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, $1 \times 10^7$ umbilical cord mesenchymal stem cells (UC-MSC) were injected into the tail vein 1 week after treatment and liver function re-assessed. DMN 10µg/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.

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

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[41].

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[12]. The more chronic disease models rely on the use of highly toxic drugs such as carbon tetrachloride (CCl₄), thioacetamide and dimethylnitrosamine (DMN)[7,8]. In addition to very high mortality rates CCl₄ - induced damage is reversible when treatment is interrupted, a limiting factor in studies where an extended therapeutic window is desirable[8,10]. 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[10]. 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 week-long 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 rules from both institutions.

In order to induce cirrhosis, animals received intra-peritoneal injections of N-nitrosodimethylamine [dimethylnitrosamine (DMN); Sigma Chemical Co., St. Louis, MO] diluted in phosphate buffer 3 times/week during 4 weeks. Alternatively, 30% hepatectomy was performed, 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[12]. The more chronic disease models rely on the use of highly toxic drugs such as carbon tetrachloride (CCl₄), thioacetamide and dimethylnitrosamine (DMN)[7,8]. In addition to very high mortality rates CCl₄ - induced damage is reversible when treatment is interrupted, a limiting factor in studies where an extended therapeutic window is desirable[8,10]. 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[10]. 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 week-long 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].

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**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, Rio de Janeiro, RJ, Brazil) assays and were carried out at the Laboratory of Biochemistry of Liver Transplantation Unit of the Department of Surgery and Anatomy, (FMERP-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.

**Reverse Transcriptase and Real Time PCR reaction**

Total RNA was extracted from hepatic tissue using IllustraRNaPure 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 (Life Technologies, Gaithersburg, MD). Protocol included 2 min at 50 °C, 10 min at 95 °C for initial denaturation and 40 cycles of 15 sec at 95 °C and 1 min at 60 °C. Primers flanking selected genes of interest were designed using Primer 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), phosphoenoxyruvatecarboxykinase (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[20,21]. In brief, umbilical veins were cannulated with a catheter and secured with cardiac cotton tape (Ethicon, Bridgewater, NJ). After thorough washing with
Moreover, hepatectomy led to a significant increase up to 60% in DMN (0/7), 18.2% (4/22) animals treated with 10 µg/g DMN died. Compared to control animals (0/22) and those treated with 5 µg/g, hepatectomy was added to the induction protocol (results not shown). No differences in the macroscopic analysis could be seen when 30% liver was not visibly different from the control group (Figure 2A). Lower dosage (~ 5 µg/g) induced only mild swelling (Figure 2B) and the yellowish liver zones, and increased rigidity (Figure 2C). Lower dosages were delivered in a 3 days/week protocol, during 4 weeks (Figure 2D). The dose to establish a chronic liver disease in Wistar rats is 10 µg/g DMN doses with or without hepatectomy indicated that the effective 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).

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 no instance we observed hallmarks of advanced hepatic fibrosis such as presence of regenerative nodules and complete loss of lobular architecture.
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 $1 \times 10^7$ 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 high DMN dose (5µg/g BW and 10µg/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 bilirubin (TB) were evaluated in serum samples from control (PBS-treated), low and high DMN dose (2-5µg/g BW and 10µg/g BW, 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 a liver 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\[25\] 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\[19,23\]. 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 CC14-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\[19\]. Finally, we chose to evaluate the results one week after treatment as MSC reportedly do not survive longer after injection into immunocompetent animals\[25\], and therefore should be able to exert their effect in this narrow timeframe.

In another study, working on similar chronic model (with CCl4) 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\[22\]. 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\[22,24\]. 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\[23\] or a single CCl4 injection\[35\] 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\[19\]. 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 and further 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.

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CONFLICT OF INTEREST
The authors indicate no potential financial conflict of interests.

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