

Bone Marrow Cells Reduce Collagen Deposition in the Rat Model of Common Bile Duct Ligation

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Abstract

Background and Aim: Transplantation of bone marrow cells (BMC) was shown to improve liver function in animal models of cirrhosis. In this work we evaluated the effects of mononuclear BMC transplantation in rats submitted to bile duct ligation (BDL), a model of cholestatic liver disease.

Methods: BDL was performed on Wistar rats, and two weeks later animals underwent a liver biopsy. At the same time, BMC group was injected with 1×10^6 mononuclear BMC and compared to untreated (BDL) and a fake-surgery (Sham) group. Animals were sacrificed two weeks later. Alkaline Phosphatase (ALP) and collagen deposition quantified by Sirius red staining were analyzed at both time points. MMP-9 expression assessed by immunohistochemistry and liver oxidative stress parameters (TBARS, SOD and catalase activity) was performed at four weeks. The effect of BMC-conditioned medium upon activated hepatic stellate cells was tested *in vitro* by MTT using GRX cells.

Results: Treated animals showed a 25% decrease in ALP levels at four weeks. Collagen deposition in untreated group at 4 weeks had values 2-fold higher, compared to those found in the biopsy. In contrast, BMC-treated rats barely increased collagen deposition after treatment ($p < 0.01$). No difference was observed in MMP-9 expression nor in oxidative stress parameters. BMC-conditioned medium was able to induce cell death on GRX cells *in vitro*.

Conclusions: A decrease in collagen deposition and a reduction in ALP levels suggest a better outcome in the treated group. The effect of BMC conditioned medium *in vitro* suggests a possible mechanism for the reduction of fibrosis observed *in vivo*.

Keywords: Cholestasis; Liver fibrosis; Cell therapy; Cell transplantation; Conditioned medium

Abbreviations: HCPA: Hospital de Clínicas de Porto Alegre; UFRGS: Universidade Federal de Rio Grande do Sul; UFCSPA: Universidade Federal de Ciências da Saúde de Porto Alegre; BMC: Bone Marrow Cell; BDL: Bile Duct Ligation; ALP: Alkaline Phosphatase; MMP-9: Matrix Metalloproteinase 9; TBARS: Thiobarbituric Acid Reactive Substances; SOD: Superoxide dismutase; MTT: 3-(4,5 dimethylthiazolyl)-2,5-diphenyl-2H-tetrazolium; CREAL: Centro de Reprodução e Experimentação de Animais em Laboratório; ICBS: Instituto de Ciências Básicas da Saúde; UEA: Unidade de Experimentação Animal; PBS: Phosphate Buffered Saline; DMEM: Dulbecco's Modified Eagle Media; DAPI: 4',6-diamidino-2-phenylindole; DNA: Deoxyribose Nucleic Acid; PCR: Polymerase Chain Reaction; Sry: Sex-determining region Y; Gapdh: Glyceraldehyde-3-Phosphate Dehydrogenase; H_2O_2 : Hydrogen peroxide; FCS: Fetal Calf Serum; CM: Conditioned Medium; SPSS: Statistical Package for Social Sciences; ANOVA: Analysis of Variance; MMP-13: Matrix Metalloproteinase 13

Introduction

Liver fibrosis, and its outcome, cirrhosis, result from an imbalance in extracellular matrix synthesis and degradation. This process is mediated mainly by activated hepatic stellate cells (HSC) undergoing a phenotypic switch from a quiescent vitamin A storing phenotype to a proliferative myofibroblast-like cell [1]. Cirrhosis represents a serious

worldwide health care problem and may be caused by genetic disorders [2], viruses [3], toxins [4], auto-immune [5,6], and cholestatic diseases [7]. Liver transplantation is a highly successful treatment for end-stage cirrhosis, with a 5-year survival rate of around 70%. However, limited availability of organs and comorbid factors are still major problems [8].

