UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

PROGRAMA DE PÓS-GRADUAÇÃO: GENÉTICA E BIOLOGIA MOLECULAR

EFEITOS PARÁCRINOS DA TERAPIA CELULAR EM MODELOS DE INSUFICIÊNCIA HEPÁTICA AGUDA UTILIZANDO MICROCÁPSULAS SEMIPERMEÁVEIS

CAROLINA URIBE CRUZ

TESE DE DOUTORADO

Porto Alegre, Brasil

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A meus pais e irmãs pelo eterno apoio.

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SUMÁRIO

LISTA DE ABREVIATURAS

AFP	Alfa-Fetoproteína
ALB	Albumina
APAP	Paracetamol
BrdU	Bromodeoxiuridina
CCl_3	Radical triclorometil
CCl_4	Tetracloreto de Carbono
CFMMO	Células da Fração Mononuclear da Medula Óssea
CK18	Citoqueratina 18
CK8	Citoqueratina 8
CMO	Células da Medula Óssea
CO	Células Ovais
CTG	Centro de Terapia Gênica
gp130	*Glycoprotein 130*
HCPA	Hospital de Clínicas de Porto Alegre
HGF	Hepatocyte Growth Factor

HP	Hepatectomia parcial
IHA	Insuficiência Hepática Aguda
IL-10	*Interleukin-10*
IL-1β	*Interleukin*-1 Beta
IL-6	*Interleukin*-6
JAK	*Janus kinase*
LPS	Lipopolissacarídeos
MYD88	*Myeloid Differentiation Factor88*
NFκ-B	*Nuclear Factor Kappa B*
SOCS3	*Suppressor of Cytokine Signaling 3*
Stat3	Signal Transducer and Activator of Ttranscription 3
TAA	Tioacetamida
TGF- β	*Transforming Growth Factor-Beta*.
TLR-4	*Toll Like Receptor4*
TNF-α	*Tumor Necrosis Factor Alfa*

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RESUMO

A insuficiência hepática aguda é resultante da perda das funções vitais do fígado em consequência da disfunção maciça do tecido hepático. Em muitos casos, o transplante de fígado é o tratamento definitivo, apesar da capacidade regenerativa do fígado. Os mecanismos da regeneração envolvem intricadas vias de regulação, com ativação de fatores de crescimento e citocinas. A terapia celular com células tronco da medula óssea tem sido proposta como alternativa terapêutica, mas seu emprego permanece no campo experimental. O presente estudo teve por objetivo investigar os efeitos parácrinos da terapia com células em microcápsulas semipermeáveis, em modelos de insuficiência hepática aguda.

Em uma primeira etapa animais submetidos a hepatectomia parcial de 90% foram transplantados com células da medula óssea (CMO) encapsuladas ou cápsulas vazias. Os resultados demonstraram um aumento na sobrevida em 10 dias nos animais tratados com CMO encapsuladas. Estudos em tempos iniciais da regeneração (em até 72 horas) demonstraram que as CMO reduziram a expressão de genes que favorecem a regeneração hepática e aumentaram a expressão de reguladores negativos da regeneração. Como consequência, houve uma diminuição da taxa de regeneração nos animais tratados. Este efeito ocorreu através de mecanismos parácrinos.

Na segunda etapa avaliamos a capacidade de diferenciação das células da fração mononuclear da medula óssea (CFMMO) encapsuladas *in vivo* e *in vitro*.

Animais submetidos a uma lesão por Tetracloreto de Carbono (CCl_4) foram transplantados com CFMMO encapsuladas. Animais sem lesão hepática foram utilizados como controle. Observou-se que, 48 horas após o transplante as CFMMO passaram a expressar genes característicos de hepatócitos. A fim de estudar melhor os mecanismos envolvidos nesta diferenciação, utilizamos CFMM encapsuladas em um modelo *in vitro* que replicasse as características do modelo *in vivo.* Assim empregamos um sistema de co-cultivo, onde as CFMMO encapsuladas eram mantidas em contato com o meio de hepatócitos com ou sem lesão por CCl_4. Nossos resultados demonstraram que em apenas 6 horas de co-cultivo *in vitro* as CFMMO encapsuladas expressaram marcadores hepáticos. Estes resultados sugerem a ocorrência de diferenciação das CFMMO em tempos muito precoces por mecanismos parácrinos.

Em conjunto, os resultados apresentados nos permitem concluir que a microencapsulação celular se mostrou uma ferramenta adequada para avaliar os processos que ocorrem na lesão hepática aguda de maneira bidirecional. Sendo assim, pudemos observar o efeito que as células da medula óssea (total ou fração mononuclear) exercem sobre o tecido hepático lesado, bem como o efeito que substâncias secretadas por este tecido têm sobre essas células.

ABSTRACT

Acute liver failure results from loss of the liver's vital functions due to major dysfunction of hepatic tissue. In several cases, liver transplantation is the definitive treatment, despite the liver's regenerative capacity. Regeneration mechanisms involve intricate regulatory pathways, with activation of growth factors and cytokines. Cell therapy with bone marrow stem cells has been proposed as a therapeutic alternative. However, its use remains in the experimental field. The objective of this study was to investigate paracrine effects of cell therapy in models of acute liver failure with the use semipermeable microcapsules.

First, animals undergoing 90% partial hepatectomy were transplanted with encapsulated bone marrow cells (BMC) or empty capsules. Results showed an increase in 10-day survival in treated animals treated with encapsulated BMC. Studies performed at earlier steps (within 72 hours) demonstrated that BMC reduced expression of genes that promote liver regeneration and increased the expression of its negative regulators. As a consequence, there was a decrease in the rate of liver regeneration in treated animals, and this effect occurred through paracrine mechanisms.

In the second study we evaluated the differentiation ability of encapsulated bone marrow mononuclear cells (BMMC) *in vivo* and *in vitro*. Animals submitted to carbon tetrachloride (CCl_4) induced acute liver injury were transplanted with encapsulated BMMC. Animals without liver injury were used as a control group. It was observed that 48 hours after transplantation, BMMC

began to express hepatocyte-specific genes. In order to better understand the mechanisms involved in this differentiation, we used encapsulated BMMC in an *in vitro* model that replicates the characteristics of the *in vivo* model. So, we employed a co-culture system, where encapsulated BMMC were kept in contact with medium from hepatocytes with or without injury by CCl_4. Our results showed that in just 6 hours of *in vitro* co-culture the encapsulated BMMC expressed hepatic markers. These results suggest the occurrence of BMMC differentiation at very early steps by paracrine mechanisms.

Taken together, results shown here allow us to conclude that cellular microencapsulation proved to be a suitable tool to assess the processes that occur in acute liver injury in a bidirectional manner. Thus, we were able to observe the effect that bone marrow cells (both total and mononuclear fraction) exert on the injured liver tissue, as well as the effect that substances secreted by this tissue have on these cells.

INTRODUÇÃO

Fígado

O fígado é o maior órgão sólido do corpo, responsável pela homeostase metabólica, uma vez que atua no metabolismo, síntese, armazenamento e redistribuição de nutrientes. É também o principal órgão de detoxificação do organismo por conversão metabólica e excreção biliar. Cerca de 80% do suprimento sangüíneo que recebe vem do intestino, através da veia porta, e o restante da artéria hepática (Taub, 2004).

O fígado está dividido em lóbulos, cujo número varia de acordo com a espécie, sendo que em humanos são 4: direito, esquerdo, caudado e quadrado. Entre as células constituintes do fígado, 80% são hepatócitos (ou células parenquimatosas). Entre as células não parenquimatosas estão as células endoteliais, células estreladas, células de Kupffer e linfócitos (figura 1). As células endoteliais proporcionam uma grande área de superfície para a absorção de nutrientes, formando sinusóides fenestrados que permitem o contato entre hepatócitos e linfócitos. Este contato pode produzir-se por meio de microvilosidades dos hepatócitos que se estendem para dentro do lúmen ou por extensões dos linfócitos que penetram no espaço de Disse (Fainboim et al., 2007) As células estreladas, localizadas no espaço de Disse, têm entre suas funções o armazenamento de vitamina A e a produção de matriz extracelular (Friedman, 2008). As células de Kupffer são a maior população de macrófagos teciduais, localizadas no espaço vascular sinusoidal do fígado, na área periportal. Esta localização permite a detoxificação do sangue, fagocitando partículas e organismos infecciosos que adentram o órgão pela circulação entero-hepática (Naito et al., 2004).

