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Efficacy of adipose tissue-mesenchymal stem cell transplantation in rats with acetaminophen liver injury☆

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Abstract **Objective** Acetaminophen intoxication is a leading cause of acute liver failure. Liver transplantation for acute liver failure is limited by the availability of donor organs. In this study, we aimed at identifying if the transplantation of adipose tissue-mesenchymal stem cells (ASCs) may exert therapeutic effects on acetaminophen-induced liver injury.
Methods ASCs were isolated from human subcutaneous tissue and were transfected with a green fluorescent protein (GFP). Sprague–Dawley rats were administrated 300 mg/kg of acetaminophen intraperitoneally and were transplanted with ASCs or vehicle. After 24 h from acetaminophen administration, rats were sacrificed. Hepatic levels of isoprostanes, 8-hydroxyguanosine (8-OHG), nitrites/nitrates and reduced glutathione (GSH) were determined as markers of oxidative stress; JNK phosphorylation and hepatic levels of inflammatory cytokines and regeneration factors were also assessed.
Results Transplantation of ASCs decreased AST, ALT and prothrombin time to the levels observed in control rats. Transplanted animals had normal plasma ammonia and did not display clinical encephalopathy. Liver sections of intoxicated rats treated with vehicle showed lobular necrosis and diffuse vacuolar degeneration; in rats transplanted with ASCs liver injury was almost absent. Transplantation of ASCs decreased liver isoprostanes, 8-OHG and nitrite–nitrates to the levels of control rats, while preserving GSH. Consistently, hepatic levels of TNF-α, MCP-1, IL-1β, ICAM-1 and phospho-JNK were markedly increased in rats treated with vehicle and were restored to the levels of controls in animals transplanted with ASCs. Furthermore, ASC transplantation increased liver expression of cyclin D1 and PCNA, two established hepatocyte regeneration factors, whereas ASCs were not able to metabolize acetaminophen *in vitro*.
Conclusion In this study, we demonstrated that ASC transplantation is effective in treating acetaminophen liver injury by enhancing hepatocyte regeneration and inhibiting liver stress and inflammatory signaling.

Abbreviations: ALF, acute liver failure; GSH, reduced glutathione; OLT, orthotopic liver transplantation; MSCs, mesenchymal stem cells; ASCs, adipose tissue mesenchymal stem cells; GFP, green fluorescent protein; 8-OHG, 8-hydroxyguanosine; BM-MSCs, bone marrow mesenchymal stem cells; PCNA, proliferating cell nuclear antigen.

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Introduction

Acute liver failure (ALF) is a life-threatening condition in which an acute liver injury can lead to coagulopathy, brain edema and in many cases to multiorgan failure; a variety of etiologies including drugs, viral infections, alcohol, metabolic, autoimmune or genetic disorders may cause acute hepatic dysfunction leading to liver failure (Lee and Stravitz, 2011). The main cause of ALF in industrialized countries is acetaminophen intoxication; although acetaminophen is a safe and widely used analgesic drug, its overdose can lead to ALF and the mortality for acetaminophen intoxication is currently impressive (Lee and Stravitz, 2011).

A number of studies in human and rodents suggest that oxidative stress plays a key role in the pathogenesis of acetaminophen-induced liver injury (Jaeschke et al., 2012). Oxidative stress is the consequence of the acute depletion of reduced glutathione (GSH) that occurs early, after 1.5 to 2 h from acetaminophen overdose, and it is caused by the generation of N-acetyl-p-benzoquinone imine (NAPQI), the toxic metabolite of acetaminophen (Jaeschke et al., 2012). This early event impairs the antioxidant defense of the liver against reactive oxygen species and reactive nitrogen species; the increase of radical species triggers lipid and protein peroxidation, and DNA oxidative damage with consequent death of hepatocytes (Jaeschke et al., 2012).

The treatment of acetaminophen intoxication is based on the use of N-acetyl-cysteine (Lee and Stravitz, 2011). However, in a number of patients with acetaminophen-induced acute liver injury, pharmacological treatment fails and patients need to undergo orthotopic liver transplantation (OLT) (Lee and Stravitz, 2011). Nonetheless, the shortage of donor organs for OLT makes the need of finding alternative therapeutic options. Recently, the transplantation of mesenchymal stem cells (MSCs) has been identified as a therapeutic tool in different types of experimental liver injuries (Sato et al., 2005; Kuo et al., 2008). MSCs are adherent, fibroblast-like, pluripotent and non-hematopoietic progenitor cells, which reside in many tissues and organs investigated so far including the bone marrow (Pittenger et al., 1999), umbilical cord (Bieback et al., 2004), placenta (In 't Anker et al., 2004), amniotic fluid (De Coppi et al., 2007) and subcutaneous adipose tissue (Zuk et al., 2001). Adipose tissue mesenchymal stem cells (ASCs) are abundant in subcutaneous adipose tissue and can be easily obtained by lipoaspiration, thus representing a fast source of MSCs in patients with critical acute diseases. The transplantation of ASCs has demonstrated therapeutic efficacy in CCl_4 (Banas et al., 2008), concanavalin A (Kubo et al., 2012) and ischemia–reperfusion (Sun et al., 2012) liver injuries. In this study, we aimed at identifying if the transplantation of ASCs may exert therapeutic effects in rats with acetaminophen-induced liver injury and the underlying molecular events.

