Journal of

Gastroenterology and Hepatology Research

Online Submissions: http://www.ghrnet.org/index./joghr/
doi:10.17554/j.issn.2224-3992.2016.05.607

Journal of GHR 2016; 5(2): 1989-1996
ISSN 2224-3992 (print) ISSN 2224-6509 (online)

ORIGINAL ARTICLE

Cirrhosis Model That Tracks Umbilical Cord Mesenchymal Cell Transplantation Using Liver Serum Parameters: A Pilot Study

Erica Oliveira Moreira, Carla de A Piccinato, Vanessa Bittencourt Pazzini, Mari Cleide Sogayar, Anna Carla Goldberg

Erica Oliveira Moreira, Hospital Israelita Albert Einstein, 627/701 São Paulo, SP, Brazil; Department of Biochemistry, Chemistry Institute, University of São Paulo, São Paulo, Brazil and Cell and Molecular Therapy Center (NUCEL), University of São Paulo, São Paulo, Brazil
Carla de A. Piccinato, Hospital Israelita Albert Einstein, 627/701 São Paulo, SP, Brazil
Vanessa Bittencourt, Hospital Israelita Albert Einstein, 627/701 São Paulo, SP, Brazil
Mari Cleide Sogayar, Department of Biochemistry, Chemistry Institute, University of São Paulo, São Paulo, Brazil and Cell and Molecular Therapy Center (NUCEL), University of São Paulo, São Paulo, Brazil
Anna Carla Goldberg, Hospital Israelita Albert Einstein, 627/701 São Paulo, SP, Brazil
Correspondence to: Anna Carla Goldberg, Hospital Israelita Albert Einstein, 627/701 São Paulo, SP, 05652-900, Brazil.
Email: goldberg@einstein.br
Telephone: +55(11)21510941 Fax: +55(11)21510273
Received: November 20, 2015 Revised: March 5, 2016
Accepted: March 9, 2016
Published online: March 22, 2016

ABSTRACT

AIMS: This study aims to evaluate a preclinical model of hepatic failure to propose an effective scheme to follow progression of mesenchymal cell treatments using serum parameters.

METHODS: Male Wistar rats ($n = 53$) received dimethylnitrosamine (DMN) or PBS (control) intraperitoneally. We evaluated liver macroscopy, histology, serum albumin (ALB), total bilirubin (TB), alanine (ALT) and aspartate (AST) aminotransferase, alkaline phosphatase (ALP), gamma globulin, and hepatic gene expression of ALB and selected regeneration markers.

RESULTS: After defining the effective DMN dose, 1×10^7 umbilical cord mesenchymal stem cells (UC-MSC) were injected into the tail vein 1 week after treatment and liver function re-assessed. DMN 10 μg/g delivered 3 days/week for 4 weeks altered liver macroscopy and histology. DMN treatment also increased ALP, ALT and TB, and significantly reduced gene expression of regeneration factors, although ALB and hepatic growth factor mRNA were not altered. Upon UC-MSC treatment, a greater percentage of rats showed reduction of AST levels (75% UC-MSC *vs* 0% PBS).

CONCLUSIONS: Selected serum parameters can predict liver function in a rodent model, but each animal must be its own control.

© 2016 The Authors. Published by ACT Publishing Group Ltd.

Key Words: Cirrhosis; Liver function; Rat model; Dimethylnitrosamine, serum parameters

Moreira EO, Piccinato CA, Pazzini VB, Sogayar MC, Goldberg AC. Cirrhosis Model That Tracks Umbilical Cord Mesenchymal Cell Transplantation Using Liver Serum Parameters: A Pilot Study. *Journal of Gastroenterology and Hepatology Research* 2016; 5(2): 1989-1996 Available from: URL: http://www.ghrnet.org/index.php/joghr/article/view/1521

INTRODUCTION

Chronic liver diseases caused by B and C hepatitis, steatosis resulting from obesity and alcohol consumption, as well as hepatocarcinomas are an important healthcare challenge, being the fifth cause of death worldwide[1]. Liver injury resulting from these affections results in fibrosis due to accumulated extracellular matrix components in the space of Disse. This increase is caused both by excessive production and failure in removal by degradation[2], which in the long run lead to widespread fibrosis, appearance of regenerative nodules, and destruction of the lobular architecture, the hallmarks of liver cirrhosis[3]. When this stage is reached, liver transplant is the standard

treatment indicated. The need of good quality organs and the ever-increasing demand for liver transplants leads to significant morbidity and mortality of patients in the waiting list[4,5].

The search for novel therapies depends on animal models that mimic, as best as possible, the physiopathology of fibrosis and cirrhosis in human patients. This issue is essential to allow one to differentiate true therapeutic improvement from natural self-regenerative processes, a recurrent feature of murine models. In addition, the more commonly used models mimic the acute forms of liver failure[6]. The more chronic disease models rely on the use of highly toxic drugs such as carbon tetrachloride (CCl_4), thioacetamide and dimethylnitrosamine (DMN)[7,8]. In addition to very high mortality rates CCl_4 - induced damage is reversible when treatment is interrupted, a limiting factor in studies where an extended therapeutic window is desirable[9,10]. In the case of TAA, a very long treatment period is needed to achieve significant fibrosis[11]. In contrast, treatment with DMN leads to a chronic and progressive hepatic fibrosis, with lower mortality rates[7,12], as DMN exerts only localized toxic effects when metabolized by CYP2A6 and CYP2E1 enzymes in the liver[13]. Treatment period, dosage, and combined partial hepatectomy as well as type of animal strain used have been shown to impact on disease outcome[14-16]. A 3-4 weeklong consecutive treatment protocol has been applied in several instances to study the mechanisms involved in the establishment of fibrosis and to test anti-fibrotic drugs[17,18].