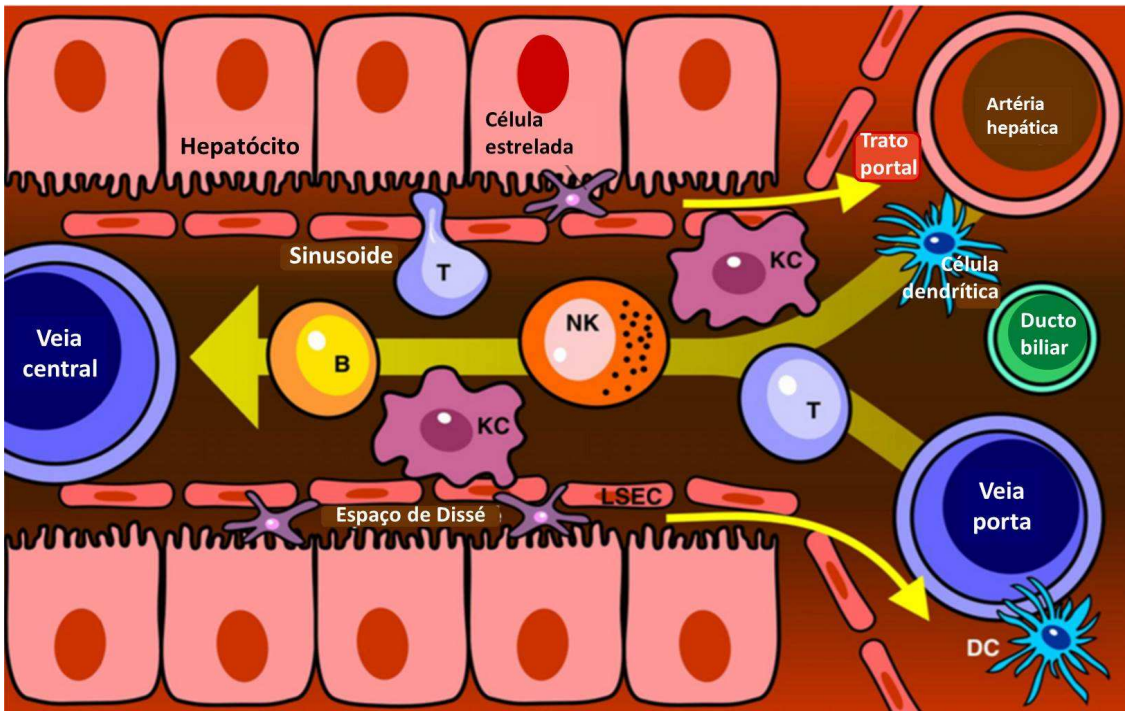

Figura 1. Arquitetura do fígado: hepatócitos, sinusóides e células do sistema imunológico. Células endoteliais formam uma monocamada no endotélio fenestrado sinusoidal. O espaço de Disse contém células estreladas hepáticas. Células de Kupffer residem dentro do espaço vascular sinusoidais do fígado, predominantemente na área de periportal. Linfócitos estão espalhados por todo o parênquima e tratos portais (Adaptado de Fainboim et al., 2007)

O hepatócito é a unidade celular funcional do fígado e realiza múltiplas funções sintéticas e metabólicas. Os hepatócitos são células epiteliais poliplóides, altamente diferenciadas, que formam estruturas tipo cordão. A subunidade funcional do fígado é o lóbulo hepático (figura 2), constituído de uma veia central rodeada por 6 tríades portais formadas pelos ductos biliares, pela artéria hepática e pela veia porta (Duncan et al., 2009; Islam and S. Lim, 2009).

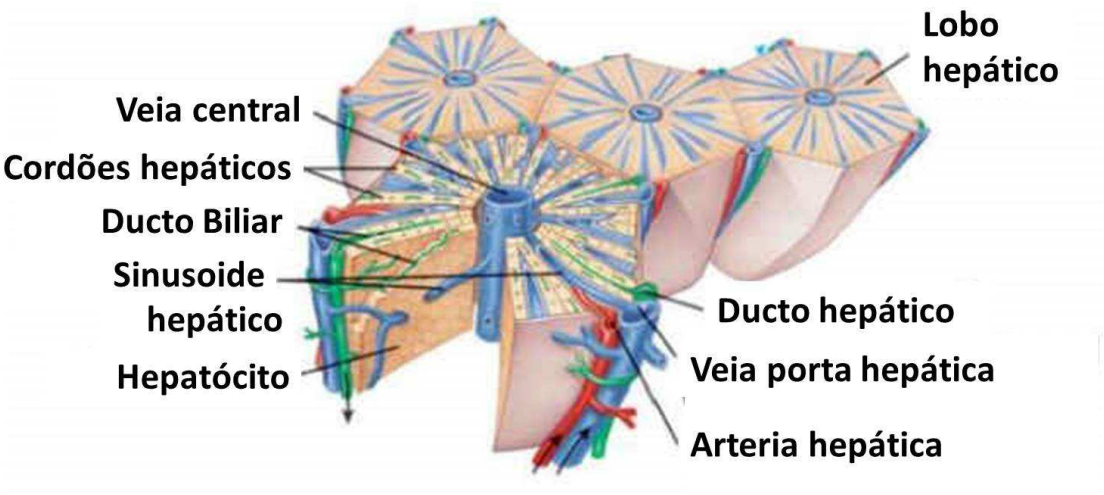

Figura 2. Esquema do lobulo hepático (Adaptado de Mohammed and Rama 2005)

Regeneração Hepática

O fato de que o fígado tem uma capacidade de regeneração extraordinária é conhecido há muitos anos. Como conta a mitologia grega, Prometeu, punido por Zeus, ficou acorrentado a uma rocha e seu fígado era comido diariamente por uma águia, porém durante a noite se regenerava fazendo com que o castigo não tivesse fim (Theise et al., 2000). É possível que a grande variedade de funções desempenhadas pelo fígado e sua importância para a homeostase do organismo tenham sido salvaguardadas por eventos evolutivos, que conferem ao fígado a capacidade de se regenerar. Este processo permite a recuperação da massa hepática perdida, sem comprometer a viabilidade do resto do organismo (Fausto, 2005).

In vivo existem três fontes distintas de células que participam na regeneração hepática. A primeira são os hepatócitos maduros, os quais respondem rapidamente ao dano hepático e constituem a primeira linha de regeneração (Overturf et al., 1997). A segunda fonte é uma população de células progenitoras especializada, residente no tecido, chamada de células

ovais (CO) em murinos e pequenos hepatócitos ou células progenitoras hepáticas em humanos (Roskams et al., 2004). Estas células estão localizadas nos canais de Herring, próximas aos ductos biliares e podem se diferenciar tanto em hepatócitos quanto em colangiócitos (células do epitélio biliar) (Beltrami et al., 2007). Esta população é ativada quando ocorre um dano crônico e extenso, em que os hepatócitos maduros não são mais capazes de responder (Viebahn and Yeoh, 2008). A terceira fonte são as células tronco originadas de fontes extra-hepáticas como, por exemplo, as da medula óssea ou outros tecidos (Banas et al., 2007b).

A característica de regeneração a partir de células diferenciadas (hepatócitos) é única do fígado. No fígado normal os hepatócitos se encontram em um estado quiescente em G0, porém, ante uma lesão têm a habilidade de proliferar entrando no ciclo celular (Taub, 2004). Na regeneração hepática o organismo lesado busca recompor a massa hepatocitária e a estrutura funcional lobular necessária à manutenção da homeostase corporal, cessando o processo regenerativo quando isso ocorre (Fausto et al., 2006).

Desde que Higgins and Anderson e colaboradores (1931) descreveram um método reprodutível de hepatectomia parcial em ratos, a capacidade regenerativa do fígado tem sido intensamente investigada (Ueno et al., 2006). Após hepatectomia parcial há uma resposta hiperplásica com a replicação de praticamente todas as células maduras nos lobos remanescentes (Taub, 2004). A hepatectomia parcial de até 70% de ressecção produz intensa regeneração e sobrevida de quase 100% em ratos (Tygstrup et al., 1996). Ressecções hepáticas mais extensas (70 a 80%) induzem insuficiência, e estão associadas a maior mortalidade (Panis et al., 1997).

Grande importância tem sido dada à patofisiologia e à resposta inflamatória do fígado lesado e necrótico (Rahman and Hodgson, 2000). Os mecanismos iniciais da regeneração hepática envolvem ativação das células de Kupffer em resposta a fatores como os lipopolissacarídeos (LPS). Alguns estudos sugerem que os LPS de origem entérica podem ser agentes estimulantes da produção inicial de citocinas pró-inflamatórias no processo regenerativo hepático (Vaquero et al., 2011a). LPS se ligam a seus receptores específicos os ,TLR-4 (*Toll Like Receptor*), que se encontram nas células de Kupffer regulando a imunidade inata e adaptativa (Fausto and Riehle, 2005; Campbell et al., 2006;). Uma vez ativados, os TLR-4 ativam a cascata de sinalização de MYD88 (*Myeloid Differentiation Factor*) envolvendo a ativação de NFκ-B (*Nuclear Factor Kappa B*), TNF-α (*Tumor Necrosis Factor Alfa*) e IL-6 (*Interleukin*-6) nas células de Kupffer (Vaquero et al., 2011c) (Figura 3). A liberação de IL-6 constitui um passo crucial na regeneração hepática, conforme demonstrado pela diminuição da sobrevida pós-hepatectomia parcial nos modelos deficientes nesta via (Wuestefeld et al., 2003). A IL-6 atua pela interação entre o seu receptor e gp130 (*Glycoprotein* 130), levando à fosforilação de Stat3 (*Signal transducer and activator of transcription* 3), que ativa genes da resposta de fase aguda, mecanismos de redução da apoptose de hepatócitos e induz a regeneração hepática (Wuestefeld et al., 2003; Taub, 2004). Após serem "ativados" por IL-6, os hepatócitos ficam receptivos a HGF (*Hepatocyte Growth Factor*), que induz à passagem de G0 a G1 iniciando o ciclo celular (Fausto and Riehle, 2005; Sudo, 2008). Este fator de crescimento é secretado como pró-HGF e depende da ativação por plasminogênio para produção da sua forma ativa que irá se ligar ao seu receptor Met

(Shanmukhappa et al., 2009). A ação concertada da IL-6 produzida pelas células de Kupffer e do HGF secretado pelas células estreladas desencadeia o processo inicial de regeneração hepática que ocorre nas primeiras horas após a lesão (Figura 3).

