-treated rats). **(B)** PCNA analysis by immunohistochemistry. Image is representative of each group: SO, TAA, TAA+MSC infusion and TAA+LYS (TAA plus cellular LYS of MSC). Magnification: 5×.
LYS: Lysates; MSC: Mesenchymal stromal cells; PCNA: Proliferating cell nuclear antigen; SO: Sham-operated; TAA: Thioacetamide.

hepatic regeneration and improving hepatic function. Others reports have demonstrated the relationship between an increase of NF-κB, lower IκBα levels and Akt phosphorylation and apoptosis inhibition [22–24]. Therefore, the infusion of MSC or MSC cell lysates improved the cellular antiapoptotic machinery of TAA-injured livers. In addition to the observed antiapoptotic effect, MSC infusion (as intact cells or as cell lysates) also increased cell proliferation and liver regeneration as shown by increased expression of cyclin D1 and PCNA proteins (Figure 6). An increase in hepatocellular proliferation is a key element to achieve a complete recovery of liver function. Other studies have shown that intact MSC promotes hepatocyte proliferation and organ repair [25] but, as far as we know, this is the first time that a similar capacity is being demonstrated for the cell lysate of MSC. A relationship between proliferation, stem cells and cancer stem cells has been established and there is growing evidence that tumoral stem-like cells often represent the cell of origin of some types of cancer [26,27]. Thus, the therapeutic use of MSC lysate constitutes an appealing alternative in order to maintain the beneficial effects of bone marrow stromal cells avoiding mutations and other harmful changes in viable transplanted stem cells.

The therapeutic potential of MSC has been demonstrated in many studies. However, in a wide proportion of these studies, only a small fraction of donor cells have been retained and detected in the receiver animals. This observation suggested that engraftment, differentiation and a direct tissue repair must be not the sole mechanism for therapeutic effects of MSC and other mechanisms should be considered. It has been reported that the ability to differentiate into other cell types is not the only characteristic that makes MSC attractive for therapeutic purposes. The intracellular content, along with secretion of growth factors, cytokines and chemokines, may constitute their most efficient mechanism leading to organ recovery and/or regeneration after injury [28]. In this regard, several studies have shown the beneficial effects of other therapeutic strategies based in MSC such as conditioned medium from MSC [19,20], secretome [29] or the administration of MSC-derived exosomes [30] and microvessicles [31]. However, the mechanisms are still not fully understood and the results remain controversial.

The data obtained in the present study support the notion that the high efficiency of lysates from MSC in reducing apoptosis and improving liver function may be due to a fast and efficient delivery of hepatoprotective molecules. Our study shows that MSC are an efficient source of IL-6, which is a known inflammatory cytokine related to hepatic regeneration and with antiapoptotic effects [32,33]. In this sense, the differences in the intracellular levels of IL-6 between MSC and MNC might be one of the causes why MNC does not exert a hepatoprotective effect after portal transplantation; however, more studies would be necessary to support this hypothesis. Therefore, a fast delivery of IL-6 via infusion of MSC cell lysates leading to a fast reduction of apoptosis and stimulation of hepatic proliferation may be other of the reasons why lysates from MSC exert a more efficient hepatoprotective effect than intact MSC. Our experiments also indicate that the effects of intact MSC or MSC cell lysates are specific for this stem cell type. In addition to IL-6 changes, we have observed that MSC contains specific miRNAs related to apoptosis and cell proliferation. miRNAs are small noncoding RNAs that control gene expression at the posttranscriptional level by binding to 3′-untranslated regions of their target mRNAs [34]. The expressions of miRNA-124a and miRNA-125b were higher in MSC than in MNC from peripheral blood, whereas miRNA-155 was reduced. Intact MSC showed high contents of miRNA-124a and -125. It is unsolved if

these miRNAs are equally bioavailable in intact MSC than in the lysates of MSC. Lysates therapy could lead to a lesser apoptosis due to a bigger and faster release of miRNAs. It has been reported that, an excess of miRNA-124a expression in HepG2 cells leads to a reduction of IκBα expression, a nuclear member of the IκB protein family that inhibits the activity of the transcription factor NF-κB [35]. Moreover, the negative NF-κB regulator TNFAIP3 is a direct target of both miRNA-125a and miRNA-125b, so in malignant B cells, the upregulation of this miRNA causes a NF-κB increase, leading to proproliferative and antiapoptotic effects [36]. Other authors have observed that miRNA-125b promotes cell survival through an increase of NF-κB after ultraviolet exposition [37]. Additionally, Tili *et al.* showed that miRNA-155 and miRNA-125b have opposite effects on the production of TNF-α [38], which is one of the main cytokines involved in the response to lipopolysaccharide and inflammation and associated with a lesser hepatic proliferation [39]. Finally, high levels of miRNA-155 have been associated to an increase in the production of proinflammatory cytokines [40] and this is related to a higher stability of TNF-α mRNA. Together, our data suggest that the fast delivery of hepatoprotective molecules, such as cytokines or miRNAs from MSC cell lysates may greatly contribute to achievement of more beneficial effects than intact MSC.