An adequate evaluation of the therapeutic effects of a cell-based therapy to treat chronic liver injury requires stable preclinical models as close as possible to the human cirrhotic condition. Here, we tested the effective scheme to induce hepatic failure in Wistar rats using conservative analysis (dosage of serum parameters of liver function), associated to histological evaluation and gene expression analysis in a DMN-model of chronic hepatic cirrhosis, more similar to the disease in human patients.Furthermore, using this model, we present a pilot study by which we tracked the therapeutic effects of human mesenchymal stem cells (MSC) derived from umbilical cord Wharton´s jelly (UC-MSC) on the levels of selected serum parameters of liver function.

MATERIAL AND METHODS

Animal models

A total of 53 male Wistar rats with approximately 150 g of body weight (BW) were enrolled in this study. Animals were housed either at the Animal Facility at the Chemistry Institute, University of São Paulo or in an American Association for Accreditation of Laboratory Care (AAALAC) certified facility at Hospital Albert Einstein, with free access to laboratory diet and water in a room with fixed temperature. All animal care and experimentation conformed to the Committee for Animal Research rulesfrom both institutions.

In order to induce cirrhosis, animals received intra-peritoneal injections ofN-nitrosodimethylamine [dimethylnitrosamine (DMN); Sigma Chemical Co., St. Louis, MO] diluted in phosphate buffer 3 times/week during 4 weeks. Alternatively, 30% hepatectomy was added to the protocol after 3 weeks and a further week of DMN treatment was added[16]. A schematic representation of the protocols employed is shown in Figure 1. Dose-response experiments compared controls (PBS injected rats; $n = 22$) versus DMN-treated rats using 2 dosages: low dose (from 2-5 μg/g; $n = 7$) and high dose (10 μg/g; $n = 22$). Body weight was measured once a week. Because 2 μg/g and 5 μg/g did not present meaningful differences, they were classified as “low DMN” group. In order to test the effects of hepatectomy plus DMN, the same DMN doses were used [sham operated ($n = 3$), low DMN ($n = 3$) and high DMN ($n = 3$)]. To test the effects of UC-MSC in the recovery of injured liver, 10 rats previously treated with DMN for 4 weeks received PBS (controls) or 1×10^7 MSC in 300 μl of PBS ($n = 5$) a week after the last DMN dose through the tail vein. At the time of sacrifice, animals were overdosed with an intra-peritoneal injection of Ketamine.

For hepatectomyand blood collection (live procedures) an anesthetic scheme consisting of an intra-peritoneal injection of 50 mg/kg Ketamine/5 mg/kg plus Xylazine was used. In all experiments, blood was collected for analyses of hepatic function and macroscopic examination of the liver was performed to visually evaluate the extent of fibrosis/cirrhosis development at the end of the experiment. Samples of liver tissue were fixed in 4% buffered formalin and embedded in paraffin for histological analysis. A subset of samples per group ($n = 5$) were snap- frozen and stored at -80℃ for gene expression studies.

Blood and histological analysis

At the end of the experimental period, up to 2 mL of blood was collected from the caudal vein or abdominal vena cava with a Hamilton 3 mL syringe and transferred to the appropriate 3.5 mL BD Vacutainer® for serum separation. After 1800xg centrifugation for 10 min at room temperature, serum was separated and frozen for further use.

Hepatic function was assessed by the analysis of biochemical serum parameters including: ALB, TB, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gammaglobulin (GGT) using standard kinetic and colorimetric (Labtest, Lagoa Santa, MG, Brazil) assays and were carried out at the Laboratory of Biochemistry of Liver Transplantation Unit of the Department of Surgery and Anatomy, (FMRP-USP), Brazil. Fixed liver tissue sections were stained with hematoxylin-eosin and Masson´s trichrome to visualize tissue architectural/morphological changes and also extension of fibrosis.

ReverseTranscriptase and Real Time PCR reaction

Total RNA was extracted from hepatic tissue using IllustraRNAspin Mini RNA isolation kit (GE Healthcare). After reverse transcription of 2 μg RNA, using SuperScript (Invitrogen, CA), cDNA was amplified using the SYBR Green PCR system (LifeTechnologies, Gaithersburg, MD). Protocol included 2 min at 50℃, 10 min at 95℃ for initial denaturation and 40 cycles of 15 sec at 95℃ and 1 min at 60℃. Primers flanking selected genes of interest were designedusingPrimer Express™ version 1.5 (Applied Biosystems, Foster City, CA) and are listed in Table 1. Genes of interest for analysis included albumin (ALB) and genes involved in liver regeneration including: tyrosine aminotransferase (TAT), phosphoenolpyruvatecarboxykinase (PEPCK), hepatic growth factor (HGF). Hypoxanthine guanine phosphoribosyltransferase (HPRT) gene expression was used as an internal reference for each individual sample. The relative gene expression was calculated from cycle threshold (Ct) using the ΔΔCt method[19].

Isolation, culture and preparation of MSC from UC

Clearance for the use of human umbilical cord mesenchymal cells was obtained from the Ethical Committees of Research in Humans at both,Hospital Universitário, University of São Paulo (USP) and Hospital Israelita Albert Einstein (HIAE). Upon informed consent, umbilical cords were processed for isolation of UC-MSC by a modified version of a previously published protocol[20]. In brief,umbilical veins were cannulated with a catheter and secured with cardiac cotton tape (Ethicon, Bridgewater, NJ). After thorough washing with

