

## Original Article

# Protective effect of mesenchymal stem cell-conditioned medium on hepatic cell apoptosis after acute liver injury

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**Abstract:** The aim of this study was to investigate the role of Mesenchymal Stem Cell (MSC) conditioned medium (CM<sub>MSC</sub>) on apoptosis of cultured mouse primary hepatocytes after *in vivo* carbon tetrachloride (CCI4)-induced acute liver injury. The acute liver injury was induced by injecting CCI4 intraperitoneally in C57/BL6 mice. Hepatocytes were isolated by liver perfusion, cultured in a defined medium to maintain their differentiation and characterized by reverse transcriptase polymerase chain reaction (RT-PCR) using the hepatic cell specific genes albumin, hepatocyte nuclear factor 4 (HNF4) and cytokeratin 18 (CK18). CM<sub>MSC</sub> was generated from cultured bone marrow-derived MSCs (BM-MSCs). BM-MSCs were positive for CD73, CD90, CD44 by flow cytometry and able to differentiate into chondrocytes, adipocytes and osteocytes. Apoptosis was evaluated by both annexin V. CM<sub>MSC</sub> were examined by flow cytometry to detect MSC-derived annexin V- and CD54/CD44-positive microparticles (MPs). In the CCI4-CM<sub>MSC</sub> treated hepatocytes, interleukin-6 (IL-6) was increased on the first day of culture compared to control and CCI4 and was followed by upregulation of fibroblast-like-protein (FGL1) expression after 48 hrs. This was associated with a significant decrease of annexin V positive CCI4-CM<sub>MSC</sub> treated hepatocytes at day 3 post plating. Recombinant IL-6 was induced FGL1 expression in hepatocytes derived from CCI4-treated mice suggesting that CM<sub>MSC</sub> which is enriched also in microparticles, attenuates CCI4-induced early apoptosis in hepatocytes through activation of FGL1.

**Keywords:** Hepatic cell apoptosis, mesenchymal stem cell-conditioned medium, FGL1, IL-6, microparticles

## Introduction

Bone-marrow derived mesenchymal stem cells (MSCs) have been shown to promote repair of damaged tissues by inhibiting apoptosis and fibrosis [1-3]. MSCs also secrete a number of bioactive factors, a number of which mediate tissue repair [1, 4, 5]. Recently MSC-derived molecules were shown to exert a therapeutic effect in fulminant hepatic failure [6].

In previous studies we have demonstrated that granulocyte colony-stimulating factor (GCSF) either on its own or by mobilizing HSCs promotes endogenous repair programs in acute and chronic liver injury [7]. Acute liver injury represents a serious condition with high mortality rate. Liver is the only organ with regenerative capacity although injury can progress to irreversible stages [8, 9]. CCI4-induced acute liver

injury is a well-known model in rodents. Hepatotoxicity is histopathologically characterized by hepatocyte necrosis as well as apoptosis [10]. Apoptosis is a process by which cells undergo nonnecrotic cellular suicide. Phosphatidylserine, one component of cell membrane phospholipids is confined to the inner leaflet of the plasma membrane. Annexin V-binding to phosphatidylserine in the outer leaflet is an early apoptotic event [11]. In an acute liver injury model, increased hepatocyte cell protection is associated with downregulation of proapoptotic signaling [12].

MSC therapy might offer a promising strategy for acute liver injury treatment, determination of treatment protocol for MSC transplantation requires improvement. Transdifferentiation of MSCs into hepatocytes seems also to be a rare event [6] while the usage of secreted factors

from MSC could exert a real beneficial effect on hepatocytes. One of the MSC derived factors is the hepatoprotective cytokine IL6 [13, 14], which regulates the acute phase response and plays a pivotal role in liver regeneration as well as the down-regulation of anti-apoptotic genes [15]. Additionally, IL-6 induces the expression of fibroblast-like-protein 1 gene (FGL1)[16] also called as hepassocin (HPS) [17] and MFREP-1 (hepatocyte-derived fibrinogen-related protein) [18] after hepatectomy or turpentine oil-induced acute phase response [19]. Therefore it has been suggested that FGL1 may be involved in the liver regeneration process [17, 20] and that the FGL1 gene is a key gene involved in repair following CCl<sub>4</sub>-hepatotoxicity [16]. Recently several studies reported that MSC-derived microparticles, which are plasma membrane-derived vesicles participate in physiological and pathological processes [21]. Furthermore, MPs facilitate intercellular communication [22] and confers protective effect against tissue injury [21] as well as accelerates hepatic regeneration [23].

We hypothesized that the CM<sub>MSC</sub>, a rich source of growth factors and paracrine mediators secreted by MSCs such as IL6, can protect hepatocytes from acute injury. FGL1, which has been shown to be induced by IL6 [17], could also play a role in hepatocyte apoptosis. Therefore, we aimed to evaluate the effect of CM<sub>MSC</sub> on hepatoprotection and modulation of apoptosis of primary hepatocytes in an acute model of CCl<sub>4</sub> induced-liver injury. Furthermore, we showed that CM<sub>MSC</sub> can increase IL6 production that may contribute to the elevated expression of FGL1 in primary hepatocytes derived from CCl<sub>4</sub>-treated mice.