Há pouco conhecimento sobre os mecanismos envolvidos na finalização do processo de regeneração hepática. Possivelmente a restauração da função e da massa hepática seja um dos controladores do processo (Fausto and Riehle, 2005). A redução dos fatores estimulantes que induziram inicialmente a regeneração hepática ocorre via expressão gênica de moléculas como o SOCS3 (*Suppressor of Cytokine Signaling 3*) e o TGF- β (*Transforming Growth Factor-beta* 1*)*. TGF- β 1 é produzido pelas células mesenquimais na maioria dos tecidos e tem ação inibidora da mitose para a maioria das células epiteliais (Michalopoulos, 1995). No fígado, TGF- β é produzido principalmente pelas células estreladas hepáticas (Ikeda et al., 1998). Durante a regeneração os hepatócitos se tornam resistentes aos efeitos inibitórios do TGF- β devido à redução da expressão de seus receptores (Michalopoulos et al., 1995, 2009), porém ao final do processo de regeneração, sua ação é restabelecida e os hepatócitos retornam ao seu estado quiescente (Campbell et al., 2001; Taub, 2004). O SOCS3 provavelmente interage com a JAK (*Janus kinase*), inibindo a fosforilação de STAT3 e potencialmente encerrando a sinalização de IL-6 (Fausto and Riehle, 2005) (Figura 3). É importante salientar que a duração desse processo de regeneração é bastante curta, com o fígado recuperando quase a totalidade da massa perdida em uma hepatectomia parcial em apenas três dias (Panis et al., 1997).

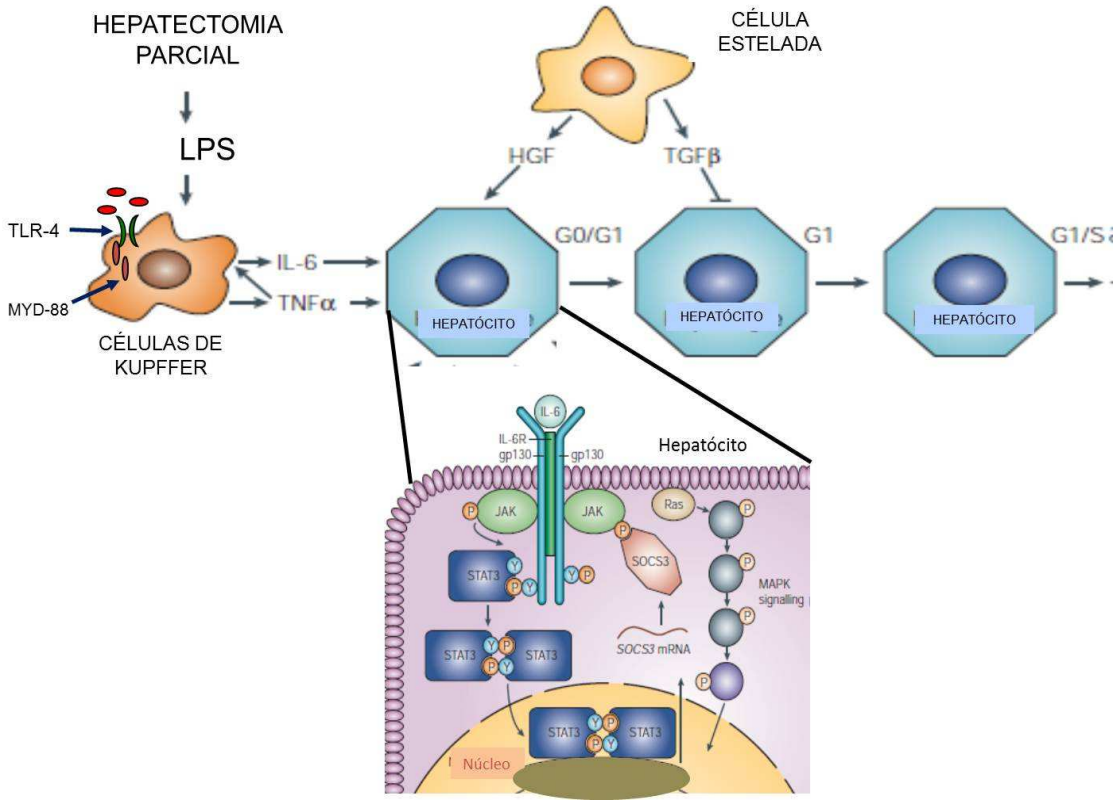

Figura 3. Vias de sinalização no processo de regeneração hepática. Modificado de Taub (2004)

Insuficiência hepática aguda

As causas da Insuficiência Hepática Aguda (IHA) incluem hepatite viral, doença hepática induzida por toxina ou drogas, erros inatos do metabolismo, isquemia, entre outras. No entanto, independentemente da etiologia, a evolução clínica de todos os casos com IHA é muito semelhante (Lee et al., 2008). Não existe nenhum tratamento médico comprovado para a IHA além do transplante hepático. Apesar da grande capacidade de regeneração do fígado, mesmo com medidas de suporte, quase 40% dos pacientes falecem por IHA, na sua maioria por falta de órgãos disponíveis (Lee et al., 2008).

A insuficiência hepática aguda (IHA), ou falência hepática fulminante, é caracterizada por uma perda repentina das funções hepáticas em um indivíduo

previamente saudável. É uma condição rara que muitas vezes leva a consequências devastadoras, sendo uma das mais difíceis emergências gastrointestinais encontradas na prática clínica. O padrão de sintomas clínicos e respostas fisiopatológicas estão associados com a rápida deterioração da função hepática normal (Sass and Shakil, 2005). A síndrome é definida pelo aparecimento de repentino de icterícia, coagulopatia, encefalopatia hepática, insuficiência renal, infecção e, em última instância, falência de múltiplos órgãos (Saliba and Samuel, 2013).

Modelos experimentais

Nos últimos 40 anos, vários modelos animais foram descritos para estudar a insuficiência hepática aguda, cada um deles refletindo um aspecto particular da doença, mas nenhum capaz de mimetizar o que ocorre nos pacientes de modo completo (Bélanger and Butterworth, 2005; Martins et al., 2008). Existem modelos químicos, cirúrgicos e virais. Como este estudo se orienta em modelos cirúrgicos e químicos, faremos uma maior descrição destes.

Modelos Cirúrgicos

Os modelos de IHA cirúrgicos podem ser divididos em três categorias: hepatectomia (total ou parcial), devascularização (total ou parcial) e modelos que são uma combinação dos anteriores (Martins et al., 2008; Tuñón, 2009). A maior limitação dos modelos cirúrgicos é a dependência da experiência e da habilidade técnica do cirurgião que pode interferir na sua reprodutibilidade (Bélanger and Butterworth, 2005; Rahman and Hodgson, 2000).

Hepatectomia parcial (HP) de 90% é o limite máximo para o modelo de regeneração hepática em animais (Makino et al., 2005). Este modelo serve como arquétipo para estudar os mecanismos que acontecem em hepatectomia estendida em humanos devido ao tratamento radical de tumores malignos no fígado ou transplante hepático, assim como na "*small for size syndrome*", na qual um receptor recebe um fígado de tamanho menor do que o ideal para o seu peso corporal (Shinohara et al., 2007; Sowa, 2008)

Nos modelos murinos, o percentual de massa hepática correspondente a lobos hepaticos está demonstrado na figura 4. No modelo de HP 90% são retirados os lobos direito, esquerdo e mediano, restando apenas os lobos caudados, que representa 10% da massa hepática (Fig. 4). Este modelo está mais relacionado a estudos de IHA do que de regeneração, que são realizados principalmente nos modelos de HP 70% (Jensen 2001; Oh et al., 2007 Chen et al., 2012; Fouraschen et al., 2012). Recentemente nosso grupo utilizou este modelo para avaliar a influência do regime anestésico na recuperação após anestesia, a sobrevivência, e os níveis de glicose no sangue após uma HP de 90% em ratos (Kieling et al., 2012).

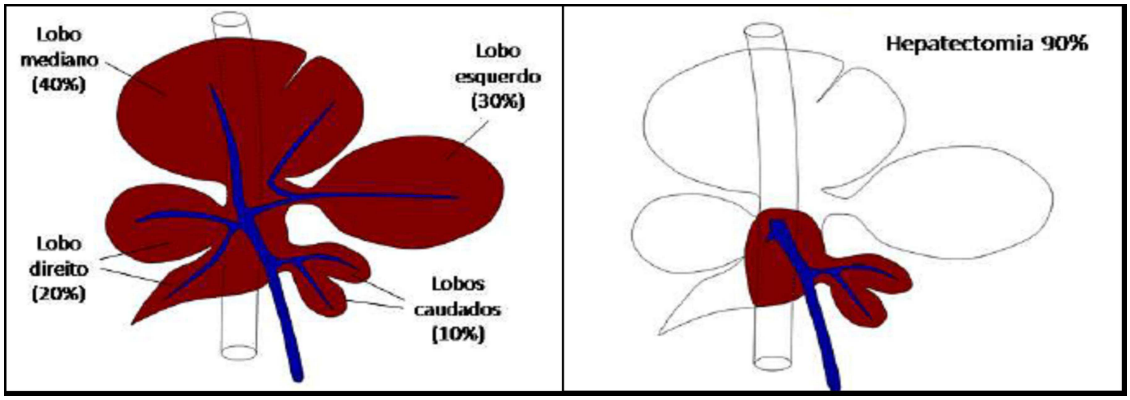

Figura 4. Esquema dos lobos ressecados em uma hepatectomia parcial de 90%. Modificado de Kieling (2012)

Modelos Químicos

O uso de agentes químicos, tais como o paracetamol (APAP), a D-galactosamina, o Tetracloreto de Carbono (CCl_4) a tioacetamida (TAA) e, mais recentemente, a concanavalina A e os LPS (Rahman and Hodgson, 2000) podem reproduzir um número importante de características clínicas da IHA, tais como hipoglicemia, encefalopatia e aumento dos níveis séricos de enzimas hepáticas.

Um modelo amplamente utilizado é a lesão causada por CCl_4 e seu efeito hepatotóxico é conhecido da década de 1930. O mecanismo de ação desta droga envolve sua metabolização ao radical triclorometil (CCl_3) através do citocromo P-450. Este se liga aos ácidos graxos das membranas celulares produzindo radicais alcóxi (R^-) e peróxi (ROO^-) e gerando peróxidos lipídicos altamente reativos (Recknagel *et al.*, 1989).