PBS, the cords were clamped at one end, perfused with 10 ml of 1 % collagenase solution, and clamped shutfor incubation at 37℃ for 1 h, with occasional massages to promote enzymatic action. The clamps were released and the collagenase-containing solution collected. The cords were then washed with 50 mL of 50% fetal bovine serum (FBS) in non-supplemented Dulbecco's Modified Eagle Medium (DMEM) followed by recovery of the cells with 5 % FBS in non-supplemented DMEM. All volumes were combined, washed by centrifuging at 450 g for 4 min,resuspended in 7 ml of DMEM supplemented with 10 % FBS, 1 % L-glutamine and 1 % and antibiotic-antimycotic solution for seeding onto 25 cm^2 tissue flasks (Corning, St. Louis, MO) and cultured at 37℃ in a humidified 5% CO_2 atmosphere. Cells were allowed to adhere for a 24 h period before receiving fresh culture medium that was changed three times a week. Passaging was carried out when 70% sub-confluence was reached maintaining a density of 4,000 cells/cm^2 in all passages. Cell dissociation was performed with TrypLE™Express for 3 min at 37℃ and the mixture inactivated by dilution in DMEM followed by brief centrifugation of the cell suspension before reseeding. All MSC were cultured up to passage 4 and then tested for their differentiation capacity into adipocytes and osteocytes and profiled for specific cell-surface markers using FACSAria (BD Biosciences, San Jose, CA), as described previously[21]. A total of 1×10^7 MSC in PBS was injected via the caudal vein.

2.5. Statistical Analysis. Results are presented as mean +/− standard error (SEM). For the dose-dependent evaluations, the statistical difference between treatments (controls and 2 levels of DMN dose) was determined by unbalanced one-way analysis of variance (ANOVA) followed by Tukey-Kramer's multiple comparison test using SAS software version 6.11 (Statistical Analysis System Institute Inc., Cary, NC, USA). Log transformation prior to test or non-parametric Kruskal-Wallis test was used when requirements of normalization were not attended. Since we carried out the above experiments in two separate animal facilities, we had to preclude local effects. Thus, the values of circulating levels of biochemical parameters (ALB, AST, ALT, GGT, TB, and ALB) were calculated as percentage values relative to the average of controls (from each experiment) to account for distinct conditions of animal facilities. Fisher´s Exact Test was used to assess improvement rates for serum levels of biochemical parameters for animals pre-treated with DMN for 4 weeks and one week after treatment with PBS (controls) or UCMSCs. We considered as "improved" those animals in which biochemical parameters showed amelioration after treatment, that is, values lower or similar to those obtained before treatment (cells) was given. All differences with a p value <0.05 were considered statistically significant.

RESULTS

Animal mortality and liver morphological analyses

Macroscopic analysis of livers after treatment with two different DMN doses with or without hepatectomy indicated that the effective dose to establish a chronic liver disease in Wistar rats is 10 µg/g delivered in a 3 days/week protocol, during 4 weeks (Figure 2). With this dosage, livers presented swelling of hepatic lobules, yellowish liver zones, and increased rigidity (Figure 2C). Lower dosage (~ 5 µg/g) induced only mild swelling (Figure 2B) and the liver was not visibly different from the control group (Figure 2A). No differences in the macroscopic analysis could be seen when 30% hepatectomy was added to the induction protocol (results not shown). Compared to control animals (0/22) and those treated with 5 µg/g DMN (0/7), 18.2% (4/22) animals treated with 10 µg/g DMN died. Moreover,hepatectomy led to a significant increase up to 60% in mortality (of 5 animals in each treatment group, only 2 survived).

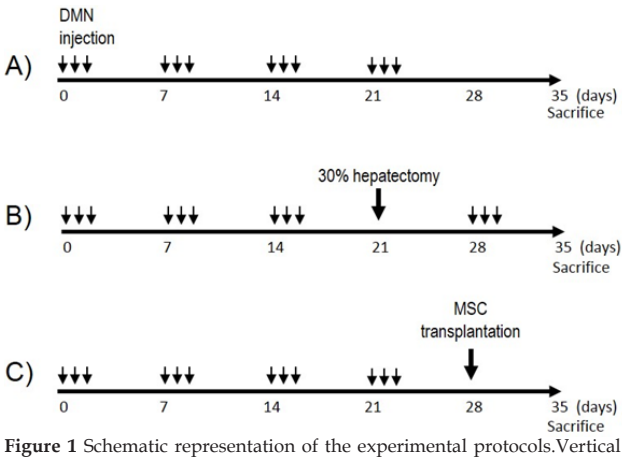

Figure 1 Schematic representation of the experimental protocols.Vertical downward arrows represent weekly intraperitoneal injections of DMN (low dose 2-5 ug/g or high dose 10 ug/g of body weight) or PBS in untreated animals (controls). Each line represents a drug-only scheme (A); a scheme that includes 30% partial hepatectomy which was carried out, followed by an additional week of treatment with DMN (B); and the latter by which umbilical cord mesenchymal stem cells (1 x 10^7 MSC) diluted in 300 µl of PBS were injected into the tail vein 1 week after a 4-week high DMN-treatment (C).

Table 1 Primers used for quantitative PCR analysis.

Gene	Oligonucleotide sequences
TAT	Sense 5′CAGCCCAATCCGAACAAG3′
	Anti-sense 5′CCAGGGCATCTTTCATGG3′
HGF	Sense5′TTTACGGCTGGGGCTACA3′
	Anti-sense5′TTGCCTTGATGGTGCTGA3′
PEPCK	Sense5′CGTTCAATGCCAGGTTCC3′
	Anti-sense5′CAGCTCGATGCCGATCTT3'
HPRT	Sense5′CAAAATGGTTAAGGTTGCAAGCT3′
	Anti-sense5′CAAACTTGTCTGGAATTTCAAATCC3′

Some differences were observed upon histological analysis. Compared to the control group (Figure 3A for HE and 3D for Masson staining, respectively), animals receiving the lower doses of 5 µg/g DMN, with (not shown) or without (Figure 3B) added hepatectomy showed spots of cell swelling, indicative of sodium and water accumulating in the cytoplasm as a first sign of hepatocyte damage. Trichrome Masson staining disclosed discrete changes in extracellular matrix notably in the vicinity of blood vessels but with preserved liver architecture (Figure 3E). Upon treatment with DMN 10 µg/g, however, independently of accompanying hepatectomy, presence of infiltrating immune cells and marked changes in liver architecture occurred (Figure 3C). Masson staining confirmed the increase in extracellular matrix deposition (Figure 3F). However, in no instance we observed hallmarks of advanced hepatic fibrosis such as presence of regenerative nodules and complete loss of lobular architecture.