Materials and methods

ASC isolation, culture and transfection

Subcutaneous adipose tissue was obtained from a 23-year-old man with no significant medical history undergoing umbilical hernioplasty. Written consent was obtained. The choice of a single young and healthy man as source of ASCs was in order to eliminate any bias related to the use of cells from different individuals, which can display different functional activity, and to exclude any situation of disease that could have affect the proliferative properties of ASCs. Adipose tissue was minced with scissors and scalpels into less than 3-mm pieces and isolation of ASCs proceeded as previously described (Banas et al., 2007). Briefly, after gentle shaking with equal volume of PBS, the mixture separated into two phases. The upper phase (containing stem cells, adipocytes and blood) after washing with PBS was enzymatically dissociated with 0.075% collagenase (type I)/PBS for 1 h at 37 °C with gentle shaking. The dissociated tissue was then mixed with an equal volume of DMEM (GIBCO-BRL, Japan) supplemented with 10% FBS and incubated 10 min at room temperature. The solution then was separated into two phases. The lower phase was centrifuged at 1500 rpm for 5 min at 20 °C. The cellular pellet was resuspended in 160 mM NH_4Cl to eliminate erythrocytes and passed through a 40 μm mesh filter into a new tube. The cells were resuspended in an equal volume of DMEM/10% FBS and then centrifuged. Isolation resulted in obtaining 7.7×10^6 of adherent cells for a primary culture from 5 g of adipose tissue (approximately; 1.0×10^5 to 4.6×10^6/1 g) after 7 to 10 days of culture. The cells were suspended in a DMEM/10% FBS plated in concentration $1–5 \times 10^6$ cells/75 cm^2. The cells with 70%–80% confluence were harvested with 0.25% trypsin–EDTA and then either repleted at 1.0×10^5 cells/60-mm dish. The phenotype of ASCs was evaluated by flow cytometry analysis (FC500 Beckman Coulter). Flow cytometry revealed that the cells isolated from the subcutaneous adipose tissue expressed stromal-associated markers CD90 and CD105 but did not express the hematopoietic markers CD34 and CD45 (Suppl. Fig. 1). In an *in vitro* experiment to establish if ASCs metabolize acetaminophen, cells were treated with 2 mM acetaminophen; NAPQI concentration was measured in the cell medium after 2, 8 and 24 h. For the *in vivo* experiment, before transplantation cells were transfected with a baculovirus-mediated transfection system for green fluorescent protein (GFP) (Invitrogen, US).

Animals and treatments

All procedures fulfilled the Italian Guidelines for the Use and Care of Laboratory Animals. Sprague–Dawley female rats were purchased from Charles River Lab (Calco, Italy). Animals were maintained in a light- and temperature-controlled facility and fed with a standard chow and water *ad libitum*. After an overnight fast, twelve rats weighing about 200 g were administrated a 300 mg/kg body weight dose of acetaminophen (Sigma, Italy) dissolved in PBS. After 2 h from acetaminophen administration, six rats received the infusion *via* the caudal vein of 200,000 cells suspended in 1 mL of saline; six rats were administrated only saline. Four rats were administrated only the vehicles, PBS and saline, and served as healthy control. ASCs were administrated 2 h after acetaminophen administration because this is the time occurring for the formation of NAPQI, and this early time point is widely used in animal studies evaluating the efficacy of a treatment for acetaminophen intoxication (Saito et al., 2010). After 24 h from acetaminophen administration, rats

were sacrificed by cardiac puncture; blood and liver samples were harvested for further analysis.

Biochemical analyses

Plasma levels of ALT, AST, ammonia and prothrombin time were determined on blood samples using a Cobas Multi-analyzer (Roche Diagnostics, UK). Isoprostanes and 8-hydroxyguanosine (8-OHG) were determined by enzyme-linked immunosorbent assay (ELISA) test (Cayman, Ann Arbor, Michigan); GSH was assessed by a GSH assay (Cayman). Nitrite/nitrates were measured colorimetrically using Griess reagent (Merck KGaA, Darmstadt, Germany), following the manufacturer's instructions. Whole liver homogenates were processed for Western blot analysis and protein levels were visualized by immunoblotting with antibodies against total-JNK and phospho-JNK (Chemicon, Temecula, CA), cyclin D1, proliferating cell nuclear antigen and β-actin (all from Santa Cruz, TX).