No Centro de Terapia Gênica do Hospital de Clínicas de Porto Alegre (CTG/HCPA) o paracetamol e o CCl_4 foram empregados em modelos animais de lesão hepática aguda (Belardinelli et al., 2008; Baldo et al., 2010;Rodrigues et al., 2012). A grande limitação desses modelos tóxicos é a imprevisibilidade da extensão da lesão hepática devido à variabilidade metabólica individual e toxicidade extra-hepática (Bélanger and Butterworth, 2005).

Novas Terapias Para a Insuficiência Hepática Aguda

O transplante de fígado de urgência é o único tratamento definitivo para a maioria dos pacientes em IHA. No entanto, a falta de doadores disponíveis limitam o uso dessa alternativa terapêutica. Por isso, nos últimos anos, tem-se buscado novas opções para o tratamento da IHA, incluindo sistemas de hemofiltração e o uso da terapia celular, seja por transplante de hepatócitos ou uso de células tronco.

O transplante de hepatócitos tem sido realizado no tratamento de pacientes com doenças crônicas (Strom et al., 1997; Dhawan et al., 2005; Khan et al., 2008) metabólicas (Ellor et al., 2008; Khan et al., 2008) e com insuficiência aguda (Habibullah et al., 1994; Bilir et al., 2000; Meyburg et al., 2008). No entanto, a limitação do emprego de hepatócitos isolados está relacionada à incapacidade de isolamento de um número suficiente de hepatócitos, bem como à impossibilidade da sua expansão em cultura (Muraca, 2011; Vosough et al., 2011).

Por outro lado, diversos estudos em modelos animais têm demonstrado o potencial da terapia celular com células derivadas de medula óssea (CMO) como alternativa terapêutica para a IHA. A medula óssea é formada por diferentes tipos celulares, entre eles as células tronco hematopoiéticas e as células tronco mesenquimais. Estas têm a capacidade de auto-renovação e diferenciação em vários tipos celulares, incluindo hepatócitos tanto *in vivo* (Lagasse et al., 2000; Liu and Chang, 2006; Quintana-Bustamante et al., 2012) c*omo in vitro* (Lee et al., 2004; Chen et al., 2007; Pournasr et al., 2011; Dong et

al., 2013). Entre os atrativos do uso das CMO estão a facilidade de isolamento e a expansão dessas células *in vitro* (Banas et al., 2007a). Além disso, a possibilidade de uso de células autólogas para a IHA eliminaria a necessidade de imunossupressão, que é uma das complicações do transplante hepático (P. A. Lysy et al., 2008).

Vários estudos em animais têm avaliado o efeito do transplante de CMO na IHA causada por agentes químicos (Jung et al., 2006; Cho et al., 2009; Jin et al., 2009; Gruttadauria et al., 2013). Na maioria deles os desfechos avaliados (sobrevida, redução da ALT, capacidade de enxertia das células e marcadores de proliferação como PCNA) são positivos nos animais tratados. Nosso grupo particularmente demonstrou o efeito benéfico do transplante da fração mononuclear de CMO em modelos de CCl_4 e paracetamol (Belardinelli et al., 2008; Baldo et al., 2010). Em modelos cirúrgicos, o transplante de CMO também tem mostrado benefícios na sobrevida e no aumento dos pesos dos lobos remanescentes nos animais tratados (Liu and Chang, 2006; Tokaiet al., 2009; Zhang et al., 2009)

Embora a utilização da CMO na IHA tenha sido vastamente estudada, o modo de atuação dessas células ainda não foi totalmente esclarecido. Vários mecanismos de ação têm sido propostos. Por um lado, sugere-se que haja um aumento do número de hepatócitos, seja por diferenciação, fusão celular ou mecanismos parácrinos, sendo esses mecanismos não excludentes (Mukhopadhyay, 2013).

Como já mencionamos as CMO tem a capacidade de originar hepatócitos sem fusão celular tanto *in vitro* como *in vivo.* Dong et al., (2013)

demonstraram a capacidade das CMO em diferenciar-se em hepatócitos *in vitro* utilizando uma combinação de citocinas e ácido valpróico. Liu et al., (2006) evidenciaram que CMO encapsuladas implantadas no peritônio de animais submetidos a HP de 90% apresentam marcadores típicos de hepatócitos, como albumina (ALB), citoqueratina 8 e 18 CK8,18) e alfa-fetoproteína (AFP). Da mesma forma, Gibran and Muhammad (2012), comprovaram que CMO transplantadas em um modelo de IHA por CCl_4 expressam albumina quatro semanas após o transplante.

Outro mecanismo para a atuação das CMO que já foi demonstrado *in vitro* e *in vivo* é a fusão celular, no qual duas células se unem para gerar um *heterocarion* (Herzog et al., 2003). Em particular, a fusão celular entre CMO e hepatócitos parece ser um mecanismo importante de acordo com os estudos de vários pesquisadores (Alvarez-Dolado et al., 2003; Wang et al., 2003; Quintana-Bustamante et al., 2012;). Alvarez-Dolado et al. (2003) utilizaram um sistema de recombinação Cre/Lox para demonstrar a fusão de CMO com hepatócitos. CMO de animais transgênicos expressando a proteína Cre foram transplantadas em ratos da linhagem Rosa26 (que expressam o gene repórter *LacZ*), nos quais o cassete de expressão se flanqueado por sítios LoxP. Por conseguinte, a expressão de LacZ é detectável apenas nas células que contêm a recombinase Cre fornecida pelo doador. Utilizando este sistema, os autores mostraram a fusão de hepatócitos (e também de células de Purkinje e cardiomiócitos) com CMO doadoras.

Contudo é importante reconhecer de que qualquer que nenhum dos mecanismos acima poderia produzir hepatócitos em número suficiente para sustentar os benefícios demonstrados nos modelos de hepatectomia parcial

(Houlihan and Newsome, 2008). Assim, a ideia de que o transplante de CMO exerce um efeito benéfico parácrino nas células hepáticas endógenas está ganhando força e é apoiada por observações em roedores e seres humanos, embora nestes últimos os estudos ainda sejam pequenos e não controlados (Almeida-Porada et al., 2010). Sabe-se que as células tronco mesenquimais secretam diversas moléculas bioativas que atuam sobre a dinâmica celular local, estimulando a regeneração das células endógenas e a recuperação dos tecidos (Caplan 2006; Meirelles et al., 2009; Zhang et al., 2009; Lin et al., 2011).

Em um modelo de lesão hepática aguda por D-Galactosamina, o tratamento com meio condicionado (meio que esteve em contato com células, fragamentos de tecido ou suplementado com soro) de células tronco mesenquimais aumentou a sobrevida e reduziu os níveis de IL-6, IL-1β (*Interleukin-1Beta*) e TNF-α e aumentou a expressão de IL-10 (*Interleukin-10*) (van Poll *et al.*, 2008).

Em um modelo de HP de 70%, Fouraschen e colaboradores (2012) demonstraram que o meio condicionado derivado de células tronco mesenquimais aumentou significativamente a regeneração hepática e a proliferação de hepatócitos. Também demonstraram que o meio condicionado regula positivamente a expressão de citocinas e fatores de crescimento relevantes para a proliferação celular, angiogênese e resposta anti-inflamatória (Fouraschen et al., 2012).

Por sua parte Zhiyong et al (2013) demonstrou que a infusão de meio condicionado derivado de células tronco mesenquimais em um modelo de

transplante aumenta a sobrevida, reduze significativamente a lesão e promove uma rápida regeneração de fígado remanescente. Além disso, a terapia com meio condicionado reduz a apoptose de hepatócitos e células endoteliais, assim como diminui a expressão de diversas citocinas pró-inflamatórias e diminui o infiltrado de neutrófilos e a ativação de células de Kupffer (Zhiyong et al., 2013)

Microencapsulação

Originalmente, as técnicas de microencapsulação foram desenvolvidas para utilização em terapia celular como forma de proteger células transplantadas da resposta imune do hospedeiro (Orive et al 2004). Essa estratégia tem sido proposta para situações como transplante de ilhotas (Holdcraft et al., 2012) ou para uso com células geneticamente modificadas para super-expressar uma proteína com efeito terapêutico (Baldo et al., 2012). As microcápsulas são constituídas por uma membrana polimérica semipermeável que circunda as células, permitindo a entrada de nutrientes e a saída de produtos protéicos terapêuticos, obtendo-se desta maneira uma distribuição sustentada da molécula desejável. O uso desta tecnologia para entrega de produtos terapêuticos de uma forma controlada e contínua no hospedeiro por células é um método potencialmente de baixo custo para o tratamento de uma ampla gama de doenças (Hernández et al., 2010; Matte et al., 2011).

Para a sua produção, as células são suspensas em um polímero que é perfundido através de um sistema encapsulador. Um fluxo de ar aplicado sobre

a ponta da agulha do perfusor produz microgotas, que ao caírem em uma solução polimerizadora (ex.: cloreto de cálcio) formam o envoltório capsular (Zimmermann et al., 2005) (figura 5). No nosso grupo, o alginato de sódio tem sido utilizado para o isolamento de linhagens celulares geneticamente modificadas (Baldo et al., 2012; Lagranha et al., 2008; Mayer et al., 2010) para a terapia celular experimental de doenças genéticas.