Animal models of chronic liver injury showed that transplantation of bone marrow cells (BMC) can improve liver function, as well as the survival rate, although through mechanisms not fully understood [9,10]. Both cell fusion and transdifferentiation into hepatocytes have been reported [11,12]. Although these events seem to be very rare (less than 0.5%), suggesting that other mechanisms are involved in liver regeneration mediated by BMC [13]. However, data on this issue were obtained in animal models of liver disease induced by toxins (as CCl_4

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or acetaminophen), whose mechanisms differ from those of cholestatic liver diseases, one of the main causes of liver failure in children [14].

Taking that into account, in this work we evaluated the effects of mononuclear bone marrow cell transplantation in rats submitted to common bile duct ligation (BDL), a model of cholestatic liver disease. We also searched for possible mechanisms which could contribute to the amelioration of liver fibrosis produced by these cells.

Methods

Animals

We used 16 female Wistar rats weighing 200-240g to perform the experiments. Male rats of the same weight were used as cell donors. All animals were obtained from the Laboratory of Animal Reproduction and Experimental Center (CREAL - ICBS /UFRGS). Rats were kept at the Experimental Animal Unit of Research Center of Hospital de Clínicas de Porto Alegre (UEA-HCPA), under controlled temperature (between 18 and 22°C) in light-dark cycles of 12 hours. Standard rat chow and water were given *ad libitum*. The study was approved by our local ethics committee and complied with National Guidelines on Animal Care.

Experimental design

In order to access all the experimental conditions, female Wistar rats were divided into three groups. Bone marrow cells (BMC) group: rats (n=6) were submitted to a common bile duct ligation surgery and two weeks later were injected with 1×10^6 BMC in 0.2 mL of phosphate buffer saline (PBS) suspension. BDL group: rats (n=5) were submitted to the same surgical procedure of BMC group, but received only 0.2 mL of PBS with no cells. Sham group: rats (n=5) were submitted to the same surgical procedure of the two first groups, but bile duct was not ligated or transected and there was no posterior intervention. All animals were sacrificed four weeks after the first surgical procedure.

Animal surgery

Rats underwent BDL at 12 weeks of age, four weeks before sacrifice. They were anesthetized with an intraperitoneal injection of Ketamine 100mg/kg (Eurofarma Lab-Brazil) 10% and 10mg/kg of Xylazine (Sespo) 2%. After performing a 3 cm incision in the abdomen, the common bile duct was ligated two times with 4-0 silk and transected on both ends [15]. Sham operation was performed similarly except that the bile duct was not ligated or transected.

Isolation and staining of BMC

Cell isolation and staining was performed as described elsewhere [16]. Briefly, male Wistar rats were sacrificed in CO₂ chambers, the femurs and tibias were isolated and whole bone marrow was flushed with DMEM (Gibco, USA). Cells were then placed onto a Ficoll Histopaque (GE-Healthcare, USA) layer and centrifuged at 800 x g for 30 minutes. Cell viability was determined by trypan blue exclusion test. DAPI [4',6-diamidino-2-phenylindole 2,7 mg/mL (Roche, Germany)] fluorescent staining was performed according to manufacturer's protocol. Cells were then counted again and adjusted to a final concentration of 5×10^6 cells/mL in PBS. Three independent Ficoll isolation procedures were analyzed by simple size/ scatter flow cytometry, aiming to characterize and count the mononuclear cell fraction, and results were described elsewhere [17].

Cell transplantation

Two weeks after BDL, animals from BMC group, were anesthetized with Ketamine and Xylazine just before transplant. A 3 cm longitudinal incision was performed, the mesenteric vein was exposed and 0.2 mL of the cell suspension (1×10^6 cells) was injected *in bolus*. To avoid bleeding, porcine collagen matrix was placed over the vein. Animals were sutured in layers and allowed to recover in the cage. BDL group was submitted to the same surgical procedure, although receiving 0.2 mL of PBS with no cells. A small liver biopsy (0.2x1.0 cm) was collected just before cell or PBS injection and fixed in 10% formalin solution for 24h before embedment in paraffin wax for histological analysis.