High performance liquid chromatography

NAPQI concentrations were determined using the Agilent 6410 Triple Quadruple Mass Spectrometer (Triple Quad MS) with an Electrospray Ionization (ESI) source (Agilent Technologies, USA). The chromatographic separation was achieved on a Gemini-NX C_{18} (50 mm × 2.0 mm, 3 μm) column. All data were acquired employing Agilent 6410 Quantitative Analysis version B.01.03 analyst data processing software. In preliminary *in vitro* experiments, in the absence of cells, we noticed that the recovery of acetaminophen from the FBS-containing medium is substantially reduced when compared to the nominal amount (the amount added to the solution). This may be due to binding to the plastic ware and FBS proteins. Thus, we measured only NAPQI to establish if ASCs metabolize acetaminophen. NAPQI was analyzed in cell medium, which was diluted in ultrapure water and an aliquot of 5 μL was injected into the HPLC column. Standard stock solutions of NAPQI was dissolved in water to obtain an exact final concentration of 20 μg/mL of NAPQI, and stored at +4 °C. Target calibration range was 0.101–20 μg/mL (LOD was 101.51 ng/mL). The flow rate was set at 0.2 mL/min. An isocratic of mobile phase in 5 min for assay (U.P. water, with 0.1% HCOOH and CAN, with 0.1% HCOOH, 90/10, v/v solution). NAPQI was ionized under positive ionization conditions. The predominant peak in the primary ESI spectra of NAPQI corresponds to the $[M - H - H_2O]^+$ ion at m/z 168, both product ion for NAPQI at m/z 126. Chromatograms were integrated and the calibration curves were plotted as the peak area of NAPQI (Suppl. Fig. 2). The coefficients of variation were less than 15%; the determination coefficient (r^2) was 0.999.

Morphological analyses

Liver samples were fixed in 10% buffered formalin and embedded in paraffin using standard techniques. Histological damage was evaluated by using hematoxylin–eosin staining. Indirect immunofluorescence for the identification of GFP^+ cells was performed on frozen liver samples. Monoclonal antibodies for human CK-18 and vimentin (both from DakoCytomation, Italy) were used on paraffin-embedded sections, following the manufacturer's instructions. All antibodies did not cross-react with rat proteins, as evidenced in previous experiments.

RNA extraction and real time PCR

Total RNA was extracted by homogenizing snap frozen liver samples in TRIzol reagent (Invitrogen, Milan, Italy). Quantitative real-time PCR was performed in 7900HT Fast Real-Time PCR System Applied Biosystems (Applied Biosystems, Foster City, CA), using the EXPRESS SYBR GreenER™ qPCR SuperMix with Premixed ROX (Invitrogen). The following primer sequences were used: TNF-α fw 5′-CGAGTGACAAGCCTGTAGC-3′ rev 5′-GGTGTGGGTGAGGAGCACAT-3′; ICAM-1 fw 5′-CCTTCCT CACCGTGTACTGG-3′ rev 5′-AGCGTAGGGTAAGGTTCTTGC-3′; MCP-1 fw 5′-CATAGCAGCCACCTTCATTCC-3′ rev 5′-TCTGCACT GAGATCTTCCTATTGG-3′; IL-1β fw 5′-AAGCTGATGGCCCTAAA CAG-3′ rev 5′-AGGTGCATCGTGCACATAAG-3′; GAPDH fw 5′-GGT GGTCTCCTCTGACTTCAACA-3′ rev 5′-GTTGCTGTAGCCAAATTC GTTGT-3′; reactions were performed in a 20 μL mixture containing cDNA, specific primers of each gene and the SYBR GreenER™ qPCR SuperMix. The specific PCR products were detected by the fluorescence of SYBR Green, the double stranded DNA binding dye. The relative mRNA expression level was calculated by the threshold cycle (Ct) value of each PCR product and normalized with that of GAPDH by using comparative $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Statistics were aided by GraphPad Prism. All results were expressed as mean ± standard deviation. Unpaired *t* test and one-way ANOVA with Bonferroni *post-hoc* analysis were used when appropriate. P values less than 0.05 were considered significant.

Results

Effects of ASC transplantation on plasma markers of liver injury

Rats intoxicated with acetaminophen and treated with vehicle displayed a marked increase in plasma levels of ALT and AST as compared with control animals (Figs. 1A, B); interestingly, rats transplanted with ASCs displayed normal plasma AST and ALT activities (Figs. 1A, B). Consistently, prothrombin time, as measured by INR, was elevated in rats treated with vehicle and was restored to the levels observed in the control group in rats transplanted with ASCs (Fig. 1C). Furthermore, animals treated with vehicle had clinical evidence of encephalopathy and a mild increase in plasma ammonia (Fig. 1D); by contrast, rats transplanted with ASCs did not display encephalopathy and had normal levels of plasma ammonia (Fig. 1D).