Bioavailability studies of TAA demonstrate presence of small amount of this drug or its metabolites in plasma and liver tissue 12 h after the injection of 600 mg/kg of TAA [41]. Thus, the MSC activity may be negatively influenced by some of the TAA still present 12 h after injection. This might partially explain differences between MSC and lysates. In addition, this might be an additional advantage of lysate therapy versus intact MSC due to TAA metabolites not affecting lysates; the harmful effects of TAA are developed in alive cells and not in lysate cells.

Further studies are warranted to fully develop this promising modality of therapy in humans. It would be interesting to know whether other methods of administration of MSC cell lysates such as intraperitoneal, local or by peripheral blood are equally effective in this and other pathologies. A future therapy with MSC cell lysates would be extremely attractive because the allogenic use of these MSC lysates might allow the setting up of a MSC cell lysate bank and the quick availability of the treatment.

Conclusion

This is the first study comparing the efficiency of lysates versus intact MSC transplantation on hepatic liver function. As compared with intact MSC, the infusion of MSC cell lysates resulted in a significantly greater improvement of liver function and cell proliferation associated with a more efficient antiapoptotic response. Our work supports the idea that MSC cell lysates may constitute a useful strategy for recovering liver function.

Future perspective

Therapies based on stem cells are increasing in number even some clinical studies with different types of stem cells are being developed with satisfactory results. However, these therapies have still some important limitations. The creation of a stem cell bank allowing a rapid use of these cells, the possibility of an allogenic use or the biosafety of these treatments are some of the pending issues of stem cell therapies. A large body of evidence relates mutation or alterations in stem cells with the origin of cancer (cancer stem cells). This relationship between cancer and stem cells suggests a more conserver position and to take more precautions. In this sense, different strategies are being proposed in order to enhance biosafety and availability of these cells. One of them led to the use of molecules

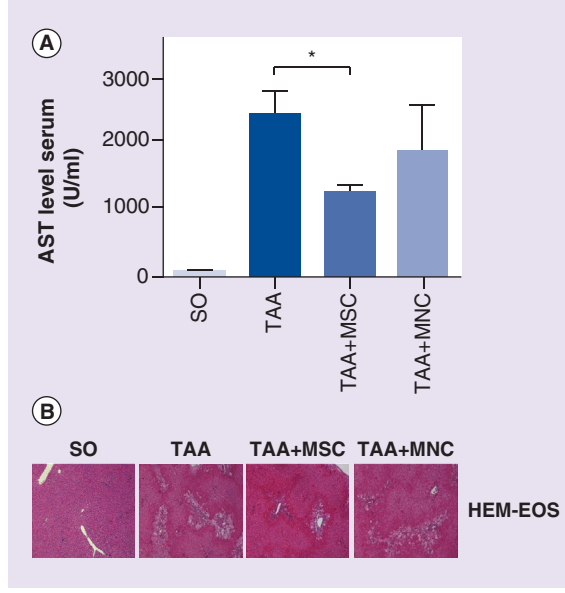

Figure 9. Hepatoprotective effects are specific of human mesenchymal stromal cells. (A) Serum levels of AST were measured after transplantation of human MNC or MSC in TAA-treated rats. Data represent the mean ± standard error (*$p < 0.05$ vs TAA-treated rats). **(B)** HEM-EOS staining after human MNC or MSC transplantation in TAA-treated rats. Images are representative of each group: SO, TAA, TAA+MSC infusion and TAA+MNC. Magnification: 5×.
AST: Aspartate aminotransferase; HEM-EOS: Hematoxylin and eosin; MNC: Mononuclear cells; MSC: Mesenchymal stromal cells; SO: Sham-operated; TAA: Thioacetamide.