### Materials and methods

#### *Animals and treatment*

Eight-week old C57BL/6 mice, maintained in 12-hour light/12-hour dark cycles in a conventional clean facility, were used in these experiments. All experiments were performed in an accredited animal facility (number EL 54 BIO 14) and complied with the Federation of European Laboratory Animal Science Associations guidelines. Acute liver damage was induced by injecting mice with 2 ml/kg CCl<sub>4</sub> (dissolved in corn oil in a ratio 1:1) intraperitoneally [24].

#### *Mouse primary hepatocyte cultures*

The liver was perfused *in situ* through the portal vein using Ca<sup>++</sup>-free HBSS (pH 7.4) based on a modified protocol published by Klaunig [25]. Perfusion was continued with Waymouth's 752/1 medium (Gibco, USA) supplemented with 0.57 mg/ml Collagenase (type IV, Sigma-Aldrich, USA). Immediately after perfusion the liver was removed and hepatocytes were cultured in fibronectin coated culture plates at a density 1×10<sup>6</sup> cells/ml in serum free Waymouth's 752/1 supplemented with 10 µg/ml insulin, 25 µg/ml transferrin, 100 µg/ml bovine serum albumin, 50 ng/ml EGF, 10 µg/ml glucagon and 10<sup>-7</sup> M dexamethasone (Sigma, USA). This medium was found to promote maintenance of hepatocyte differentiation. At the indicated time points (1, 3, 4 and 6 days) the supernatants were harvested and, after cell debris removal, stored at -20°C until used.

#### *Culture of MSCs and preparation of MSC-conditioned medium*

Bone marrow cells from femurs and tibiae of C57/BL6 mice were plated at a density of 10<sup>6</sup> cells per cm<sup>2</sup> in a-MEM medium containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, USA) [26, 27]. The cells were then incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> and non adherent cells were removed by changing the culture medium. The medium was changed every 4 days while adherent cells were harvested by trypsinization and re-plated. After 4-5 passages culture supernatants (CM<sub>MSC</sub>) were collected, centrifuged for 10 min at 1000 g to remove cell debris and stored at -80°C until used.

#### *Characterization of MSC*

**Flow cytometry:** Samples were analyzed by a flowcytometer (BD FACSCalibur 4 Color). Cells were washed, pelleted, resuspended in PBS and then incubated with anti-CD45-PE, anti-CD73-PE, anti-CD90-FITC, anti-CD44-PE antibodies (BD, USA). Specific isotype controls were used for background staining. All monoclonal antibodies (mAbs) used for flow cytometry in this study were purchased from BD Pharmingen.

**Differentiation assays:** For adipogenic differentiation, cells were seeded at a concentration of

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$2,5 \times 10^4/\text{cm}^2$  in a 6-well plate. After 24 hrs of culture, adipogenic medium containing a-MEM, 10% FBS, (Gibco, USA) 10 ng/ml insulin and  $1 \times 10^{-8}$  M dexamethasone (Sigma, USA) was added and cells were grown for 3 weeks with medium replacement twice per week. Adipogenesis was detected by Oil Red O staining.

For osteogenic differentiation, cells were seeded at a concentration of  $2,5 \times 10^4/\text{cm}^2$  in a 6-well plate. After 24 hrs of culture, osteogenic medium containing a-MEM, 50  $\mu\text{g}/\text{ml}$  L-ascorbic acid-2 phosphate, 10 mM glycerol 2-phosphate disodium salt,  $1 \times 10^{-8}$  M dexamethasone (Sigma, USA) and 200  $\mu\text{g}/\text{ml}$  rBMP-2 (R&D, MN, USA) was added and cells were grown for 3 weeks with medium replacement twice per week. Osteogenic differentiation was detected by Alizarin red staining.

For chondrogenic differentiation, cells were grown in a micromass culture supplied with chondrogenic medium ( $0,2 \times 10^6/\text{tube}$ ). Chondrocytes were grown in a-MEM supplemented with, 1% FBS, 6,25  $\mu\text{g}/\text{ml}$  insulin, 50 nM L-ascorbic acid 2-phosphate and 10 ng/ml TGF- $\beta$ 3 (R&D, MN, USA). Medium was replaced twice a week and chondrogenic differentiation was detected by Alcian blue staining [23].

### *Annexin V assay*

Viability of hepatocyte cultures was monitored by FACS analysis using annexin V-propidium iodide staining. Cultured hepatocytes were detached, centrifuged, suspended in PBS and stained with annexin V-FITC and propidium iodide (BD Pharmingen, CA, USA). Apoptotic cells were identified as an annexin V-positive/propidium iodide-negative population. Analysis was performed using the FACScalibur cytometer (BD, USA) using FACs Diva 6 software.