Effects of ASC transplantation on liver histology

Rats intoxicated with acetaminophen and treated with vehicle displayed diffused vacuolar degeneration, due to mitochondrial damage, and necroinflammatory foci in the lobular area (Fig. 2B). Lobular necrosis was absent in rats transplanted with

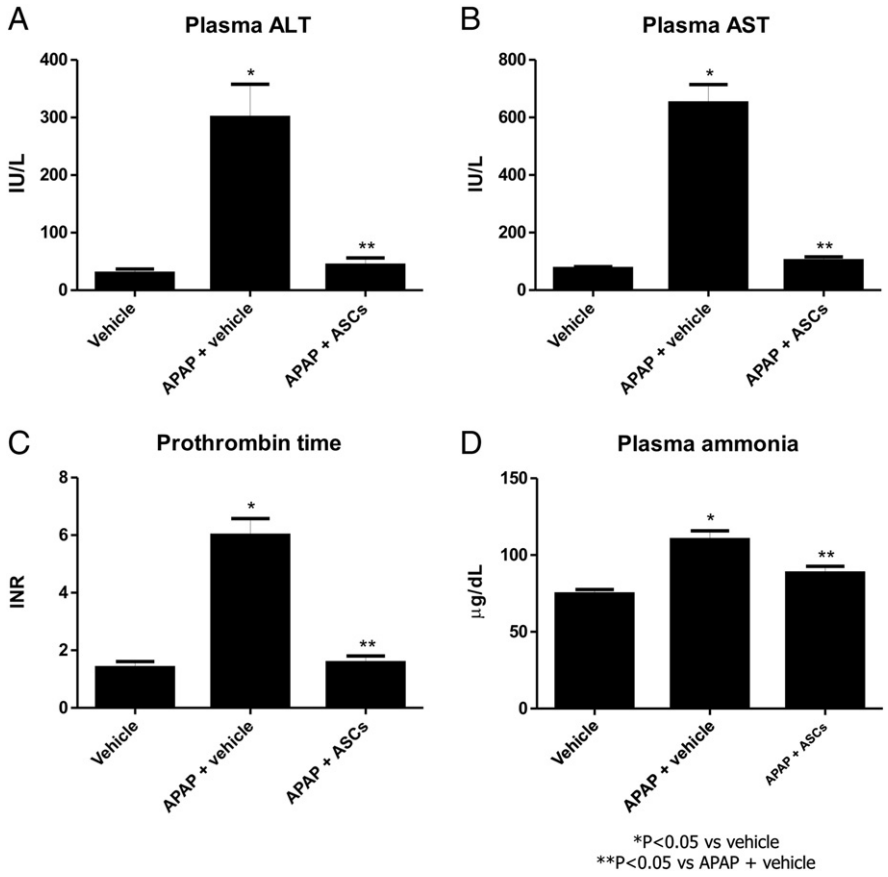

Figure 1 Effects of ASC transplantation on plasma markers of liver injury. (A, B) Rats intoxicated with acetaminophen and treated with vehicle displayed a marked increase in plasma levels of ALT and AST as compared with control animals; rats transplanted with ASCs displayed normal plasma ALT and AST activities. (C,D) INR and plasma ammonia were elevated in rats with acetaminophen overdose treated with vehicle and were restored to the levels observed in the control group in rats transplanted with ASCs. *P < 0.05 *vs* vehicle; **P < 0.05 *vs* APAP + vehicle.

ASCs (Fig. 2C) and only mild vacuolar degeneration was focally observed. Moreover, mitotic hepatocytes were observed in liver sections of rats transplanted with ASCs (Fig. 2C), whereas these were not present in control rats (Fig. 2A). Immunofluorescence for GFP revealed the presence of ASCs in liver sections of transplanted rats (Fig. 2D). In most sections, ASCs presented a shaped fibroblast-like morphology (Fig. 2D); only, in few sections, ASCs appeared with a round epithelial-like morphology (Fig. 2D). Immunohistochemistry with a monoclonal antibody against human vimentin, a typical mesenchymal marker, was positive in most liver sections from transplanted rats (Fig. 2E), whereas immunohistochemistry for human CK-18 revealed the presence of only rare ASCs undergoing differentiation (Fig. 2F), suggesting that ASCs exert a therapeutic effect by maintaining their mesenchymal phenotype.