Histological analysis

Hematoxylin-Eosin as well as Sirius red staining were performed on the liver biopsies specimens obtained at the time of BDL surgery and at the sacrifice, 2 and 4 weeks later. Immunohistochemical staining for matrix metalloproteinase 9 (MMP-9) was performed in paraffin sections using the antibody from Santa Cruz Biotechnology (SC 6840). MMP-9 expression was graded as null, moderate or intense. Liver histological analysis was performed by a pathologist blind to the treatment groups. Presence of DAPI-stained transplanted cells was evaluated in unstained liver sections under fluorescent microscopy in high power fields.

To assess the extent of collagen deposition we used a computerized image analysis system, carrying out a technique adapted from Masseroli, et al. [18] Adobe Photoshop CS3 was used to calculate the percent area of positively-stained tissue for Sirius Red, evaluating 10 high-power fields (400X) from each liver section of each animal. The positive red staining was assessed as an amount of pixels, which was divided by the total amount of pixels from each image. Results were expressed as percentage of red stained area.

Y chromosome detection

DNA extraction was performed on liver samples collected at sacrifice. PCR for *Sry* gene was performed using *Gapdh* as internal control, as described by Baldo, et al. [17].

Liver test of cholestasis (alkaline phosphatase)

On two and four weeks after BDL, blood was collected into a heparinized glass capillary from the retro-ocular sinus for the analysis of alkaline phosphatase (ALP), which was measured using commercial kits (Boehringer Mannheim) [19].

Lipid peroxidation assay

Frozen liver specimens were used to determine oxidative stress parameters. Liver lipid peroxidation was determined by measuring the concentration of thiobarbituric acid reactive substances (TBARS). The amount of aldehydic products generated by lipid peroxidation was quantified using 3 mg of protein per sample. The samples were incubated at 90°C for 30 min after adding 500 µL of 0.67% thiobarbituric acid in 10% trichloroacetic acid, then centrifuged at 4°C at 2000 x g for 15 minutes. Spectrophotometric absorbance was determined in the supernatant at 535nm [20].

Antioxidant enzymes activity

Livers were homogenized in ice-cold phosphate buffer (KCl 140 mM, phosphate 20 mM, pH 7.4) and centrifuged at 14,000 x g for 10 min. Catalase (EC 1.11.1.6) activity was determined by measuring the

exponential disappearance of H_2O_2 at 240 nm and was expressed as U/ g protein [21]. Cytosolic superoxide dismutase (SOD; EC 1.15.1.1) was assayed according to Misra and Fridovich (1972) at 30°C [22]. The rate of auto-oxidation of epinephrine, which is progressively inhibited by increasing amounts of SOD in the homogenate, was monitored spectrophotometrically at 560 nm. The amount of enzyme that inhibits epinephrine auto-oxidation at 50% of the maximum inhibition was defined as 1 U of SOD activity.

In vitro effects of BMC upon activated hepatic stellate cells

Bone marrow cells were isolated as described previously and plated at 1×10^5 cell/cm² in 6-well plate and cultured in DMEM medium supplied with 10% of FCS and 1% Penicillin/Streptomycin (normal medium). Forty-eight hours after that, the medium was collected, centrifuged and filtered using 0.22 μ m filters. This conditioned medium (CM) was then added in a proportion of 1:2 with normal medium to GRX cells (CR001, obtained from the Rio de Janeiro Cell Bank, Rio de Janeiro, Brazil), plated at 2×10^3 cells/cm² in 96-well plates. A control group received normal medium. Five days after treatment, cell viability was assessed to verify the effects of CM using MTT [3-(4,5 dimethylthiazolyl)-2,5-diphenyl-2H-tetrazolium], as described by Balestrin et al. [23]. Results were shown as percentage, considering the control group as 100% of living cells.