### *Microparticle analysis*

Microparticles were identified based on size determined by the 0.9  $\mu\text{m}$  beads (BIOCYTEX, France) in a plot side scatter. The scatter characteristics based in 0.9  $\mu\text{m}$  beads size was assessed by the logarithmic amplification of FCS and SSC signals. MSC supernatant was incubated with Annexin-FITC, anti-CD54-PE and anti-CD44-PE antibodies (BD, USA). The number of positive events for anti-CD54-PE, anti-

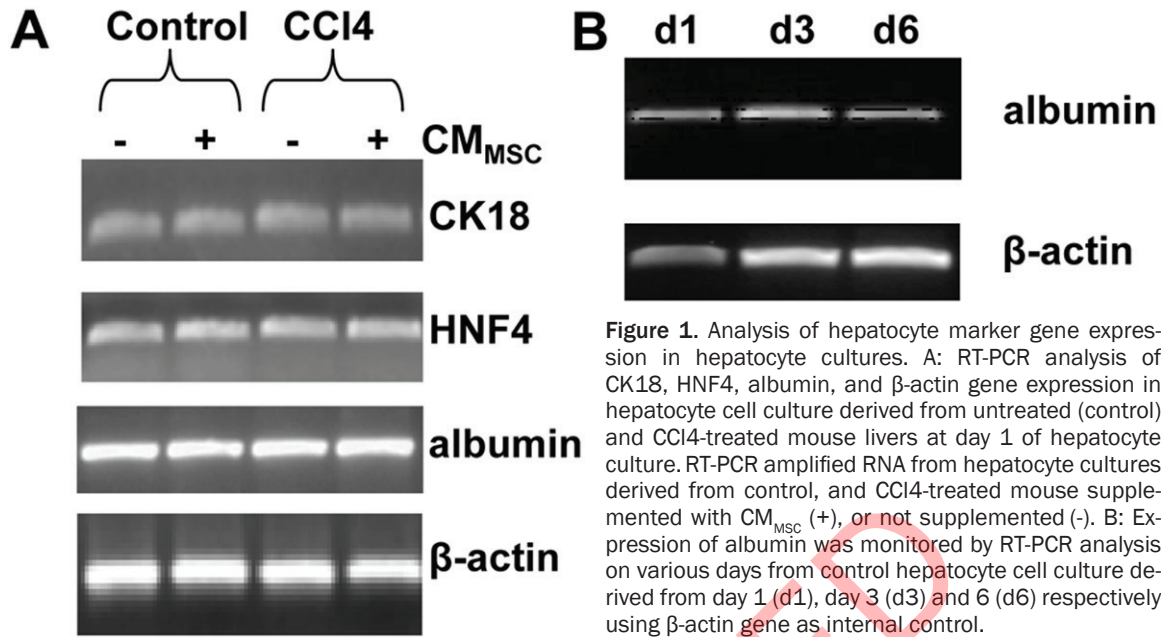
CD44-PE antibodies and Annexin-FITC was calculated after accumulation of one hundred five thousands and seven hundred beads (105700) from Cytocount (ZEBRA BIOSCIENCE, NL). A 530/30 band pass filter was used for FL1 fluorescence to measure binding of Annexin V FITC. A 585/42 band pass filter was used for FL2 fluorescence to measure binding of PE-labeled antibodies. Data were analyzed using FACS Diva 6.1.2 software (BD, USA).

### *RT-PCR*

Total RNA was isolated using Trisure (Bioline, UK) according to the manufacturer's instructions. For cDNA synthesis 3  $\mu\text{g}$  RNA were used as template in RT-PCR. The following primer sequences were used: albumin, 5'-GCATGAA-GTTGCCAGAAGACATCC-3' (forward) and 5'-TCT-GCAGTTTGCTGGAGATAGTCG-3' (reverse); HNF-4, 5'-AAATGTGCAGGTGTTGACCA-3' (forward) and 5'-GCTGTCCTCGTAGCTTGACC-3' (reverse); cytokeratin, 18 5'-CGATACAAGGCACAGATG-GA-3' (forward) and 5'-CTTCTCCATCCTCCAGC-AAG-3' (reverse);  $\beta$ -actin 5'-TGGAATCCTGTGGC-ATCCATGAAAC-3' (forward) and 5'-TAAAACGCA-GCTCAGTAACAGTCCG-3' (reverse).

### *Gene expression using the comparative method ( $\Delta\Delta\text{CT}$ )*

Total RNA was extracted using Qiagen RNeasy Mini kits (Qiagen, Germany) from the hepatocyte culture. Quantitative real-time PCR (qRT-PCR) was used to determine the FGL1 gene expression at different days. For gene expression analysis, pre-designed gene-specific primer pairs were used (Primer Express 3.0 program, Applied Biosystems, Foster City, USA). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene served as endogenous control. The reverse transcription reaction was performed with 2  $\mu\text{g}$  of total RNA, random hexamer primers and the AMV reverse transcriptase (Promega, Madison, WI) using SYBR-Green PCR master mix (SA Biosciences, Foster City, USA), the manufacturer's instructions and a 7500 Fast Real-Time PCR System (ABI, USA). Each PCR reaction was performed in triplicate and the average threshold cycle (ct) was used for the relative quantity (RQ) calculation after normalization to GAPDH. For qRT-PCR analysis of the mRNA populations the following primers were used FGL1-F (5'-CTCAGGTGCACCAGCTT-GAG-3'), FGL1-R (5'-TCCGATCCTTATCCAGAAA-



**Figure 1.** Analysis of hepatocyte marker gene expression in hepatocyte cultures. **A:** RT-PCR analysis of CK18, HNF4, albumin, and  $\beta$ -actin gene expression in hepatocyte cell culture derived from untreated (control) and CCl<sub>4</sub>-treated mouse livers at day 1 of hepatocyte culture. RT-PCR amplified RNA from hepatocyte cultures derived from control, and CCl<sub>4</sub>-treated mouse supplemented with CM<sub>MSC</sub> (+), or not supplemented (-). **B:** Expression of albumin was monitored by RT-PCR analysis on various days from control hepatocyte cell culture derived from day 1 (d1), day 3 (d3) and 6 (d6) respectively using  $\beta$ -actin gene as internal control.