Effects of ASCs on markers of liver oxidative stress, inflammation and regeneration

Rats intoxicated with acetaminophen displayed a three-fold increase in hepatic levels of isoprostanes as compared with control healthy animals (Fig. 3A), indicating significant lipid peroxidation. Interestingly, rats transplanted with ASCs had isoprostane levels similar to the control group (Fig. 3A). Similarly, rats with acetaminophen intoxication presented a marked increase in liver 8-OHG as compared with control healthy animals (Fig. 3B); rats transplanted with ASCs had 8-OHG levels not different from the control group (Fig. 3B), indicating DNA protection exerted by ASCs. Consistently with isoprostanes and 8-OHG values, animals with acetaminophen overdose presented increased levels of hepatic nitrite/nitrates, a marker of nitrosative stress, as compared to control rats (Fig. 3C). Strikingly, in animals transplanted with ASCs, nitrite/nitrates were comparable to the control group (Fig. 3D). Furthermore, acetaminophen intoxication leads to a marked increase of phospho-JNK expression whereas the levels of total JNK were not significantly modified. In rats transplanted with ASCs, p-JNK expression was restored to the levels observed in the control group (Fig. 3E). Consistently, in the liver, gene expression of inflammatory cytokines was induced by acetaminophen intoxication (Figs. 4A–D). In agreement with histological findings, ASC transplantation decreased the gene expression of TNF-α, MCP-1, IL-1β and ICAM-1 to the levels observed in healthy controls (Figs. 4A–D). Furthermore, animals transplanted with ASCs displayed increased expression of cyclin D1 and PCNA (Figs. 5A–C), indicating that these cells exert hepatoprotective effects by promoting liver regeneration. To confirm that ASCs exerted

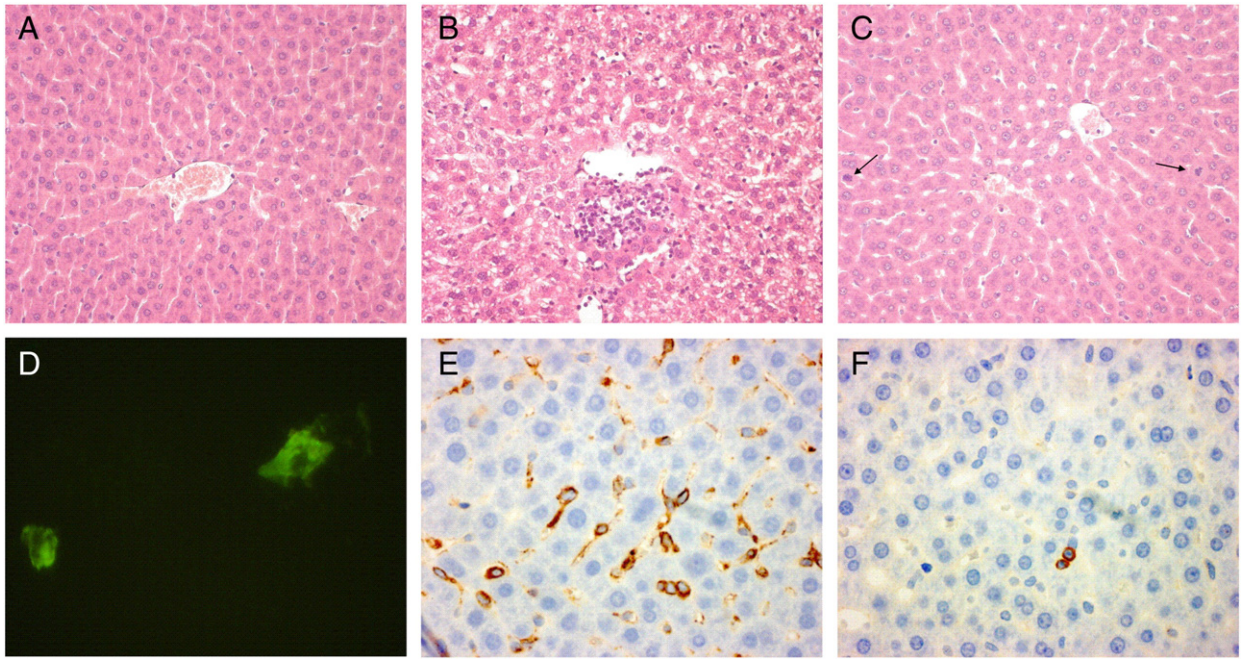

Figure 2 Effects of ASC transplantation on liver histology. (A) Hematoxylin–eosin stained liver sections from healthy rats showing normal morphology; (B) liver sections from rats with acetaminophen intoxication and treated with vehicle showing lobular necrosis and ballooning degeneration; (C) liver sections from animals transplanted with ASCs showing the absence of necroinflammation and mitotic hepatocytes (arrows). (D) Indirect immunofluorescence on frozen liver sections from the group transplanted with ASCs showing the presence of two GFP+ cells, one with a typical fibroblast-like phenotype (right of the panel), one with a round epithelial-like phenotype (left). (E) Immunohistochemistry with a monoclonal antibody against human vimentin, a typical mesenchymal marker, was positive in liver sections from transplanted rats; (F) immunohistochemistry for human CK-18 revealed the presence of only rare ASCs undergoing differentiation. [Magnification: 20× (A,B,C); magnification 40× (D,E,F)].

hepatoprotective effects not by metabolizing acetaminophen, we treated *in vitro* these cells with 2 mM acetaminophen; HPLC revealed undetectable NAPQI levels in cell medium at any time point (data not shown), thus indicating the absence of acetaminophen conversion by ASCs.