Hepatic gene expression and serum parameters after induction of liver cirrhosis with the high DMN dose of 10 µg/g BW

When we determined that the higher DMN dose without hepatectomy was the more effective scheme to produce cirrhosis in Wistar rats by macroscopic and histological evaluations, we followed up on the study with further analyses. Firstly, we analyzed the expression of selected genes in the liver of rats submitted to the high, low or no DMN (Figure 4). Hepatic ALB mRNA expression showed no reduction in animals receiving high DMN as compared to low DMN treated rats or controls. Expression of genes involved in liver regeneration such as TAT and PEPCK presented decreased

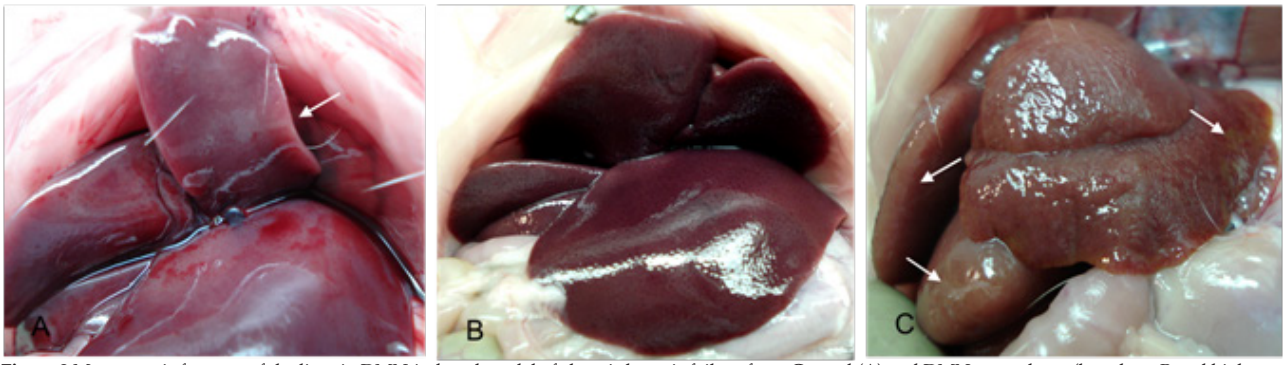

Figure 2 Macroscopic features of the liver in DMN-induced model of chronic hepatic failure from Control (A) and DMN-treated rats (low dose, B and high dose, C). Rats subjected to long-term DMN administration exhibited prominent cirrhotic aspect. Commonly encountered features were hepatic edema, increased liver size with yellowish aspect and enlarged borders (arrowheads).

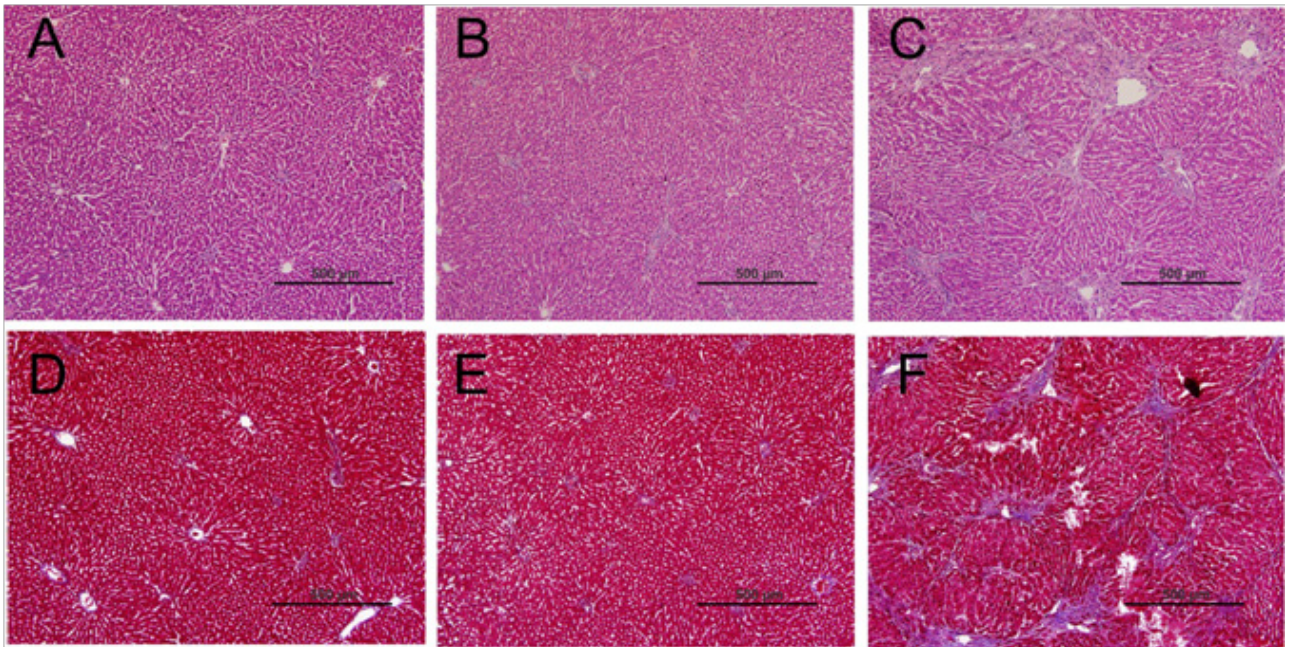

Figure 3 Histological features of liver in DMN-induced model of chronic hepatic failure. Photomicrographs of liver biopsies stained by HE from normal liver (from control rats, A), rats subjected to long-term 5ug/g of (low) DMN administration (B),rats subjected to long-term 10ug/g of (high) DMN administration. Photomicrographs D, E and F are liver biopsies stained by Trichrome Masson with the same treatment groups, respectively. In high DMN treatments, tissue architecture was compromised and lymphocytic infiltrate is present, especially around portal space.

expression in the high DMN treatment group when compared to controls (both $P = 0.04$). The low dose animals also showed reduced hepatic TAT expression ($P = 0.03$) with no difference to those of high dose animals. However, another gene that is tightly linked to liver regeneration, HGF, did not present significant differences between DMN groups and controls.