CTG-3') and GAPDH-F (5'-TGTGTCGTCGTGG-ATCT-3'); GAPDH-R (5'-TTGCTGTTGAAGTCGCA-GGAG-3').

Total RNA was extracted from IL6-stimulated hepatocytes (100 ng/ml) (R&D, USA). Each sample was analysed using one-step qRT-PCR protocol according to the manufacturer's instructions (Kappa Biosystems, USA). FGL1 and GAPDH reference gene sequence has been amplified in triplicate wells for 45 cycles on the same plate.

#### Determination of IL6

Interleukin 6 (IL-6) concentration in hepatocyte culture supernatants was determined by enzyme-linked immunosorbent assay using the Quantikine mouse IL-6 kit (R&D, MN, USA) in accordance to manufacturer's instructions.

#### Statistical analysis

Results are reported as the mean  $\pm$  SEM. Multiple comparisons were performed by the two-way analysis of variance (ANOVA). *P* values < 0.05 were considered significantly different.

### Results

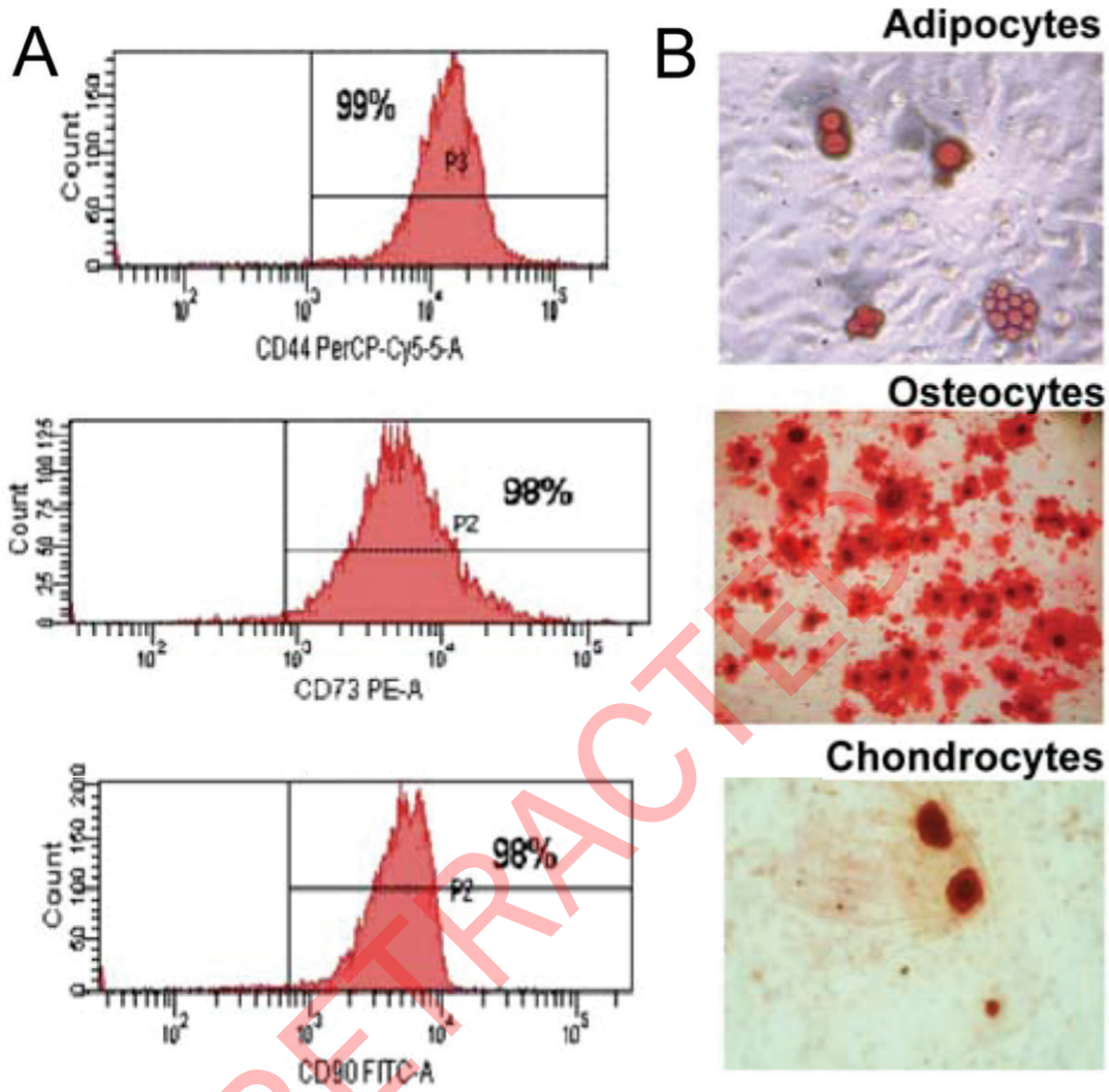
#### Gene expression of liver specific markers in hepatocytes