Discussion

In the current study, we explored the effect of ASC transplantation in rats with acetaminophen-induced liver injury. Acetaminophen intoxication is a leading cause of ALF in industrialized countries and the mortality is currently high (Lee and Stravitz, 2011). The shortage of grafts for OLT determines the need of novel therapies for ALF. There is evidence that the transplantation of hepatocyte-like cells derived from adult stem cell of various origin may represent an effective strategy for the treatment of acute or chronic liver diseases (Soltys et al., 2010). However, in our opinion the use of undifferentiated ASCs is ideal in critical acute conditions as in ALF because of their ready availability and unrestricted potential to propagate. In fact, ASCs are superior to bone marrow MSCs (BM-MSCs) and umbilical-MSCs in terms of colony frequency and show also higher proliferation capacity as compared to BM-MSCs (Kern et al., 2006). The need for long-time period of culture makes the use of hepatocyte-like cells unreliable for the management of ALF. Furthermore, there is evidence that the transplantation of undifferentiated MSCs is more effective than the transplantation of hepatocyte-like cells in rats with CCl_4-induced liver failure because of greater resistance to oxidative stress (Kuo et al., 2008).

In this study, ASC transplantation was able to counteract the appearance of coagulopathy and encephalopathy, which are the two main clinical features of ALF (Lee and Stravitz, 2011). Transplanted animals had levels of transaminases, INR and ammonia comparable to those of healthy control rats and did not displayed brain edema (data not shown). Consistently with clinical findings, we evidenced lobular necrosis and inflammation in vehicle-treated animals, whereas transplanted animals presented only mild injury. With respect to the phenotype of ASCs in the host liver, immunostaining with human monoclonal antibodies suggested that ASCs exert their therapeutic role by maintaining their fibroblast-like phenotype almost at this early time point. Nonetheless, in few sections ASCs presented an epithelial morphology and expressed epithelial markers as CK-18; thus, it is not possible to exclude that in a longer period of observation ASCs can undergo hepatogenic differentiation although, in previous experiments with four weeks of administration of BM-MSCs in CCl_4-treated mice, only a small percentage of MSCs underwent hepatocyte-like differentiation (Sato et al., 2005). The presence of mitotic nuclei in liver sections of transplanted animals and the increase of cyclin D1 and PCNA suggest a trophic activity of ASCs which is in agreement with previous data in mice treated with CCl_4 and transplanted with ASCs (Banas et al., 2008). Trophic activity and immunomodulation are considered key features