Statistical analysis

Statistical analysis was performed using SPSS (Statistical Package for Social Sciences) version 14.0. ANOVA for repeated measures and Duncan's test were performed to detect changes among the groups when parameters were analyzed at 2 and 4 weeks. One way ANOVA and Duncan's test were used to compare biochemical oxidative stress parameters at 4 weeks. Student t-test was performed for MTT analysis. A value of $p < 0.05$ was considered statistically significant.

Results

Animal model

Rats who underwent BDL presented features of jaundice, such as yellow ears and tail. Portal hypertension was evidenced by dilatation of the splenic veins observed at the time of cell injection and at sacrifice. Sham rats presented none of these features throughout the study.

Liver test of cholestasis (alkaline phosphatase)

In the Sham group we found normal ALP levels both at two (64.2±16.5 U/L) and four weeks (57.4±12.4 U/L). In the BDL group ALP presented a 4-fold increase, with mean values of 261.5±30.9 U/L (two weeks) and 241.8±40.1 U/L (four weeks). A similar increase was observed in BMC group at two weeks (239.0±41.0 U/L), while a 25% decrease in four weeks (179.1±51.6 U/L) was observed compared with BDL group ($p < 0.05$).

Histological analysis and cell tracking

Two weeks after BDL, rats presented moderate levels of bile duct proliferation, with mild fibrosis associated to injury areas and inflammatory infiltrate. Histological appearance was similar in BDL and BMC rats, and strikingly different from the sham group. Four weeks after BDL, an additional increase in bile duct proliferation could be observed in both groups (BDL and BMC). Sham group presented normal histological aspects and no inflammatory activity could be observed (Figure 1).

Prior to injection, isolated BMC were stained with DAPI. Two weeks after, DAPI-positive cells could still be visualized in livers from treated animals. Cells were located on liver parenchyma mostly as isolated cells, and some of them assumed a hepatocyte-like phenotype (Figure 2A). An increased number of stained cells could be visualized near areas of bile duct proliferation areas (Figure 2B). Positive results for the *Sry* PCR confirmed the presence of male-derived cells in the liver (Figure 2C). Fluorescent cells in livers from BDL and Sham groups were not observed, and PCR for *Sry* was also negative (data not shown).

Assessment of the extent of collagen deposition

Collagen deposition quantified by Sirius red staining was evaluated in livers from Sham, BDL, and BMC groups after two and four weeks after BDL. We did not observe differences at two weeks between BMC and LDB rats (data not shown). At four weeks, collagen levels in BDL group presented values almost twice as high as they did at two weeks (Figure 3D). On the other hand, BMC-treated rats presented levels barely increased, compared with 2 weeks ($p < 0.01$). Sham animals always had the lowest collagen levels, as expected. Figure 3A-C shows a slide representative of each group at 4 weeks.

MMP-9 expression

MMP-9 expression was evaluated by immunohistochemistry (Figure 4). MMP-9 was undetectable in the sham group; however it increased to moderate levels on both BDL and BMC groups, without difference between them using the semi-quantitative score.

Oxidative stress parameters

Lipid peroxidation, SOD and catalase activities were assessed in rat livers (Table 1). We were able to show that BDL induced a significant