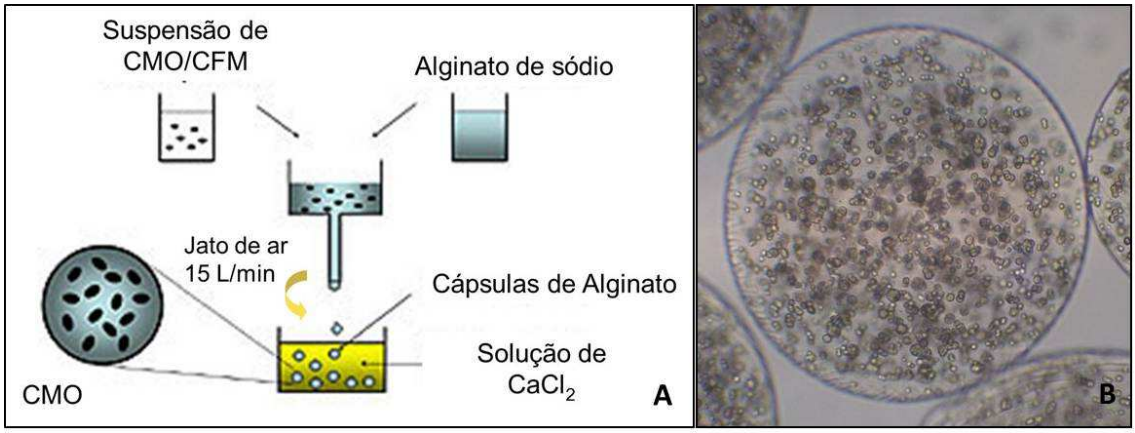

Figura 5. Encapsulação celular. A: esquema do processo de encapsulação. B: microgotografia de uma microcápsula contendo células derivada da medula óssea

No caso da IHA, a microencapsulação tem sido utilizada em vários estudos com modelos químicos ou cirúrgicos. Alguns estudos demonstraram aumento da sobrevida em animais tratados com hepatócitos microencapsulados (Aoki et al., 2005; K. Hamazaki et al., 2002; Mai et al., 2005; T. Rahman et al., 2005; Sgroi et al., 2011) assim como CMO encapsuladas (Liu and Chang, 2006; Z. Liu and Chang, 2005; Z. C. Liu and Chang, 2009)

A microencapsulação também pode ser utilizada para estudar os mecanismos parácrinos da terapia celular, ao permitir o isolamento da CMO ao mesmo tempo em que permite que essas células recebam os estímulos do

órgão lesado. Nesse sentido, é um modelo mais completo do que o uso de meio condicionado, no qual as CMO são mantidas nas condições ideais de cultivo *in vitro*. Este modelo, além de permitir a comunicação bi-direcional entre o fígado lesado e as CMO encapsuladas, também permite que diferentes tipos celulares sejam encapsulados separadamente, para avaliar a comunicação entre as células da CMO e qual o tipo de célula responsável pela resposta observada. Finalmente, a utilização de CMO encapsuladas representaria um passo adicional na segurança de uma aplicabilidade clínica desta metodologia, ao permitir que as células fossem recuperadas em caso de um evento adverso.

OBJETIVOS

Objetivo geral:

Estudar os efeitos parácrinos da terapia celular em dois modelos de insuficiência hepática aguda utilizando microcápsulas semi-permeáveis.

Objetivos específicos:

Avaliar em modelo cirúrgico de hepatectomia de 90% o efeito parácrino das células da medula óssea na sobrevida em 10 dias.

Avaliar no modelo acima o efeito parácrino das células da medula óssea nos processos precoces de regeneração hepática (até 72 horas).

Avaliar a diferenciação *in vivo* e *in vitro* das células da fração monuclear da medula óssea em células tipo hepatócitos em modelo de insuficiência hepática aguda por Tetracloreto de Carbono.

Artigo Nº 1

Encapsulated whole bone marrow cells improve survival in rats after 90% partial hepatectomy

A ser submetido para a revista *Liver International*

Encapsulated whole bone marrow cells improve survival in rats after 90% partial hepatectomy

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ABSTRACT

The use of bone marrow cells has been suggested as an alternative treatment for acute liver failure (ALF). In this study, we investigate the paracrine effect of encapsulated whole bone marrow cells (WBM) in a model of ALF. WBM were encapsulated in sodium alginate and implanted in Wistar rats submitted to 90% partial hepatectomy (PH). Control group received empty capsules (EC). The survival rate was assessed during 10 days (n=33). Another group of animals were euthanized in 6, 12, 24, 48 and 72 hours after PH (n=4-6/group/time point). Liver regeneration rate and the expression of genes related to liver regeneration were analyzed. The survival rate was higher for the WBM group than for EC group (p=0.002). Liver weight increased gradually after surgery and showed no differences between groups until 72 hours, when WBM group showed a lower regeneration rate compared to EC group (p=0.003). Gene expression levels related to the early phase of liver regeneration (*Tnf-α, Nfκ-B* and *Il-6)* were decreased in WBM group at 6 hours post 90% PH ($p<0.05$), whereas genes related to the progress of liver regeneration (*Socs-3, Hgf* and *Tgf-β)* were increased in the WBM group at 12, 24 and 48 hours compared to EC group ($p<=$ ou $=0.050$). In summary, we show that encapsulated WBM cells increase survival in a model of 90% PH by reducing the expression of genes involved in liver regeneration and increasing those responsible to end hepatocyte division.

INTRODUCTION

Acute liver failure (ALF) is characterized by the sudden loss of liver function that results in jaundice, coagulopathy and hepatic encephalopathy in a previously healthy individual. If not treated it can lead to renal and multiple organ failure, coma, and death (Chung et al., 2012). Orthotropic liver transplantation is the treatment of choice for ALF although the lack of a suitable donor in a short period of time can limit the success of this therapy (Canbay et al., 2011). In addition to that, the lifelong use of immunosuppressant after the transplant possesses side effects at short and long term (Mahadeb et al., 2009; Strauss et al., 2009). These observations and the high costs of the procedure and its complications have led to the search for alternative approaches to ALF that do not include liver transplant.

The use of bone marrow-derived cells in regenerative medicine has grown in the past years. Their efficacy has been shown in animal models of both chronic (Lyra et al., 2010; Ghanem et al., 2012) and acute liver disease (Belardinelli et al., 2008; Tokai et al., 2009; Baldo et al., 2010). They present several advantages when compared to hepatocytes as they are readily available, and can be expanded *in vivo* or *in vitro* (Almeida-Porada et al., 2010). In addition, the use of autologous cells would eliminate the need for immunosuppressants (Lysy et al., 2008). The mechanisms by which these cells exert their beneficial effect on liver regeneration are not completely well understood but may involve an increase in the number of hepatocytes either by transdifferentiation, fusion, and/or the secretion of paracrine factors that stimulate cell division, inhibit apoptosis or modulate local and systemic

inflammatory state (Houlihan and Newsome, 2008; Almeida-Porada et al., 2010).

Several pro-inflammatory factors are involved in the early phase of liver regeneration. After partial hepatectomy, the increased amounts of enteric lipopolysaccharides (LPS) that bind to TLR-4 (Toll Like Receptor 4) on Kupffer cells activate the MYD88 (Myeloid Differentiation Factor) pathway and trigger the activation of NFk-B (Nuclear Factor Kappa B), and the release of TNF-α (Tumor Necrosis Factor-α) and IL-6 (Interleukin-6) (Vaquero et al., 2011c). IL-6 plays a key role in liver regeneration, activating acute phase genes and priming hepatocytes to growth factors (Wuestefeld et al., 2003; Taub, 2004). HGF (Hepatocyte Growth Factor) then stimulates hepatocytes to pass from G0 to G1, initiating the cell cycle (Fausto and Riehle, 2005; Sudo et al., 2008). The increase in molecules such as SOCS3 (Suppressor of Cytokine Signaling 3) and TGF-β1 (Transforming Growth Factor-beta*)* contribute to the decrease in stimulating factors and the halt of liver regeneration (Nelson Fausto and Riehle, 2005; Michalopoulos, 2007).

After partial hepatectomy, there is a complex remodeling of the liver tissue with a transient disruption of the lobular architecture (Wack et al., 2001). Agglomerates of poorly vascularized hepatocytes are formed in the periportal area before invasion of sinusoidal cells (Michalopoulos, 2007; Ninomiya et al., 2010). Some authors have suggested that at the early stages of liver regeneration a very fine tuning in the rate of proliferation of parenchymal and non-parenchymal cells is needed. Ninomiya et al. (2010) showed that a slowed hepatocyte regeneration rate increased the survival in a model of 90% partial hepatectomy.

Our goal was to investigate the paracrine effect of bone marrow cells on the expression of genes related to liver regeneration and to test if this effect would have consequences in the regeneration rate and survival of rats after 90% partial hepatectomy.

METHODS

Animals

Two-month-old male Wistar rats, weighing 310.5 ± 33 g were housed under controlled temperature (between 18 and 22°C) in light–dark cycles of 12 h with free access to water and standard chow at Experimental Animal Unit at Hospital de Clinicas de Porto Alegre (HCPA). Handling, care and processing of animals were carried out according to regulations approved by our local Ethics Committee and complied with National Guidelines on Animal Care.

Experimental Design

Rats were randomly divided in two groups. Control group (n=15) received empty capsules (EC) and treated group received encapsulated whole bone marrow cells (WBM, n=11). Survival was observed for up to 10 days after 90% partial hepatectomy (PH). An additional set of animals was sacrificed at 6, 12, 24, 48, and 72 hours after 90% PH (n=4-6/group/time point) to evaluate the early effects of treatments. Another 33 animals without liver injury were used as donors of bone marrow-derived cells and 5 animals were used as normal controls (liver without injury).