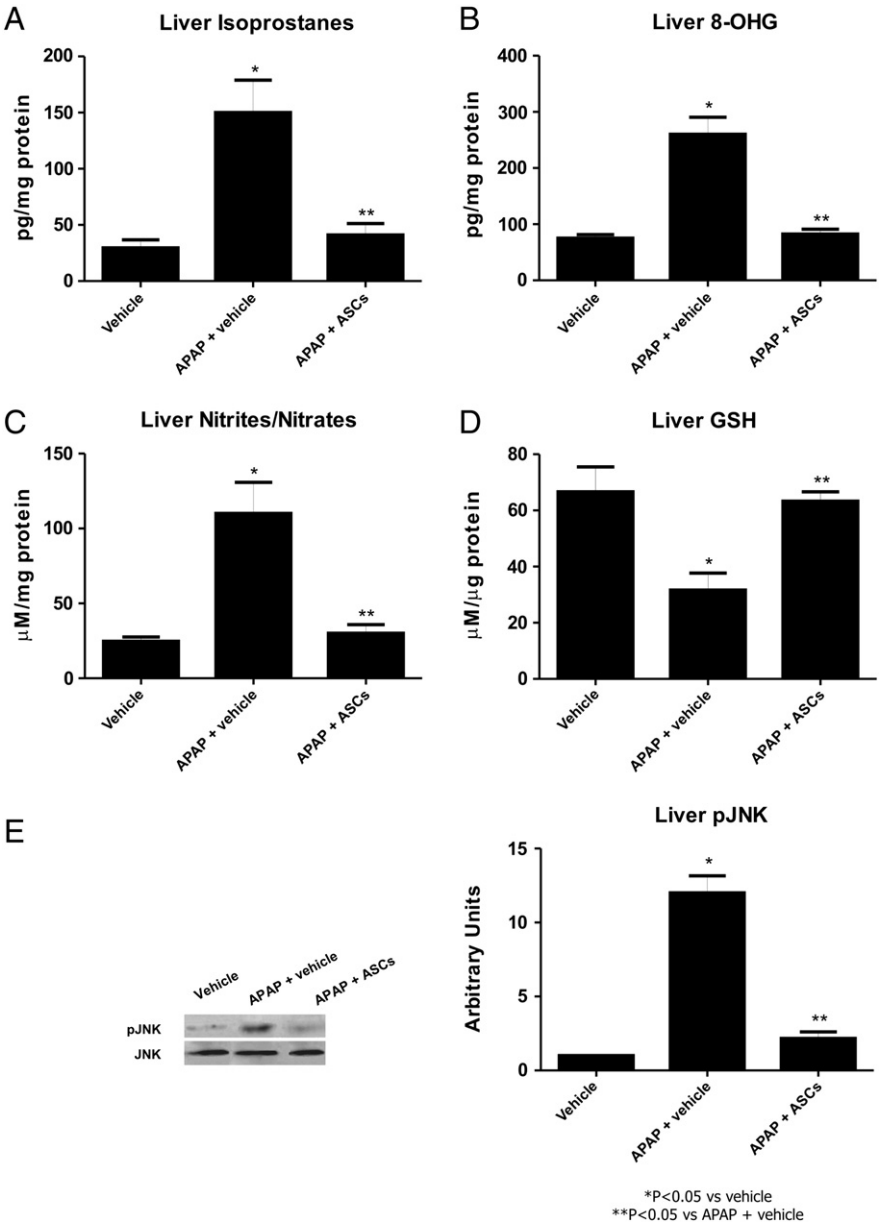

Figure 3 Effects of ASC transplantation on liver oxidative stress. (A–D) Rats intoxicated with acetaminophen displayed a marked increase in hepatic levels of isoprostanes, 8-hydroxyguanosine (8-OHG) and nitrites/nitrates as compared with control healthy animals and decreased GSH levels, indicating significant oxidative–nitrosative stress and impaired antioxidant defense; ASC transplantation exerted potent antioxidant effects restoring isoprostanes, 8-OHG, nitrites/nitrates and GSH to the levels of control group. (E) Representative Western blot and densitometric analysis showing that acetaminophen intoxication induced JNK phosphorylation whereas the levels of total JNK were not significantly modified. In rats transplanted with ASCs, p-JNK expression was restored to the levels observed in the control group. *P < 0.05 *vs* vehicle; **P < 0.05 *vs* APAP + vehicle.

of MSCs and lack of immunogenicity of MSCs was demonstrated in chronic models of liver injury (Sato et al., 2005).

Regarding the molecular basis underlying the effects of ASC transplantation, we demonstrated that these cells markedly reduce oxidative stress in the liver of animals with acetaminophen overdose. Oxidative stress plays a pivot role in acetaminophen hepatotoxicity (Knight et al., 2002; James et al., 2003; Cover et al., 2005). Here, we show that rats transplanted with ASCs did not display increased levels of isoprostanes, the most sensitive *in vivo* marker of oxidative stress (Basu, 2008), neither of 8-OHG, a marker of DNA damage, indicating that ASCs protected the rats from acetaminophen-induced oxidative stress. Consistently, ASC transplantation decreased nitrite/nitrates, which are markers of nitrosative stress, to the levels observed in healthy controls. This appears relevant because nitrogen radicals, particular peroxynitrite, exert a role in acetaminophen toxicity especially through DNA fragmentation (Das et al., 2010). Interestingly, ASCs transplantation was able to inhibit JNK activation. JNK signaling regulates the onset of hepatocyte death both in human and murine paracetamol hepatotoxicity (Gunawan et al., 2006; Henderson et al., 2007; Ghosh