RT-PCR analysis of RNA isolated from cultured primary hepatocytes derived from untreated

animals (control) or animals treated with CCl<sub>4</sub>, revealed strong expression of the hepatocyte specific genes cytokeratin 18, HNF4 and albumin at day 1 of culture (**Figure 1A**). Furthermore, time course experiments revealed stable albumin gene expression up to six days post culture initiation (**Figure 1B**). In addition, Northern analysis revealed stable levels of albumin or  $\alpha$ 1-antitrypsin mRNA levels in mouse primary hepatocytes cultured for 7 days that were reduced by day 9 (data not show). These data indicate that mouse primary hepatocytes maintain their differentiation under the culture conditions used. CM<sub>MSC</sub> from bone marrow-derived cells, exhibiting characteristic MSCs immunophenotype and differentiation capacity (**Figure 2A and B**), did not affect cytokeratin 18, albumin and HNF4 gene expression (**Figure 1A**).

#### Effect of CM<sub>MSC</sub> on hepatocyte apoptosis

MSCs secrete a variety of growth factors and cytokines that inhibit hepatocellular death. To examine whether CM<sub>MSC</sub> affect early apoptosis of hepatocytes, annexin V levels were determined in cultures of control or CCl<sub>4</sub> treated hepatocytes. To discriminate between apoptotic and necrotic cells the relative amount of the annexin V and propidium iodide (PI)-stained cells in the population were determined. Cultured hepatocytes derived from mice following a 3-day CCl<sub>4</sub> treatment and addition of CM<sub>MSC</sub> in culture medium resulted in significant



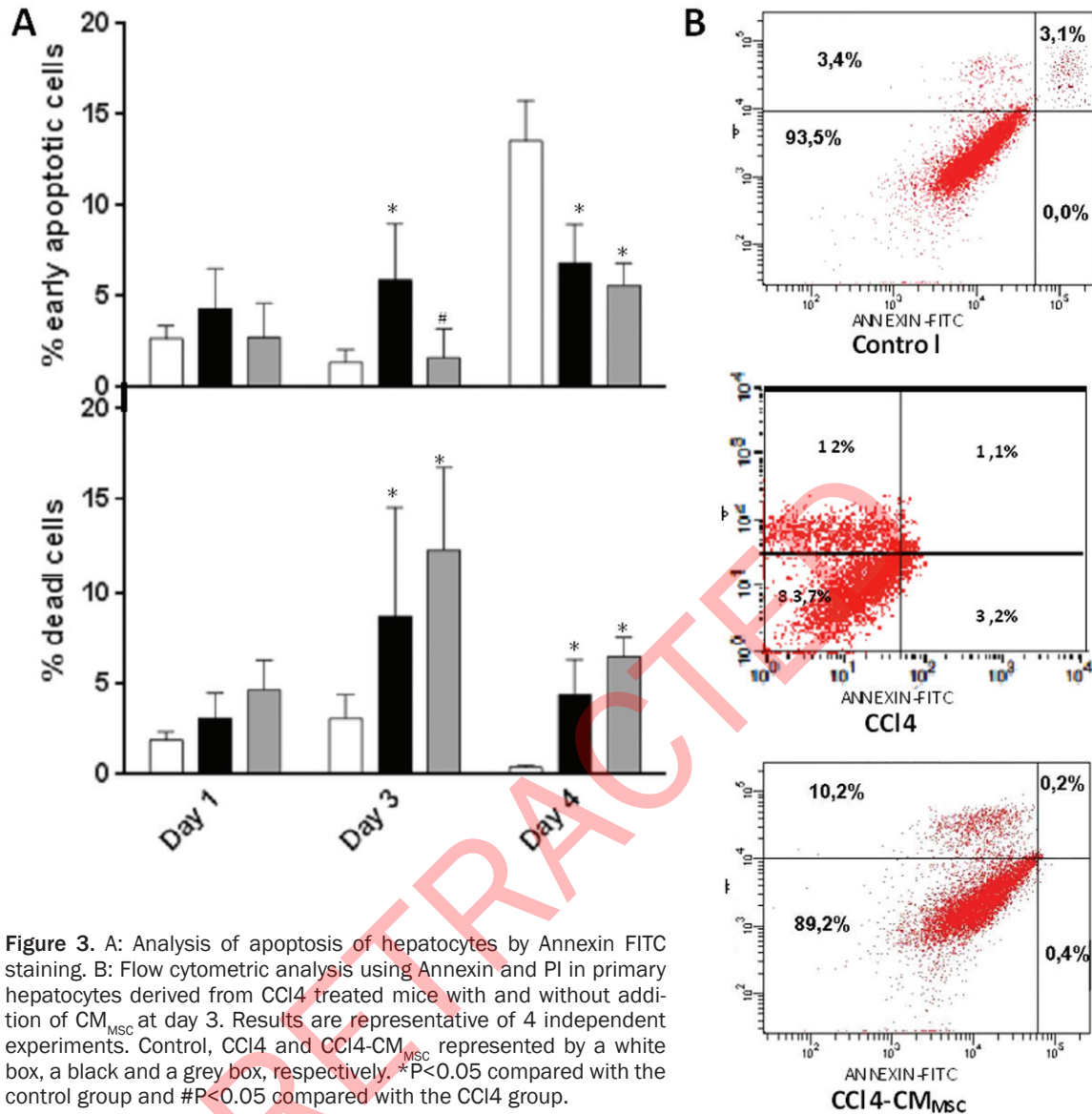
**Figure 2.** MSC characterization. A: Flow cytometric analysis of MSC cells. The cells at passage 4 were labeled with the CD44-Cy5 antibody, CD73-PE and CD90-FITC against the CD45-PE. Values show the percentage of positive cells. B: Assays for MSC differentiation. MSCs were cultured with lineage-specific media for 3 to 4 weeks to induce cell differentiation. Differentiation into adipocytes, osteocytes and chondrocytes was detected by oil Red O, alizarin red and alcian blue, respectively. Original magnification x 20 for adipocytes and chondrocytes and x 10 for osteocytes.