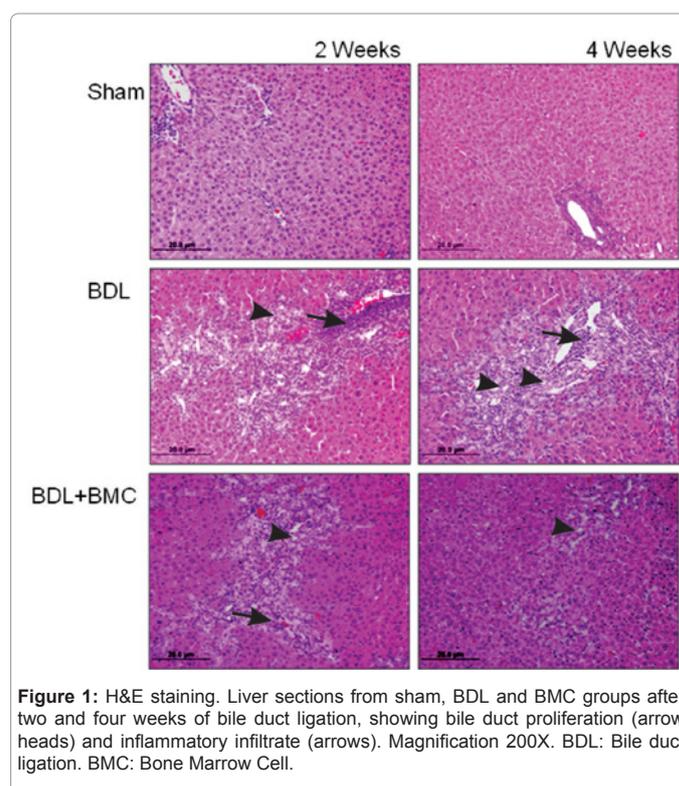


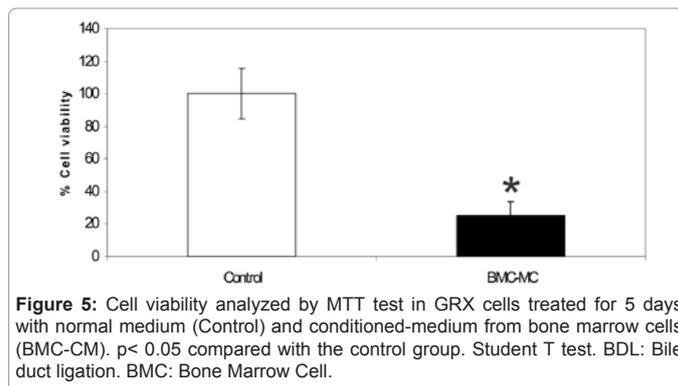
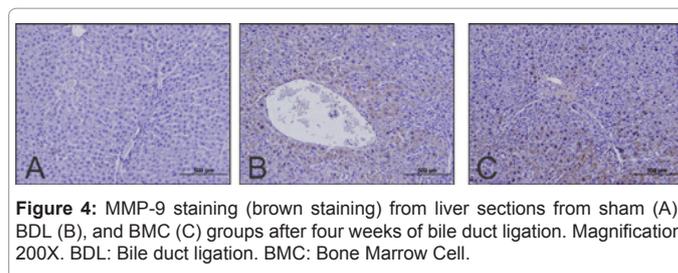
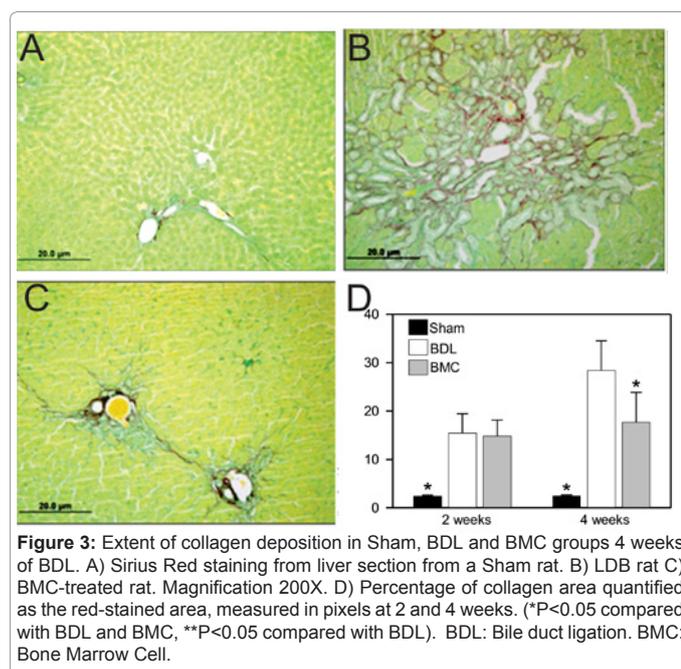
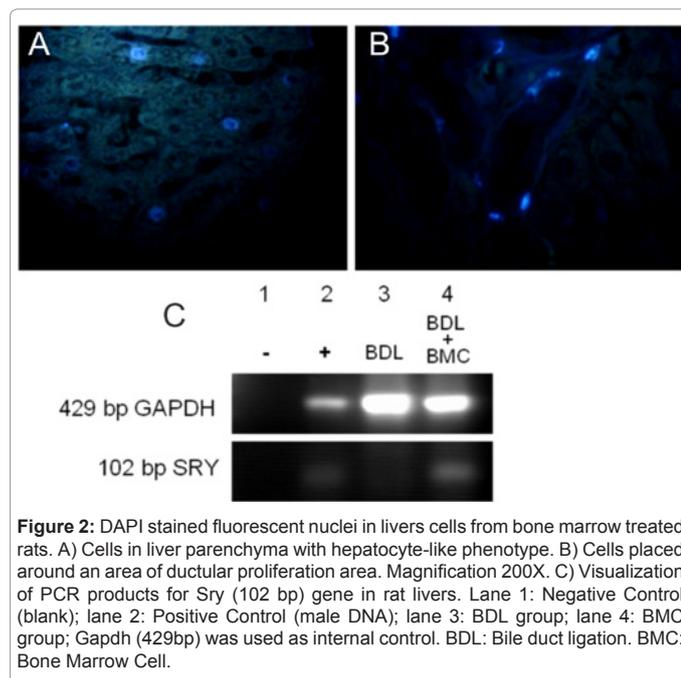
Figure 1: H&E staining. Liver sections from sham, BDL and BMC groups after two and four weeks of bile duct ligation, showing bile duct proliferation (arrow heads) and inflammatory infiltrate (arrows). Magnification 200X. BDL: Bile duct ligation. BMC: Bone Marrow Cell.