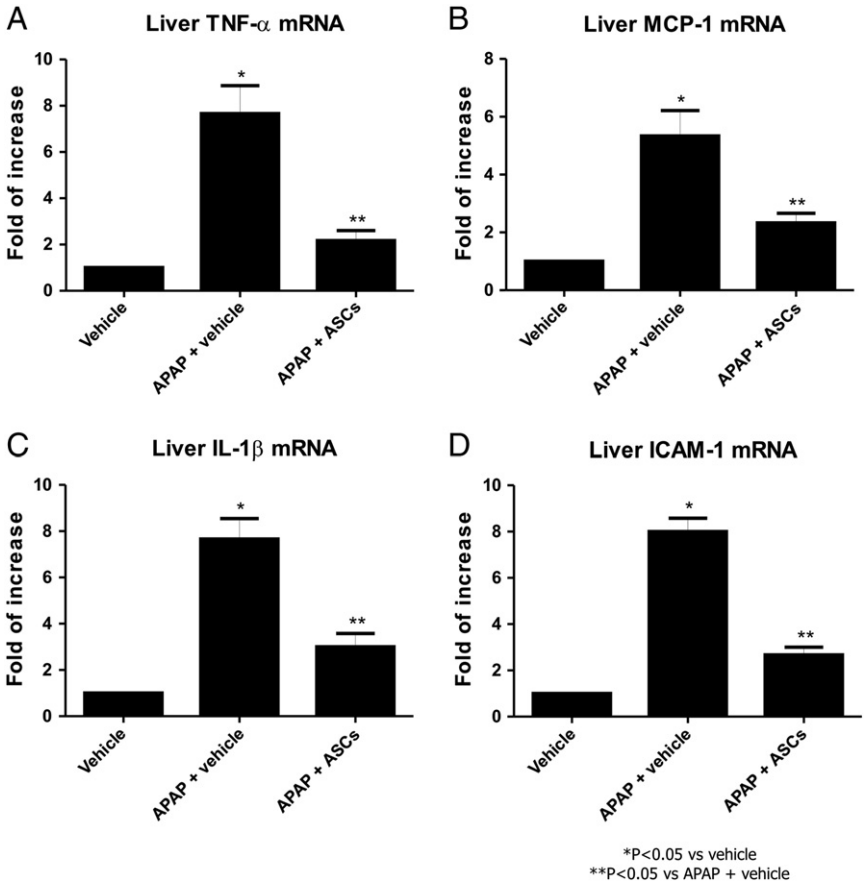

Figure 4 Effects of ASC transplantation on liver inflammatory cytokines. (A–D) Gene expression of TNF-α, MCP-1, IL-1β and ICAM-1 was induced by acetaminophen intoxication. In agreement with histological findings, ASC transplantation decreased the gene expression of inflammatory cytokines to the levels observed in healthy controls. *P < 0.05 *vs* vehicle; **P < 0.05 *vs* APAP + vehicle.

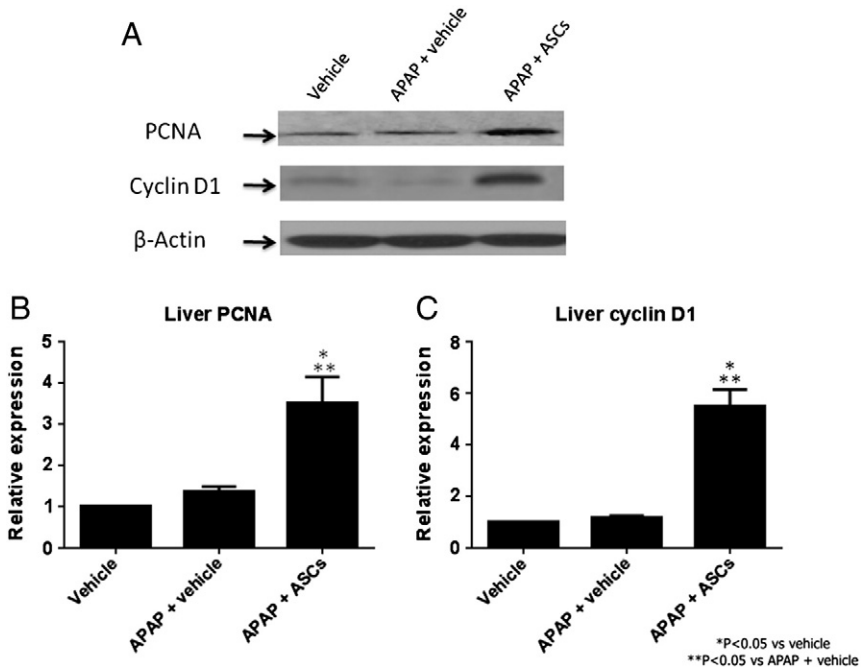

Figure 5 Effects of ASC transplantation on liver regeneration. (A–C) Representative Western blot and densitometric analysis showing a marked increase of liver cyclin D1 and PCNA in animals transplanted with ASCs as compared with rats treated with vehicle. *P < 0.05 *vs* vehicle; **P < 0.05 *vs* APAP + vehicle.

et al., 2010). JNK inhibition is not protective in CCl_4-mediated or anti-Fas antibody mediated hepatic injury (Gunawan et al., 2006) suggesting specificity for the role of JNK in acetaminophen hepatotoxicity.

In conclusion, in this study we demonstrated for the first time that ASC transplantation is effective in treating acetaminophen-induced liver injury. ASCs engraft in the injured liver where they enhance hepatocyte regeneration and inhibit stress and inflammatory signaling. Further animal studies are needed to elucidate the complex molecular events underlying ASC hepatoprotective effects. In our opinion, these findings may provide a rationale for the use of ASCs in the clinical management of patients with acetaminophen intoxication.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2013.07.003.

Authors' contribution

FS conceived and designed the study, performed the experiments and wrote the manuscript; IB and LP performed the experiments and critically reviewed the manuscript; CP performed experiments; GLV performed the experiments and critically reviewed the manuscript.

Competing interests

None.

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