decrease of Annexin V positive cells/PI(-) ( $p < 0.01$ ) compared to CCl<sub>4</sub> treated hepatocytes suggesting that CM<sub>MSC</sub> prevented activation of early apoptosis at days 3 ( $p < 0.001$ ) (Figure 3A and B). At day 3, the percentage of dead cells in CCl<sub>4</sub> and CCl<sub>4</sub>-CM<sub>MSC</sub> annexin V (+)/PI(+) was higher compared to control cells. At day 4, an increase of annexin V(+)/PI(-) was observed in CCl<sub>4</sub>-CM<sub>MSC</sub> and control groups. CCl<sub>4</sub> and CCl<sub>4</sub>-CM<sub>MSC</sub> annexin (+)/PI(+) cells were significantly higher compared to the con-

trol cells in contrast to CCl<sub>4</sub> and CCl<sub>4</sub>-CM<sub>MSC</sub> early apoptotic cells (Figure 3A). These results indicate that CM<sub>MSC</sub> decreases early apoptosis rate in hepatocytes derived from CCl<sub>4</sub> treated mice.

#### Characterization of microparticles formed by MSC

MPs are membrane vesicles originating from blebbing membranes of apoptotic cells and can



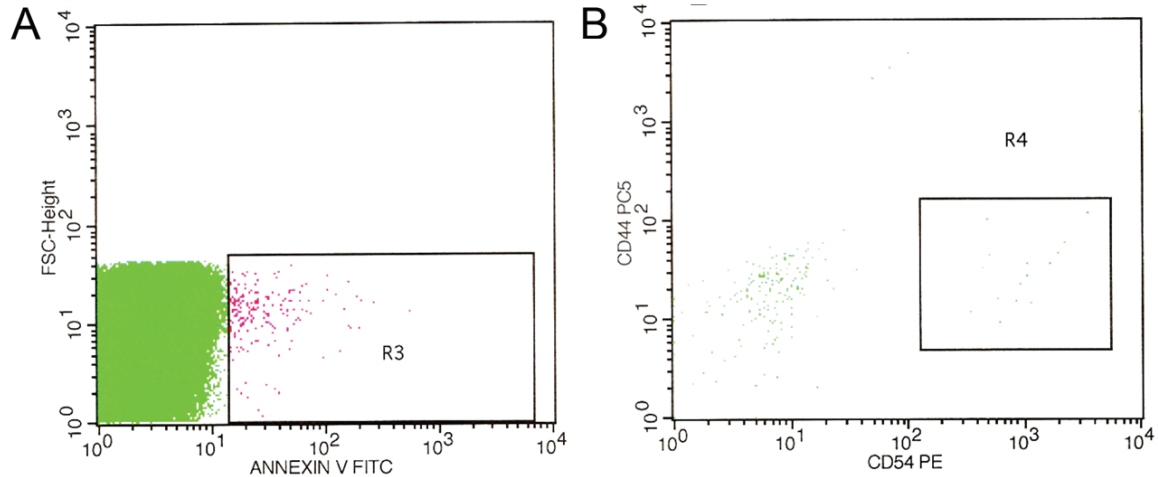
**Figure 3.** A: Analysis of apoptosis of hepatocytes by Annexin FITC staining. B: Flow cytometric analysis using Annexin and PI in primary hepatocytes derived from CCI4 treated mice with and without addition of CM<sub>MSC</sub> at day 3. Results are representative of 4 independent experiments. Control, CCI4 and CCI4-CM<sub>MSC</sub> represented by a white box, a black and a grey box, respectively. \*P<0.05 compared with the control group and #P<0.05 compared with the CCI4 group.

be identified by annexin V staining. These microparticles may contain surface glycoproteins and other molecules. The identification of MPs was assessed by flow cytometry based on size (scatter characteristics) and membrane marker expression. Small percentage of CM<sub>MSC</sub>-MPs expressed phosphatidylserine as demonstrated by binding of Annexin V localized within (R3) region (**Figure 4A**). Flow cytometry analysis showed 7,46% of annexin V-positive MPs reacted with Ab against CD44 and CD54 localized within the (R4) region (**Figure 4B**). These data demonstrate that MPs are generated by MSC and express membrane antigen CD54 and CD44.