increase in TBARS levels and a reduction in SOD activity, compared with sham animals ($p < 0.05$). However, cell therapy was not able to reduce lipid peroxidation or increase SOD activity in BMC group. Catalase activity was not different among groups.

	Sham	BDL	BMC
Liver TBARS (nmol/ mg prot)	0.23 ± 0.07	0.67 ± 0.02*	0.87 ± 0.49*
Liver SOD (U/mg)	302.71 ± 173.31	137.82 ± 63.94*	57.55 ± 12.10*
Liver CAT (nmol/ mg prot)	0.65 ± 0.45	1.07 ± 1.16	0.88 ± 0.72

Results are expressed as mean ± s.d for 3-6 animals/group.* $p < 0.05$ compared to sham group, Oneway ANOVA and Duncan's test post hoc.

Table 1: Oxidative stress parameters in rats four weeks after common bile duct ligation.



In vitro effects of BMC upon activated hepatic stellate cells

To verify if the BMC secrete cytokines that can act upon stellate hepatic cells, we analyzed the effects of adding conditioned medium (CM) from BMC on the activated hepatic stellate cell lineage GRX. MTT test revealed that CM increased cell mortality compared with controls ($p < 0.05$) (Figure 5).

Discussion

The use of bone marrow cells [24] or its fractions [25] has been proposed as a new therapeutic approach in chronic liver failure. Bone marrow cells are readily available, becoming an appealing alternative to liver transplantation. In this work we tested the effects of bone marrow mononuclear cell transplantation in a rat model of chronic liver disease induced by common bile duct ligation. We were able to demonstrate an apparent arrest in the progression of collagen deposition in the treated animals, but not a reversal of bile duct proliferation.