Next, serum parameters were evaluated in DMN-treated rats and controls from 5 independent experiments (Figure 5). Although parameters were obtained from control age- and initial weight-matched rats, there was a reduction of 13% in body weight after DMN treatment when compared to non-treated control rats ($P = 0.006$; data not shown). Values exhibited a great variability even when comparison was carried out between animals, control or treated, in the same experiment. While there was heterogeneity in values, a distinct difference, exclusively for ALP, TB, and ALT, could be noticed between controls and high DMN-treated animals. These markers were significantly increased in response to high DMN (respectively, $P < 0.0001$, $P = 0.01$ and $P = 0.02$). Other parameters varied widely and did not show significant differences among experimental and control groups.

Evaluation of serum parameters following treatment with UC-MSC

Characterization of cells in the fourth passage confirmed that over > 95% were plastic adherent, presented a fibroblastic morphology, and were positive for the expected flow cytometry markers (Figure 6).

To access the effectiveness of treating DMN-induced chronic cirrhosis in rats with UC-MSC, animals received 1×10^7 cells or PBS via the caudal vein 1 week after DMN treatment was completed. Blood was collected on the first day of the experimental injection and a week after treatment to evaluate liver function by measuring ALP, AST, ALT, ALB, GGT, and TB (Table 2). Although there was no clear differences in serum levels between groups (data not shown), a significantly greater percentage of animals showed improved AST (75% *vs* 0%, UC-MSC-treated vs. PBS-treated rats, $p = 0.04$). A percentage of rats decreased ALT (50% *vs* 0%) and ALP (75% *vs* 20%) levels compared to PBS-treated rats, but differences were not statistically significant. However, rats that improved ALT also improved AST and ALP (data not shown) levels. In addition, 75% of UC-MSC treated animals improved TB levels. It should be noted that 3 PBS-control rats also presented spontaneous recovery of TB,

GGT, and ALB levels. No difference was observed between UC-MSC injected animals and PBS-treated animals regarding average levels per group of any of the parameters, probably due to the widely fluctuating levels of each parameter ($P < 0.05$).

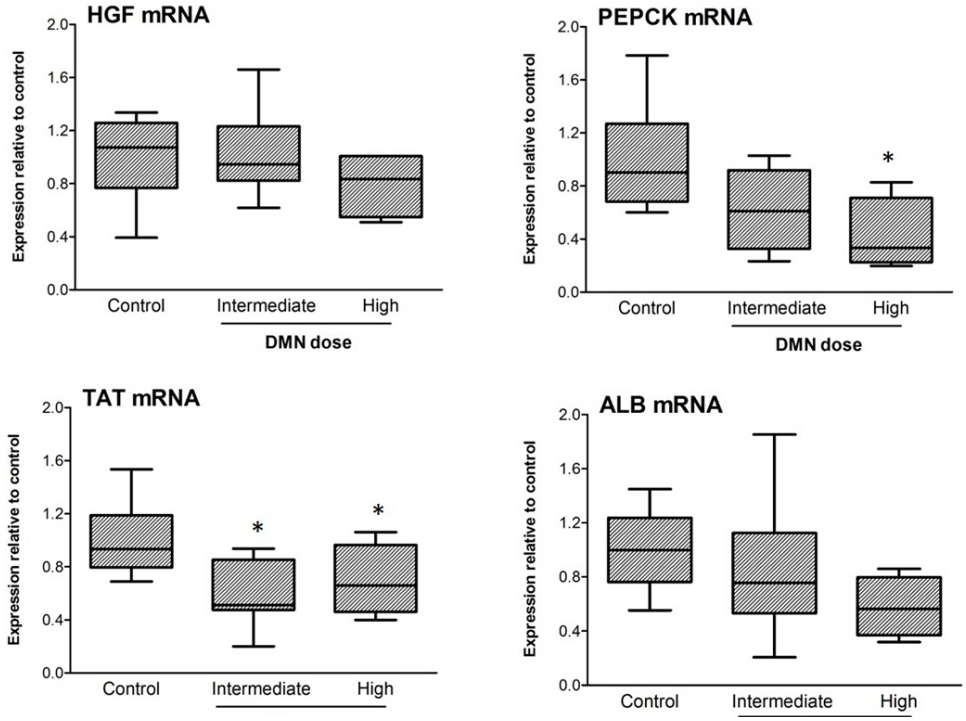

Figure 4: mRNA expression analysis of selected hepatic genes. The expression of selected hepatic genes, albumin (ALB), hepatic growth factor (HGF), phosphoenolpyruvate carboxykinase (PEPCK), and tyrosine aminotransferase (TAT) was evaluated in liver samples from control (PBS-treated), low and and high DMN dose (5ug/g BW and 10ug/g BW, respectively) by RT-real time PCR. The averaged fold change by group was calculated in relation to control (PBS-treated rats) from each experiment (n=5). Error bars represent the SEM for the average fold change. Statistical differences between control and groups were identified by *: $P<0.05$.