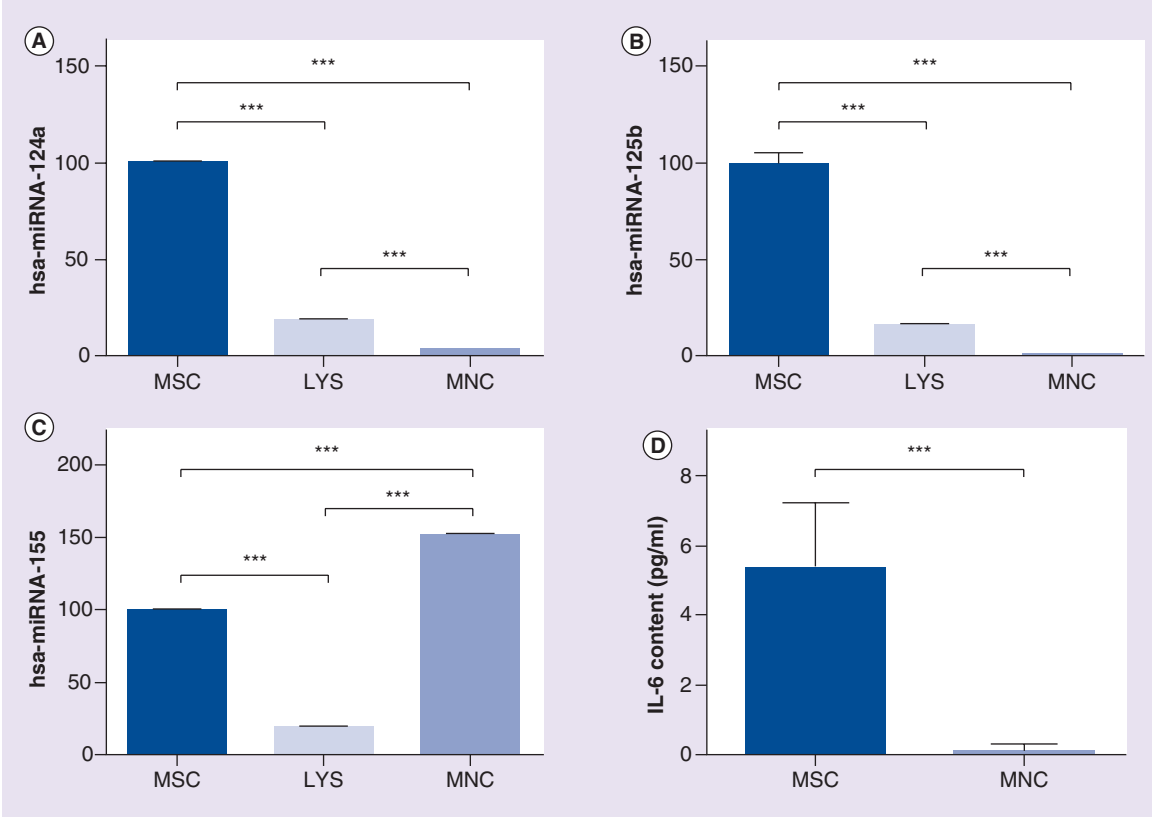

Figure 10. Mesenchymal stromal cells contain specific miRNA and cytokines. Comparison of the miRNA content **(A)** miRNA-124a, **(B)** miRNA-125b and **(C)** miRNA-155 in MSC, LYS and MNC. **(D)** Intracellular content of IL-6 after sonication of 3×10^6 MSC or 3×10^6 MNC.
***$p < 0.001$.
LYS: Lysates; MNC: Mononuclear cells; MSC: Mesenchymal stromal cells.

derived from MSC. Secreted medium of MSC may be an interesting alternative due to the capability to be stored and be applied in an allogenic way. Our study shows for the first time that the MSC lysate may be useful in the reduction of acute liver failure. MSC lysates might be equally effective in the treatment of musculoskeletal pathologies such as osteoarthritis or degenerative chondropathies. MSC cell lysate may be easily stored and the possible allogenic use in animal and human must be tested.

This alternative might suppose a revolution in the therapies based in stem cells due to:

- Evidence of beneficial effects;
- Allows storage and a rapid availability;

Executive summary

Mesenchymal stromal cell lysates may be an effective therapy for the treatment of acute liver failure

- Mesenchymal stromal cell (MSC) lysates improve hepatic architecture and reduce specific markers of hepatic necrosis and apoptosis.
- MSC lysates contain specific miRNA related to apoptosis and cell cycle and high levels of IL-6, which may achieve and enhance hepatic regeneration.
- In comparison with intact MSC, MSC lysates are more effective in reducing acute hepatic injury.

The use of MSC lysates opens a new scene for regenerative medicine with important advantages

- The use of MSC lysates opens a new scene for regenerative medicine with the following important advantages:
  - Allowing the use of an allogenic source of MSC;
  - Setting up of MSC lysate bank;
  - Accelerating the effects of MSC;
  - More biosafety avoiding the possible harmful effects of MSC (mutation and cancer stem cells).

- An allogenic use;
- A reduction of potential cancer stem cell generation.

In conclusion, these strategies might constitute the more immediate future for regenerative medicine based in stem cells.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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