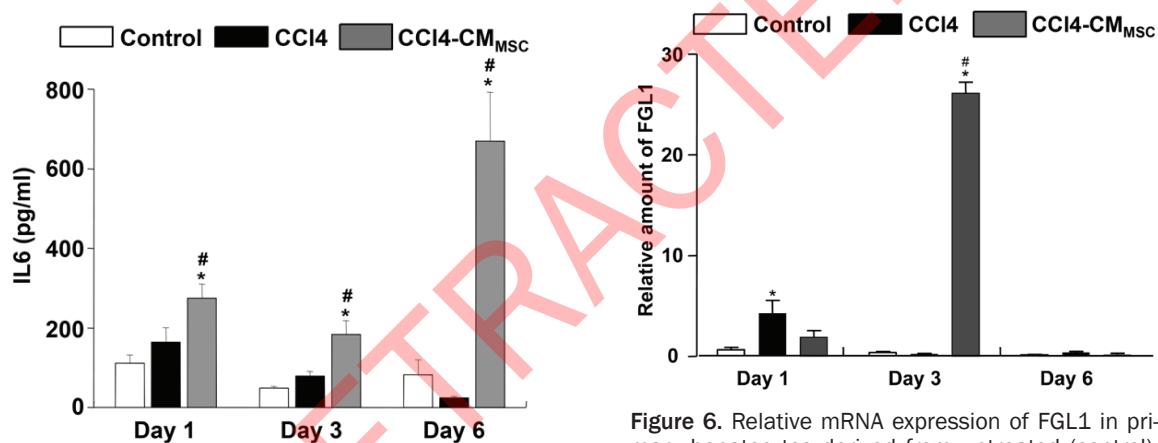
*IL-6 is highly secreted in the CCI4-CM<sub>MSC</sub> of primary hepatocytes*

Under stress conditions in the liver, IL-6 is a major regulator of the acute phase response participating also in liver regeneration [28]. Under similar conditions, MSCs also produce IL-6 [5, 6]. To determine IL-6 production in supernatants of hepatocyte cultures, we performed enzyme-linked immunosorbent assay. In MSC-conditioned medium, the IL-6 concentration was 319,8 ± 40,95 pg/ml. The data presented in **Figure 6** show that IL-6 production is increased in hepatocytes treated with CCI4 and CCI4-CM<sub>MSC</sub> compared to control hepatocytes

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**Figure 4.** Detection of microparticles from  $CM_{MSC}$ . A: Microparticles isolated from MSC supernatants were stained using CD54-PE and CD44-Cy5 antibodies and (B) microparticles were stained with annexin V and detected by flow cytometry.



**Figure 5.**  $CM_{MSC}$  induced IL6 secretion in hepatocytes. Cell supernatant was collected and analyzed by ELISA for IL6. Values are means  $\pm$  SEM. At least 3 animals were examined in each group. Control, CCI4 and CCI4- $CM_{MSC}$  represented by a white box, a black and a grey box, respectively. \*, # $P < 0.05$  compared with the control and CCI4 groups respectively.

**Figure 6.** Relative mRNA expression of FGL1 in primary hepatocytes derived from untreated (control), CCI4-treated mice and CCI4-treated mice supplemented with  $CM_{MSC}$  and was analysed by real time PCR. Values are expressed relative to GAPDH expression. Total RNA was extracted at days 1, 3 and 6 from primary hepatocytes and was analysed by real time PCR. Values are the mean  $\pm$  SD of triplicate measurements \*, # $P < 0.05$  compared with the control and CCI4 groups respectively.

on days 1, 3 in culture ( $p < 0.001$ ). IL-6 production was significantly higher in CCI4- $CM_{MSC}$  compared to CCI4 cells (day 1 vs day 6  $p < 0.001$ , day 6 vs day 3  $p < 0.01$ ) (Figure 5). This finding indicates that  $CM_{MSC}$  augmented IL6 production.

### FGL1 expression in primary hepatocytes

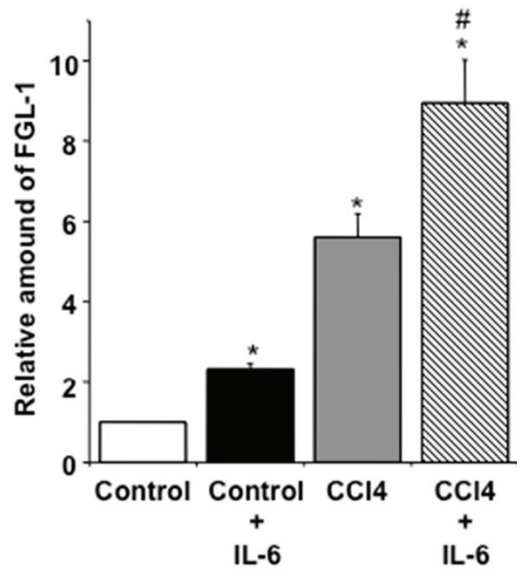
It was recently reported that IL-6 induces expression of the mitogenic factor FGL1 *in vitro*, which is produced by parenchymal hepatocytes [15, 17]. Relative quantification by real

time PCR of FGL1 showed a marked increase by day 3 in hepatocytes derived from CCI4-treated mice and exposed to  $CM_{MSC}$ , followed by a significant decline on culture day 6 (Figure 6). In hepatocytes derived from control livers, levels of FGL1 expression were relatively low.