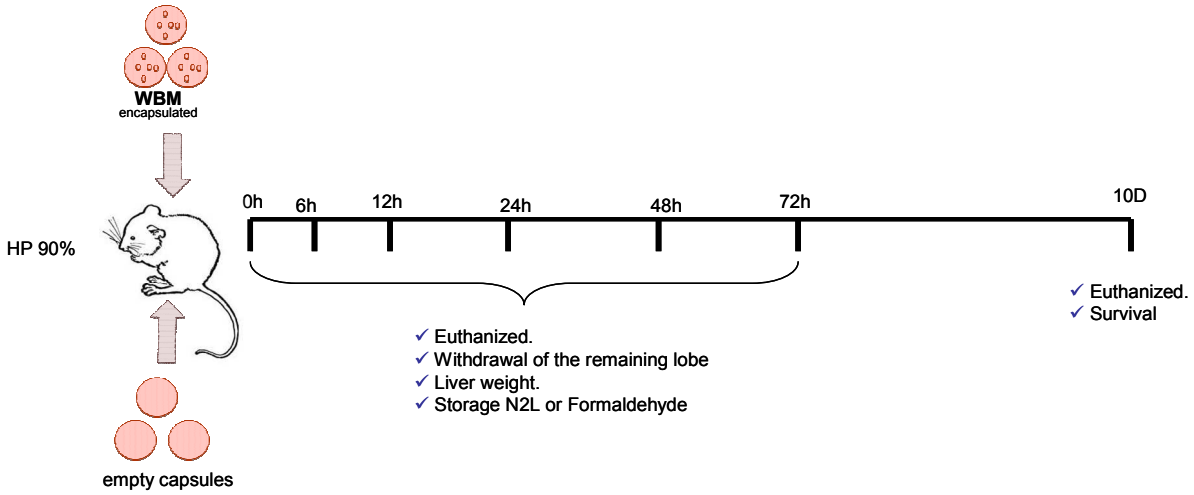

Figure 1. Experimental design.

Isolation of Whole Bone Marrow Cells

In a sterile environment, the femurs and tibias were isolated and WBM from each bone was flushed with 3 mL complete medium: DMEM (Dulbecco's Modified Eagle Medium. LGC, Brazil) supplemented with 10% fetal bovine serum (Gibco, USA), 1% penicillin/streptomycin (Gibco, USA). Cell viability was determined by Trypan's Blue exclusion.

Cell Encapsulation

Cell encapsulation was performed according to our laboratory protocol, previously described (Lagranha et al., 2008). Briefly, WBM cell were mixed with 1.5% sodium alginate (Sigma-Aldrich, USA) in complete medium and extruded through a Encapsulation Unit, type J1 (Nisco, Switzerland), attached to JMS Syringe Pump. Droplets were sheared off with an air flow of 5 L/min delivered to the tip of a 27 G needle and the rate of infusion was 40 mL/h. The droplets

fell into a bath of 125 mM $CaCl_2$ and ionically cross-linked with Ca_2^+ to form solid spherical hydrogel beads containing embedded WBM cells. In each well a volume of 2 mL of alginate suspension, containing $3x10^7$ WBM cells/animal. WBM cell encapsulation was carried out under sterile conditions. For control group, 2 mL of empty capsules were produced using the same approach, although without cells. The resulting capsules were maintained under normal cell culture conditions with complete medium at 37ºC and 5% CO_2 for 24h prior to administration.

Surgical Procedure and Capsules Transplantation

Ninety percent hepatectomy was performed by a single operator as described by Gaub and Iversen (1984). In brief, the left lateral (30%), left median (40%), and right superior lobes (20%) were removed, leaving only the caudate lobes. Hepatectomy was carried out under isoflurane (Forane®, Abbott SA, Argentina) anesthesia (Kieling et al., 2012). Immediately after 90% PH, and before complete suture, microcapsules (either empty or containing WBM cells) were placed into the peritoneal cavity and glucose was supplemented i.p. (5% of body weight). Postoperatively, animals were given i.p. glucose (5% of body weight) until day seven and received 20% glucose in their drinking water and standard chow *ad libitum*.

Euthanasia

Euthanasia was performed in CO_2 chambers at the 6, 12, 24, 48 and 72 h or at 10 days. Immediately after death the liver was removed, weighed and part was flash frozen in liquid nitrogen and the rest was placed in 10% buffered formalin.

Histology analysis

Paraffin-embedded liver specimens were cut in 4 μm sections and stained with hematoxylin and eosin (H-E). Microscopic liver aspect and presence of necrotic areas and apoptosis were observed by an expert pathologist.

Liver Regeneration Rate

The liver regeneration rate was calculated as follows: Liver Regeneration Rate (%) = 100 x [C- (A–B)]/A, where A is the estimated liver weight before 90% PH, B is the excised liver weight at the time of PH and C is the weight of the regenerated liver at the time of sacrifice (L. Zhang et al., 2012).

Quantitative Real-Time PCR

Total RNA was extracted from 50 mg of liver tissue using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Two micrograms of RNA were reverse-transcribed using High Capacity cDNA Reverse

Transcription Kit (Life Technologies, USA). Gene expression was measured using TaqMan® assays (Life Technologies, USA) for genes involved in hepatic regeneration (table 1). The percentage of a test RNA to that of β-actin was calculated by subtracting the cycle to reach the threshold (CT) for a gene from the CT for a separate assay using β-actin assay to determine the ΔCT, and the formula: percent β-actin = (100) × $2^{\Delta CT}$ (Baldo et al., 2011). The percent β-actin for hepatectomized animals was divided by the percent β-actin in normal animals to determine the ratio of the gene in both treatments after 90% PH to normal rats.

Table 1: TaqMan® (Life Technologies, USA) ID assays for genes analyzed in this study.

Gene Symbol	Assay ID
Act-b	Rn00667869_m1*
Hgf	Rn00566673_m1*
Il-6	Rn01410330_m1*
Myd88	Rn01640049_m1*
NfkB	Rn01399583_m1*
Socs3	Rn00585674_m1*
Tgf-b	Rn01475963_m1*
Tlr-4	Rn00569848_m1*
Tnf-a	Rn00562055_m1*

Statistical analysis

Results were expressed as means ± SD or medians when required. Statistical differences were assessed by Student t test and for non-parametric variables Mann-Whitney was used. The survival rate was analyzed by Kaplan-Meier curve. The comparison of survival rates in different groups was tested by the log rank test. P values less than 0.05 were considered statistically significant.

RESULTS

Survival Rate

Overall survival rate was observed during 10 days after hepatectomy. The survival rate was higher for the WBM group (63.6%) than for EC group (6.7%) (p=0.002). Animals in WBM group died predominantly during the first three days, whereas in the other group deaths occurred over time after surgery (figure 1). Therefore, the remaining analyses were performed in the first 72 h post 90% PH.

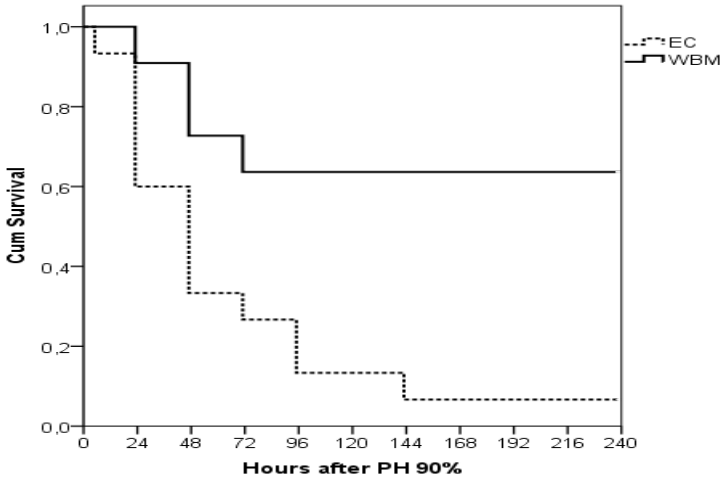

Figure 1. Spontaneous survival according to therapeutic regimen in rats after 90% partial hepatectomy. EC (Empty capsule), WBM (Whole Bone Marrow). Log rank=0.002.

Histology analysis

Microscopic liver aspect was analyzed by H-E staining at different time points. At the first 24 hours, no differences were observed between groups (figure 2). However, at 48 hours livers from the EC group presented a much higher degree of hydropic degeneration, seen as large vacuoles in the cytoplasm, than those from WBM group. At 72 hours, both groups were different, presenting small regular vacuoles that suggested some degree of microvesicular steatosis.