Histological alterations and an increase in ALP levels confirmed the damage produced by the bile duct ligation in rats. Additionally, a small biopsy taken at 2 weeks after surgery confirmed that the BDL and BMC groups were identical regarding the extent of liver damage at the time of cell transplantation. This observation, not usual in similar studies, is important since it reduced any bias of model variability.

The presence of bone marrow cells in livers from treated animals was accessed by fluorescent DAPI staining and confirmed by PCR for detection of male derived cells. Fluorescent analysis revealed that most cells were placed in areas of bile duct proliferation. Whether these cells are attracted to the areas of increased tissue damage and/or are transdifferentiating into cholangiocytes we could not determine. Other groups did not verify significant stem cell transdifferentiation into cholangiocytes in alpha-naphthylisothiocyanate-induced cholestasis [26]. Thus, injected cells may be responding to cytokine release and therefore concentrating in areas of tissue damage.

A decrease in collagen deposition and a reduction in ALP levels in the BMC group suggest a better outcome in the treated groups. The anti-fibrogenic capacity of bone marrow cells was shown a few years ago [27], but the mechanisms by which these cells can ameliorate liver function still remain obscure. For example, Higashiyama et al. demonstrated that macrophages may have a key role in reducing liver fibrosis after BMC transplantation by secreting matrix metalloproteinases, mainly MMP-9 and MMP-13 [28]. However, our results do not fully support this hypothesis, since MMP-9 expression, as visualized by immunohistochemistry in treated rats was not different from BDL group. Unfortunately, it was not possible for us to analyze other MMPs, so that the overexpression of other collagenases cannot be completely discarded.

Other studies [29,30] suggested that both mesenchymal stem cells and bone marrow mononuclear cells can ameliorate tissue damage by reducing oxidative stress in the recipient animals. Therefore, we accessed the levels of lipid peroxidation and of two antioxidant enzymes aiming to verify if changes in oxidative stress represented an active mechanism in our model. Lipid peroxidation was increased after BDL. However, cell therapy was not able to decrease TBARS levels, and no differences were found on both SOD and catalase activities after treatment. These findings suggest that transplanted cells are not reducing oxidative stress in this case, although antioxidant substances have been shown to decrease oxidative stress levels in BDL rats and improve liver function [15].

On the other hand, several studies have recently suggested that the main mechanism of action for stem cells is through the secretion of paracrine factors [17,31] that can have an effect on injured tissues. To test this hypothesis we performed an *in vitro* study using a myofibroblast cell line (GRX). Activated myofibroblasts are the main cells responsible for collagen deposition during liver fibrotic process [32]. Conditioned medium from BMC was able to induce cell death on GRX cells, suggesting that this could be a possible mechanism for the reduction of fibrosis observed *in vivo*. This toxic effect seems to be specific for this cell type, since it was not observed on primary hepatocytes [17]. Further experiments will be performed using a transwell approach aiming to strengthen these results and identify possible molecules responsible for this effect.

In summary, these results suggest that bone marrow cells may be a potential alternative for the treatment of liver fibrosis. The animal model used here has some peculiar features, since the bile duct is ligated and transected during surgery. It means that this is not a reversible model and a complete restoration of the liver architecture cannot be expected as tissue damage remains constant. Second, and maybe more important, bone marrow cells are transplanted into this environment of constant tissue damage. Our *in vitro* studies suggest that a possible mechanism for the reduction of fibrosis observed *in vivo* is the induction of death in collagen-producing cells, thus reducing the progression of collagen deposition. This induction could be performed by secretion of paracrine factors, which still need to be identified in order to ensure the safety and efficacy of this therapeutic approach.

A decrease in collagen deposition and a reduction in ALP levels suggest a better outcome in the treated group. This may suggest that bone marrow cells comprise a potential alternative for the treatment of

liver fibrosis. The effect of BMC conditioned medium *in vitro* suggests a possible mechanism for the reduction of fibrosis observed *in vivo*. Further studies regarding the mechanisms by which these cells operate in liver are needed to ensure the safety of such a therapeutic approach.

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