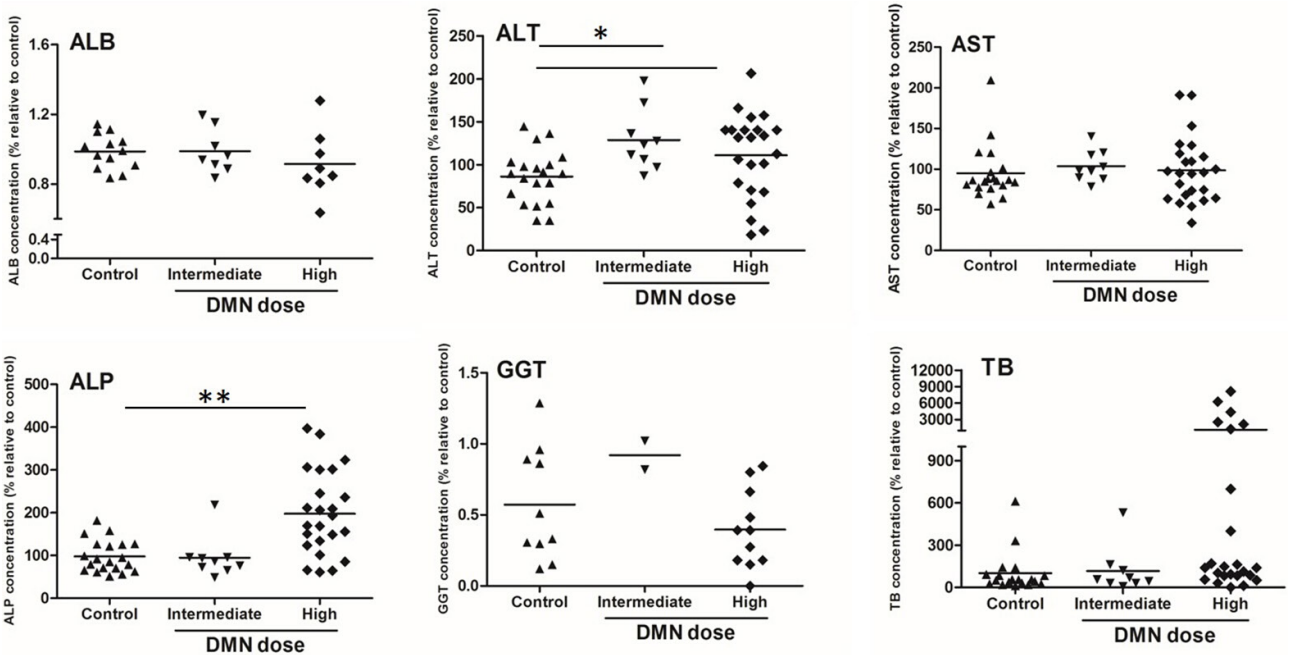

Figure 5 Panel of serum levels of biochemical parameters involved with liver function. The circulating levels of albumin (ALB), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma globulin (GGT), and total bilirrubin (TB) were evaluated in serum samples from control (PBS-treated), low and high DMN dose (2-5ug/g BW and 10ug/gBW, respectively). Error bars represent the SEM for the average levels. Statistical differences between groups were identified by *: $P < 0.05$, **: $P < 0.001$.

DISCUSSION

Here we present data on aliver failure pre-clinical model using the non-isogenic Wistar rat. The results shown in the present study reinforce the ability of DMN models to produce animals with compromised liver function but with controlled levels of mortality. We further corroborate and extend previous studies that used the same model by stressing the importance of evaluating individual responses in the follow up of new therapies (such as cell-therapies) aiming to treat hepatic failure. When only DMN treatment was used, the number of deaths was very low with a mortality of just 18% in the group treated with 10 μg/g. In spite of the less harsh treatment, fibrosis and inflammatory cells were clearly discernible by histology. After evaluating the most effective dose of DMN to induce chronic liver failure we tracked the outcome of UC-MSC transplantation using a conservative analysis, that is, dosage of serum markers of liver function, using each animal as its own control. Of all parameters analyzed, ALP, TB, and ALT levels were significantly altered after treatment with DMN, even though there was a considerable overlap between control and DMN-treated animals. This result is similar to another study[17] using the same DMN protocol in a different animal strain (Sprague-Dawley rats) where ALT did increase significantly in all treated animals when compared to controls. In addition, the markers of hepatic regeneration we tested by real time PCR were either unchanged or lower, an indication that as disease worsens, regeneration processes are either low or absent. Taken together, the histological and macroscopic features of the liver, mortality rates, and gene expression data support the association of serum parameters with progression of cirrhosis in the present rodent model.

Studies carried out in chronic liver disease models have produced controversial results, with positive results in some studies[22], but not in others[10,23]. These studies tried to establish adequate follow-up protocols and some measured serum parameters of liver function, but only at the endpoint. However, these animal models are highly toxic and result in extensive mortality of the animals, and few papers clarify this issue, showing only results of surviving animals. In our study, we opted to use DMN 10 μg/g to induce a chronic disease that we deemed to be more similar to human cirrhosis, using a protocol very similar to that employed by Zhao[22]. This group however, treated Fisher rats during 6 weeks, which resulted in extremely high mortality rates, of 90% in untreated and 40% in MSC-treated animals. The lower mortality, still quite high, was considered to be one of the indications of improved liver function. In fact, when we added hepatectomy to the protocol our mortality rates were also high, which is not ideal. Thus, we removed this procedure from the ensuing experimental protocols.