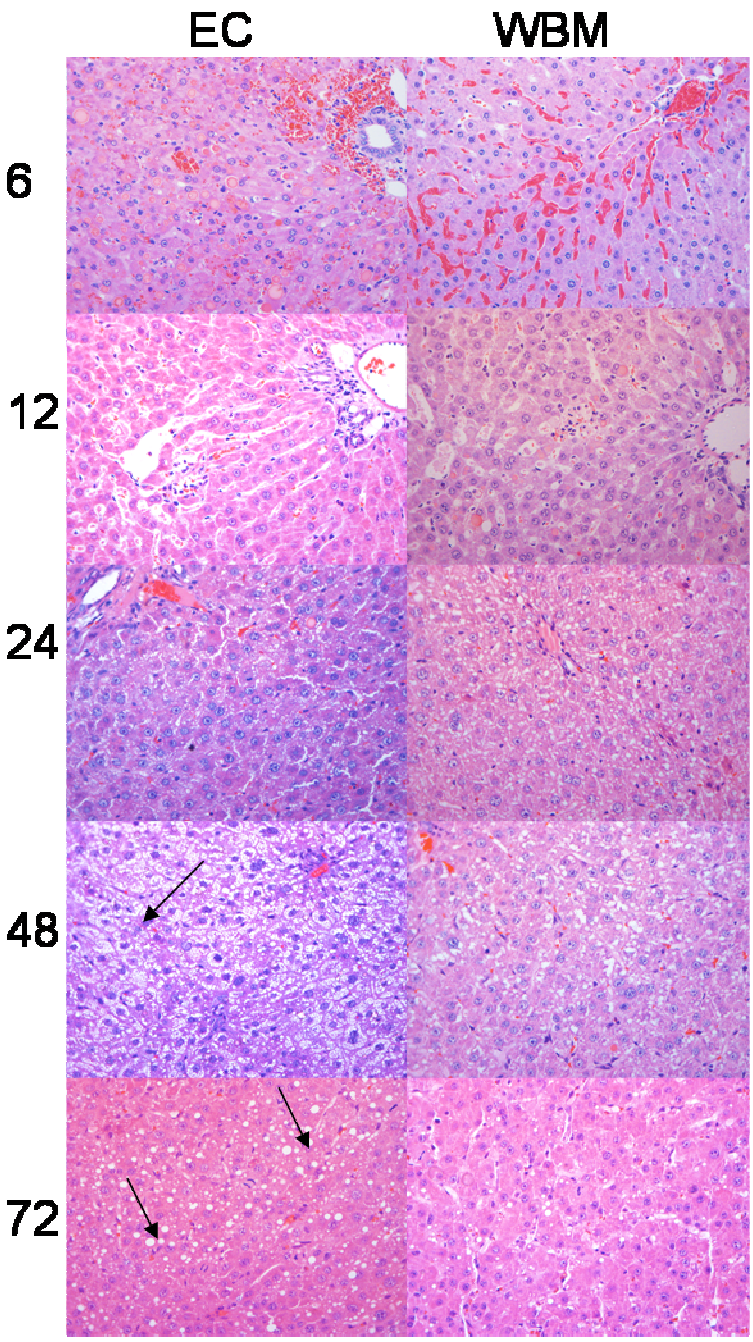

Figure 2. Liver sections from EC and WBM groups at different time points (hours) after 90% partial hepatectomy. Arrows show hydropic degeneration at 48h and steatosis at 72h. EC (Empty capsule), WBM (Whole Bone Marrow). H-E staining, 40X magnification.

Liver regeneration rate

Liver weight is usually used to determine the liver regeneration rate in the remnant lobe. Liver weight increased gradually after surgery, without differences between groups at 6, 12, 24 or 48 hours. However, as shown in figure 3, at 72 h WBM group showed a lower regeneration rate compared to EC group (44% *vs.* 59%, p=0.003).

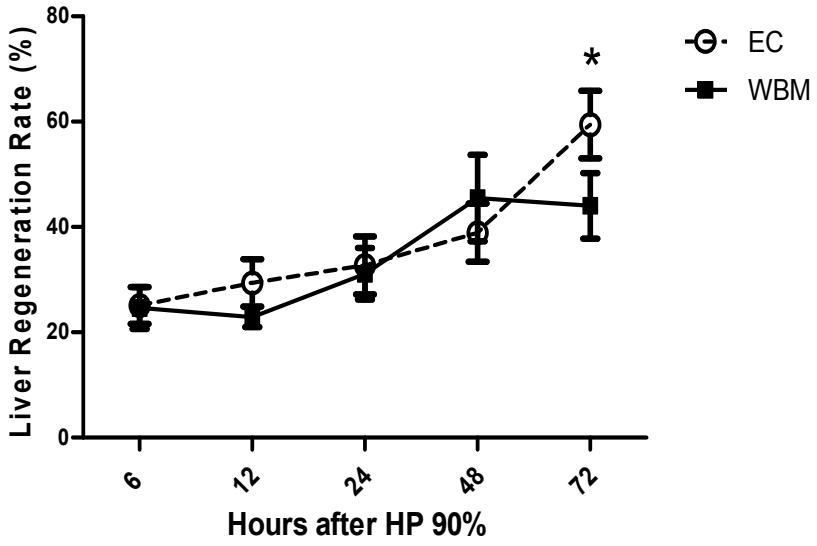

Figure 3 - Liver regeneration rate after 90% partial hepatectomy. WBM = encapsulated whole bone marrow cells; EC = empty capsules group. Values are expressed as means ± SD. *p=0.003, Student's t test.

Expression of genes involved liver regeneration

Since treated animals seemed to have a lower regeneration rate at 72 hours, we then assessed the expression levels of genes related to liver regeneration, especially those related to the IL-6 pathway.

First we assessed the expression levels of genes related to the early phase of liver regeneration. The expression of *Tnf-α* (p=0.013) and *Nfκ-B*

(p=0.011) was markedly decreased in WBM group at 6 hours post 90% HP (figure 4A and 4B). As a result, the expression of *Il-6* was also decreased (p=0.046) in the WBM group compared to EC group (figure 4C). Interestingly, LPS receptor (*Tlr-4*) and its mediator (*Myd88*) showed no differences in gene expression between groups 6 hours after 90%HP (figure 4D and 4E).

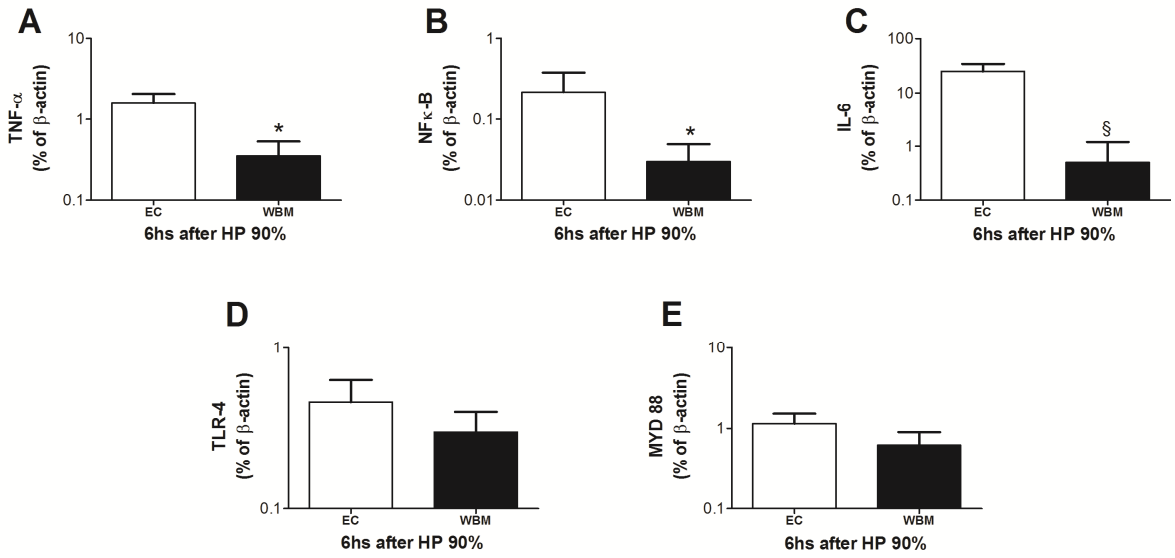

Figure 4. Liver gene expression of *Tnf-a* (A), *Nfκ-B* (B), *Il-6* (C), *Tlr-4* (D) and *Myd88* (E) 6 hours post 90% partial hepatectomy. WBM = encapsulated whole bone marrow cells; EC = empty capsules group. Values are expressed as means ± SD. Student-t test was performance, *p≤0.013 and §p=0.046. Graphs shown in log scale.

We then analyzed genes related to the progress of liver regeneration. At 12, 24 and 48 hours after 90% PH other genes were also differently expressed between WBM and EC groups. *Socs-3*, which inhibits signaling via IL-6 was increased in the WBM group at 12 and 24 hours after 90% PH (p≤0.05, figure 5A). *Hgf* was slightly increased in WBM only 24 hours after 90% PH (p=0.046,

figure 5B), whereas the expression of *Tgf-β* was increased in WBM group in 12-48hrs (p≤0.028, figure 5C).

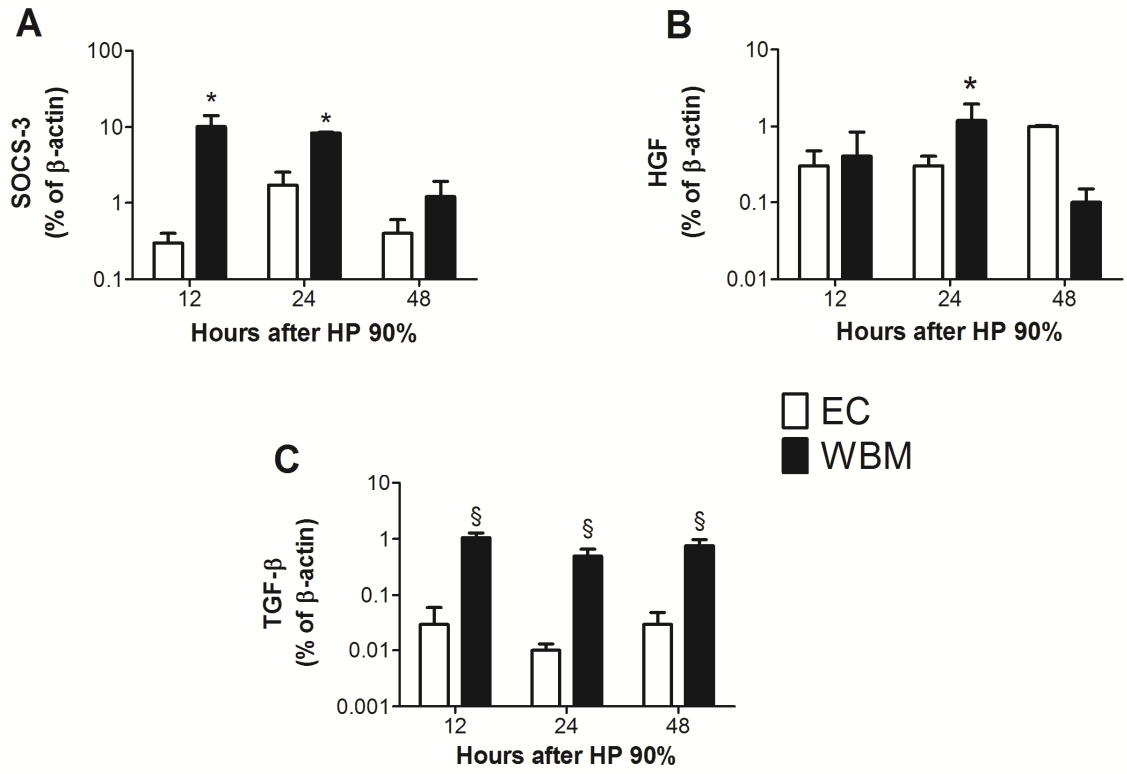

Figure 5. Liver gene expression of *Socs3* (A), *Hgf* (B), and *Tgf-β* 12, 24 and 48 hours post 90% partial hepatectomy. WBM = encapsulated whole bone marrow cells; EC = empty capsules group. Values are expressed as means ± SD. Student-t test was performance, *p≤0.05 and §p≤0.028. Graphs shown in log scale.