### IL6 induces FGL1 expression in primary hepatocytes

In order to assess the effect of IL6 in FGL1 expression in mouse primary hepatocytes,

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**Figure 7.** Total RNA was extracted from primary hepatocytes after 24 hrs treatment with recombinant IL6 at the concentration of 100 ng/ml for 24 hrs. Control, CCI4 and CCI4-CM<sub>MSC</sub> represented by a white box, a black and a grey box, respectively. Values are the mean  $\pm$  SD of triplicate measurements \*, #P<0.05 compared with the control and CCI4 groups respectively.

cells exposed to recombinant IL-6 at the concentration of 100 ng/ml for 24 hrs after dose response analysis (data not shown). The experiment demonstrate that there is an induction of FGL1 mRNA by IL-6 within 24 hours compared to cells derived from control mice but there is stronger induction in cells derived from CCI4-treated mice (**Figure 7**). These results indicate that FGL1 is activated at early stages of acute hepatocyte injury.

### Discussion

This study demonstrates the early antiapoptotic activity of BM-MSC conditioned medium (CM<sub>MSC</sub>) on apoptosis induced in mouse primary hepatocytes by CCI4 and indicates that this activity is mediated by IL-6 and FGL1 overproduction. Our findings suggest that CM<sub>MSC</sub> contains factors that might be used as a therapeutic tool in acute liver injury.

There are reports in the literature showing that paracrine factors released by MSCs exhibit protective organ-actions [29, 30]. The present work explored putative factors in a mouse model of CCI4-induced acute liver injury. Hepatocytes were isolated and cultured in the pres-

ence and absence of CM<sub>MSC</sub>. CM<sub>MSC</sub> significantly decreased the number of cultured hepatocytes undergoing early apoptosis following in vivo exposure to CCI4 with no significant difference at the percentage of dead cells. This indicates that CM<sub>MSC</sub> delays CCI4-induced apoptosis.

MSC-derived molecules modulate hepatocellular death and regeneration in a D-galactosamine-induced rat model [2]. Further evidence suggests that CM<sub>MSC</sub>-derived microparticles induces proliferation and apoptosis resistance in hepatocytes [23]. In our study, we detected few annexin V-positive microparticles released by MSCs. Flow cytometry analysis showed that 7,8% of annexin V-positive binding MPs co-expressed CD54/CD44 meaning that MP may contain signaling molecules which can regulate apoptosis. In parallel, there are annexin V-negative MPs, which may activate cell proliferation and tissue regeneration [21]. Further studies will be necessary to clarify the precise mechanism of action of microparticles.

MSCs as well as hepatocytes produce IL6 [13, 14]. IL6 has a hepatoprotective function by preventing CCI4 toxicity [14, 15]. In our experiments, IL6 levels were increased in CCI4-CM<sub>MSC</sub> hepatocytes. Hepatocytes are triggered to release IL6 especially in CCI4-CM<sub>MSC</sub> cells through autocrine or paracrine function of IL6. The significantly higher IL6 levels in CCI4-CM<sub>MSC</sub> hepatocytes compared to control and CCI4 hepatocytes on the first day of culture indicate that IL6 is derived from CM<sub>MSC</sub>. Subsequently, IL6 was increased simultaneously with a decrease of early apoptosis suggesting that it might promote survival. However, the increased IL6 levels on the last day of culture contributes to the progression of apoptosis. Therefore IL6 regulates the apoptosis of hepatocytes derived from CCI4-treated mice.

Our study reinforces previous observation that IL6 induces FGL1 expression [17]. IL6 induces FGL1 expression in hepatocytes derived from control and CCI4-treated mice. The FGL1 induction was higher in hepatocytes from CCI4 treated mice compared to control. FGL1 has been characterised mainly as a mitogenic and survival factor [19, 31] although there are indications that the human FGL1 suppress proliferation in hepatocellular carcinoma cells [17]. Our data indicate that FGL1 gene expression was upregulated when early apoptosis (culture day



3) was inhibited in CCl<sub>4</sub>-CM<sub>MSC</sub> hepatocytes with a subsequent decrease on the last day (culture day 6). Thus, it would be reasonable to postulate that IL6 and FGL1 contribute to the antiapoptotic effect of CM<sub>MSC</sub> treatment noticed on culture day 3. Taken together this data suggest that IL6 contained in CM<sub>MSC</sub> delays apoptosis.

The downstream factors in IL-6 signal transduction pathways, nuclear factor for IL6 (NF-IL6) as well as signal transducer and activator of transcription 3 (STAT-3) show conserved DNA binding sites at the 5' flanking region of FGL1 in rats [19]. The upregulation of FGL1, which occurred when early apoptosis (culture day 3) was inhibited, may suggest that IL6 modulates the apoptotic process in acute liver injury.

Taken together, our observations indicate that BM-MS-C conditioned medium contains factors that could exert a potential therapeutic benefit in CCl<sub>4</sub>-induced acute hepatocyte injury disease.

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#### Declaration

All authors declare no conflict of interest.

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