The cell-therapy study presented here may be regarded as a pilot study considering the small sample size. Yet we were able to detect significant differences of selected biochemical parameters of liver function. However, such markers varied greatly between individuals. In the present study, we followed the effects of treatment with human UC-MSC on serum parameters, aiming to avoid the sacrifice of the experimental animals and enabling longer follow-up without the mortality that usually accompanies these liver failure models. Our results evidenced that part of the animals did show improved liver function, confirmed by a reduced level of AST. Moreover, of the animals treated with MSC at least 50% showed improved ALP, ALT, and AST levels. Conversely, one out of five controls also decreased their ALP values, although none showed change in their AST and ALT levels. The commonly used parameters of total bilirubin, ALB, and GGT decreased to normal values in both treated and untreated animals and hence did not represent a good marker of liver status. One of the studies using syngeneic bone marrow MSC in CCl4-induced chronic disease did measure albumin and ALT to assess liver function but also noticed that after a few weeks parameters improved regardless of treatment with MSC[10]. Finally, we chose to evaluate the results one week after treatment as MSC reportedly do not survive longer after injection into immunocompetent animals[23], and therefore should be able to exert their effect in this narrow timeframe. In another study, working on similar chronic model (with CCl_4) that leads to a more moderate damage, no improvement in fibrosis nor the presence of syngeneic MSC in the liver could be detected after one week[23]. The fact that some of our UC-MSC treated animals did improve their functional parameters is indicative that the time-period was sufficient to detect changes.

Mesenchymal stem cells obtained from bone marrow and eventually from adipose tissue have been previously tested in tissue regeneration protocols in different preclinical models[22,24]. The autologous nature of these cell sources explains these choices. However, when considering treatment for human patients, obtaining bone marrow is not exempt from risks due to the invasive procedure and donor age leads to diminished numbers of the cells obtained[25,26]. UC-MSC though allogeneic in nature are an interesting alternative with no important issues concerning donor age in addition to their well-known immunomodulatory and proliferation capacities[27,28]. In acute and fulminant liver failure animal models, undifferentiated MSC infusions have exhibited some capacity for homing and tissue repair, since they are able to escape immune recognition and to inhibit the activation and differentiation of immune cells[22,29-32]. Studies have shown no results, positive results after 7 days[33], or only 2 to 4 weeks later[34], including a progressive improvement in the fibrosis index[30]. Treatment of acute liver failure caused by partial hepatectomy[35] or a single CCl_4 injection[36] has suggested that bone marrow MSC undergo myofibroblast differentiation contributing therefore to progression of fibrosis.

Taken together, an overview of the results obtained by several different groups indicates that correct evaluation of liver parameters in preclinical liver disease models is needed and that results obtained may simply reflect in inherent variability of the experimental animals, as already previously noted[37]. In the experiment where data are collected before and after the treatment, the individual variability of serum parameters is evident, though macroscopically the disease is present in all animals. Increasing the number of animals tested clearly exposes this variability and shows that tracking of disease treatments should be interpreted with caution.

CONCLUSIONS

To conclude, though serum changes after 7 days could be detected in part of the animals submitted to cell therapy, we believe that longer term follow-up andfurther validation studies with greater number of animals are necessary to truly evaluate benefits of such therapy. Following individual changes in serum liver parameters such as ALT, AST, and ALP could be an effective way to do it. Thus, in our view, in addition to a wider spectrum of functional liver serum parameters, animals need to have measurements done at different experimental time points, with each animal being its own control, in order to distinguish true effects from the self-tissue regeneration commonly seen in rodent models.

ACKNOWLEDGEMENTS

We would like to thank Maria Elisa J. de Souza, Clarice F. Franco

and Maria Aparecida N. C. Picinato for helping with serum parameters measurements at the Laboratory of Biochemistry of Liver Transplantation Unit of the Department of Surgery and Anatomy, (FMRP-USP, Brazil). The work was supported by the governmental agencies: CNPq (573578/2008-7) and FAPESP (07/54260-2).

CONFLICT OF INTEREST

The authors indicate no potential financial conflict of interests.

REFERENCES

1. Williams R. Global challenges in liver disease. *Hepatology* 2006; 44: 521-6.
2. Hernandez-Gea V, Friedman SL. Pathogenesis of liver fibrosis. *Annual review of pathology* 2011; **6**: 425-56.
3. Pinzani M, Rosselli M, Zuckermann M. Liver cirrhosis. *Best practice & research Clinical gastroenterology* 2011; **25**: 281-90.
4. Prince MI, Hudson M. Liver transplantation for chronic liver disease: advances and controversies in an era of organ shortages. *Postgraduate medical journal* 2002; **78**: 135-41.
5. Vulcano DS, Carvalhaes MA, Bakonyi Neto A. Evaluation of nutritional indicators and body composition in patients with advanced liver disease enrolled for liver transplantation. *Acta cirurgica brasileira / Sociedade Brasileira para Desenvolvimento Pesquisa em Cirurgia* 2013; **28**: 733-9.
6. Kuhlmann WD, Peschke P. Hepatic progenitor cells, stem cells, and AFP expression in models of liver injury. *International journal of experimental pathology* 2006; **87**: 343-59.
7. Starkel P, Leclercq IA. Animal models for the study of hepatic fibrosis. *Best practice & research Clinical gastroenterology* 2011; **25**: 319-33.
8. Nakamura T, Torimura T, Iwamoto H, et al. Prevention of liver fibrosis and liver reconstitution of DMN-treated rat liver by transplanted EPCs. *European journal of clinical investigation* 2012; **42**: 717-28.
9. Issa R, Zhou X, Constandinou CM, et al. Spontaneous recovery from micronodular cirrhosis: evidence for incomplete resolution associated with matrix cross-linking. *Gastroenterology* 2004; **126**: 1795-808.
10. Quintanilha LF, Mannheimer EG, Carvalho AB, et al. Bone marrow cell transplant does not prevent or reverse murine liver cirrhosis. *Cell transplantation* 2008; **17**: 943-53.
11. Guerra RRT, M.R.; Aloia, T.P.A.;Dagli, M.L.Z.;Hernadez-Blasquez, F.J. A novel chronic Cirrhosis TAA-induced model in rats. *Brazilian Journal of Veterinary Pathology* 2010; **3**: 9-16.
12. Jezequel AM, Mancini R, Rinaldesi ML, Macarri G, Venturini C, Orlandi F. A morphological study of the early stages of hepatic fibrosis induced by low doses of dimethylnitrosamine in the rat. *Journal of hepatology* 1987; **5**: 174-81.
13. Chowdhury G, Calcutt MW, Guengerich FP. Oxidation of N-Nitrosoalkylamines by human cytochrome P450 2A6: sequential oxidation to aldehydes and carboxylic acids and analysis of reaction steps. *The Journal of biological chemistry* 2010; **285**: 8031-44.
14. Ying TS, Sarma DS, Farber E. The sequential analysis of liver cell necrosis: inhibition of diethylnitrosamine- and dimethylnitrosamine-induced acute liver cell death by posttreatment with diethyldithiocarbamate. *The American journal of pathology* 1980; **99**: 159-74.
15. Hyon MK, Kwon E, Choi HJ, Kang BC. Dimethylnitrosamine-induced liver fibrosis and recovery in NOD/SCID mice. *The Journal of veterinary medical science / the Japanese Society of Veterinary Science* 2011; **73**: 739-45.
16. Ozawa S, Uchiyama K, Nakamori M, et al. Combination gene therapy of HGF and truncated type II TGF-beta receptor for rat liver cirrhosis after partial hepatectomy. *Surgery* 2006; **139**: 563-73.
17. He JY, Ge WH, Chen Y. Iron deposition and fat accumulation in dimethylnitrosamine-induced liver fibrosis in rat. *World journal of gastroenterology : WJG* 2007; **13**: 2061-5.
18. Choi JH, Hwang YP, Choi CY, Chung YC, Jeong HG. Anti-fibrotic effects of the anthocyanins isolated from the purple-fleshed sweet potato on hepatic fibrosis induced by dimethylnitrosamine administration in rats. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* 2010; **48**: 3137-43.
19. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; **25**: 402-8.
20. Secco M, Zucconi E, Vieira NM, et al. Multipotent stem cells from umbilical cord: cord is richer than blood! *Stem Cells* 2008; **26**: 146-50.
21. Deus GC, Normanton M, Hamerschlak N, et al. Isolation and characterization of mesenchymal stem cells obtained from reusable and disposable bone marrow collection filters. *Einstein (Sao Paulo)* 2012; **10**: 296-301.
22. Zhao DC, Lei JX, Chen R, et al.Bone marrow-derived mesenchymal stem cells protect against experimental liver fibrosis in rats. *World journal of gastroenterology : WJG* 2005; **11**: 3431-40.
23. Popp FC, Slowik P, Eggenhofer E, et al. No contribution of multipotent mesenchymal stromal cells to liver regeneration in a rat model of prolonged hepatic injury. *Stem Cells* 2007; **25**: 639-45.
24. Parekkadan B, van Poll D, Suganuma K, et al. Mesenchymal stem cell-derived molecules reverse fulminant hepatic failure. *PloS one* 2007; **2**: e941.
25. Wagner W, Ho AD, Zenke M. Different facets of aging in human mesenchymal stem cells. *Tissue engineering Part B, Reviews* 2010; **16**: 445-53.
26. Janzen V, Forkert R, Fleming HE, et al. Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. *Nature* 2006; **443**: 421-6.
27. Wang HS, Hung SC, Peng ST, et al. Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord. *Stem Cells* 2004; **22**: 1330-7.
28. Li M, Ikehara S. Bone-marrow-derived mesenchymal stem cells for organ repair. *Stem cells international* 2013; **2013**: 132642.
29. Burra P, Arcidiacono D, Bizzaro D, et al.Systemic administration of a novel human umbilical cord mesenchymal stem cells population accelerates the resolution of acute liver injury. *BMC gastroenterology* 2012; **12**: 88.
30. Ali G, Masoud MS. Bone marrow cells ameliorate liver fibrosis and express albumin after transplantation in CCl(4)-induced fibrotic liver. *Saudi journal of gastroenterology : official journal of the Saudi Gastroenterology Association* 2012; **18**: 263-7.
31. Carvalho AB, Quintanilha LF, Dias JV, et al. Bone marrow multipotent mesenchymal stromal cells do not reduce fibrosis or improve function in a rat model of severe chronic liver injury. *Stem Cells* 2008; **26**: 1307-14.
32. Yan Y, Xu W, Qian H, et al. Mesenchymal stem cells from human umbilical cords ameliorate mouse hepatic injury in vivo. *Liver international : official journal of the International Association for the Study of the Liver* 2009; **29**: 356-65.
33. Cho KA, Woo SY, Seoh JY, Han HS, Ryu KH. Mesenchymal stem cells restore CCl4-induced liver injury by an antioxidative process. *Cell biology international* 2012; **36**: 1267-74.
34. Nasir GA, Mohsin S, Khan M, et al. Mesenchymal stem cells and Interleukin-6 attenuate liver fibrosis in mice. *Journal of translational medicine* 2013; **11**: 78.
35. Baertschiger RM, Serre-Beinier V, Morel P, et al. Fibrogenic potential of human multipotent mesenchymal stromal cells in injured liver. *PloS one* 2009; **4**: e6657.

36. di Bonzo LV, Ferrero I, Cravanzola C, et al.Human mesenchymal stem cells as a two-edged sword in hepatic regenerative medicine: engraftment and hepatocyte differentiation versus profibrogenic potential. *Gut* 2008; **57**: 223-31.
37. Sancho-Bru P, Najimi M, Caruso M, et al. Stem and progenitor cells for liver repopulation: can we standardise the process from bench to bedside? *Gut* 2009; **58**: 594-603.

Peer reviewers: Andrea M.P. Romani, Department of Physiology and Biophysics, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, OH, 44106-4970, USA; Ming-Hua Zheng, MD, Associate Professor, Department of Infection and Liver Diseases, Liver Research Center, The First Affiliated Hospital of Wenzhou Medical College, No.2 Fuxue lane, Wenzhou, 325000, China.