DISCUSSION

In the present study we showed that encapsulated WBM cells increase survival in a model of 90% PH by reducing the synthesis of inflammatory cytokines in the liver and thus diminishing the pace of liver regeneration through the secretion of paracrine factors. Our results corroborate the findings

of Liu & Chang (2005; 2006; 2009), who showed that encapsulated WBM cells increased survival in rats after 90% PH. They suggest that secretion of growth factors especially by mesenchymal cells present in WBM would stimulate liver regeneration (Liu and Chang, 2009). However, unlike these previous reports, we have not seen an increase in *Hgf* expression after treatment and liver regeneration rate was lower in treated animals. Analysis of inflammatory cytokines in the early phase after 90% PH suggested that in our model liver regeneration was rather decreased than enhanced by factors secreted by WBM cells.

A similar observation was made by Ninomiya et al. (2010) who suggested that the abrupt regenerative response after PH causes a derangement of the lobular architecture the is damaging to hepatocytes. In their work, the deceleration of liver regeneration increases survival rate after 90% PH, as seen in the present study. At 10 days post-surgery, WBM group survival rate was 63% compared to 6.7% in EC group.

When evaluating the expression of inflammatory cytokines IL-6, TNF-α and NFκ-B that are pivotal for initiating liver regeneration (Garcea and Maddern, 2009), they were all decreased in WBM group 6 hours after 90% PH. We then hypothesized that this reduction could be, at least in part, due to a decreased signaling by Kupffer cells. It is known that after partial hepatectomy Kupffer cells are overloaded with enteric antigens and that LPS-binding to TLR4 triggers the regenerative process (Vaquero et al., 2011b). However, no differences in expression of TLR-4 and its mediator MYD88 were detected between groups. It is worth noticing that such differences may have occurred at earlier time points and therefore were not detected by this study.

Consistent with this decrease in genes related to the promotion of early regenerative phase, HGF was also not increased in WBM group, except at 24 hours post 90% PH. On the other hand, the expression of TGF-β, a HGF inhibitor (Arakawa et al., 2009) was markedly increased in WBM group between 12 and 48 hours. In addition to that, the expression of SOCS3, an important negative regulator of IL-6 that blocks STAT3 phosphorylation (Riehle et al., 2008;Alison et al., 2009), was also increased in WBM group. Taken together, these data suggests that encapsulated WBM are increasing survival by decreasing liver regeneration rate.

One important limitation of this study is that animals were killed at distinct time points instead of analyzed at the time of their natural deaths. This means that one cannot determine if a given animal killed at six hours post-hepatectomy would have survived for the ten-day period or would day in the next few hours. This may explain the wide distribution in the results we obtained. However, having someone to collect the livers of animals in a timely fashion as they decease would be impractical.

It is also important to notice that this study was performed in non-syngeneic Wistar rats, simulating a real situation in which heterologous WBM cells could be used in patients with ALF. The immobilization and isolation of cells within the capsules protect them from the host immune system (Matte et al., 2011; Orive et al., 2003). This may be a reason why in the works of Liu & Chang (2005, 2006 and 2009) a beneficial effect was observed only with encapsulated cells. It may also explain the lack of efficacy reported by Tokai et al. (2009) in a model of 70% PH.

In summary the results presented here show that encapsulated WBM cells reduce the expression of genes involved in liver regeneration, such as TNF-α, NFκ-B, IL-6, and HGF, and increase those responsible to end hepatocyte division, as TGF-β and SOCS-3. In spite of that, these cells increase survival in a model of 90% PH in rats, which suggests that a slower regeneration rate may be beneficial for survival after massive hepatocyte loss. The mechanisms involved in this complex gene regulation need to be further studied.

REFERENCES

Almeida-Porada, G., Zanjani, E.D., Porada, C.D., 2010. Bone marrow stem cells and liver regeneration. Experimental hematology 38, 574–80.

Arakawa, Y., Shimada, M., Uchiyama, H., Ikegami, T., Yoshizumi, T., Imura, S., 2009. Beneficial effects of splenectomy on massive hepatectomy model in rats. Hepatol Res 39, 391–7.

Baldo, G., Giugliani, R., Uribe, C., Belardinelli, M.C., Duarte, M.E.S., Meurer, Luíse, Da Silveira, T.R., Matte, U., 2010. Bone marrow mononuclear cell transplantation improves survival and induces hepatocyte proliferation in rats after CCl(4) acute liver damage. Digestive diseases and sciences 55, 3384–92.

Baldo, G., Wu, A., Knutsen, G., Frang, K., Mecham, R., Liu, Y., Wu, X., Atkinson, J., Ponder, K., 2011. Pathogenesis of aortic dilatation in mucopolysaccharidosis VII mice may involve complement activation. Mol Genet Metab 104, 608–619.

Belardinelli, M.C., Pereira, F., Baldo, G., Tavares, M.V., Kieling, C.O., Meurer, Luise, Giugliani, R., Matte, U., 2008. Adult derived mononuclear bone marrow cells improve survival in a model of acetaminophen-induced acute liver failure in rats. Bone 247, 1–5.

Canbay, A., Tacke, F., Hadem, J., Trautwein, C, Gerken, G., Manns, M., 2011. Acute liver failure: a lifethreatening disease. Dtsch Arztebl Int 108, 714–20.

Chung, R.T., Stravitz, R.T., Fontana, R.J., Schiodt, F. V, Mehal, W.Z., Reddy, K.R., Lee, W.M., 2012. Pathogenesis of liver injury in acute liver failure. Gastroenterology 143, e1–7.

Fausto, N., Riehle, 2005. Mechanisms of liver regeneration and their clinical implications. Journal of hepato-biliary-pancreatic surgery 12, 181–9.

Garcea, G., Maddern, G.J., 2009. Liver failure after major hepatic resection. Journal of hepato-biliary-pancreatic surgery 16, 145–55.

Gaub, J., Iversen, J., 1984. Rat liver regeneration after 90% partial hepatectomy. Hepatology 4, 902–4.

Ghanem, L.Y., Nosseir, M.M., Lotfi, A. a, Mohamed, A.S., Ibrahim, R. a, Hassanein, M.H., Mansour, E., Makhlouf, M.M., Fouad, Y.M., El-Khayat, H.R., 2012. Hematopoietic stem cell mobilization into the peripheral circulation in patients with chronic liver diseases. Journal of digestive diseases 13, 571–8.

Houlihan, D.D., Newsome, P.N., 2008. Clinical advances in liver, pancreas, and biliary tract. Gastroenterology 438 – 450.

Islam, S., Lim, S., 2009. Stem cells in liver regeneration , fibrosis and cancer : the good , the bad and the ugly. Journal of Pathology 1, 282–298.

Kieling, C.O., Backes, N.A., MaurerLucyk, R., Uribe Crus, C., Bersch, A.O., Matte, U., 2012. The effects of anesthetic regimen in 90 % hepatectomy in rats 1 Os efeitos do regime anestésico na hepatectomia de 90 % em ratos. Acta Cirúrgica Brasileira 27, 702–706.

Lagranha, V.L., Baldo, G., Giacomet de Carvalho, T., Burin, M., Saraiva-Pereira, M.L., Matte, U., Giugliani, R., 2008. In vitro correction of ARSA deficiency in human skin fibroblasts from Metachromatic Leukodystrophy patients after treatment with microencapsulated recombinant cells. Metab Brain Dis 23, 469–484.

Liu, Z.C., Chang, T., 2005. Transplantation of bioencapsulated bone marrow stem cells improves hepatic regeneration and survival of 90% hepatectomized rats: a preliminary report. Artif Cells Blood Substit Immobil Biotechnol 33, 405–10.

Liu, Z.C., Chang, T., 2009. Preliminary study on intrasplenic implantation of artificial cell bioencapsulated stem cells to increase the survival of 90% hepatectomized rats. Artificial cells, blood substitutes, and immobilization biotechnology 37, 53–5.

Liu, Z.C., Chang, T.M., 2006. Transdifferentiation of bioencapsulated bone marrow cells into hepatocyte-like cells in the 90% hepatectomized rat model. Liver Transplantation 12, 566–572.

Lyra, A.C., Soares, M.B.P., Da Silva, L.F.M., Braga, E.L., Oliveira, S. a, Fortes, M.F., Silva, A.G.P., Brustolim, D., Genser, B., Dos Santos, R.R., Lyra, L.G.C., 2010. Infusion of autologous bone marrow mononuclear cells through hepatic artery results in a short-term improvement of liver function in patients with chronic liver disease: a pilot randomized controlled study. European journal of gastroenterology & hepatology 22, 33–42.

Lysy, P., Campard, D., Smets, F., Najimi, M., Sokal, E.M., 2008. Stem cells for liver tissue repair: current knowledge and perspectives. World journal of gastroenterology: WJG 14, 864–75.

Mahadeb, P., Gras, J., Sokal, E., Otte, J., Lerut, J., Detaille, T., 2009. Liver transplantation in children with fulminant hepatic failure: The UCL experience. Pediatr Transplant 13, 414–20.

Matte, U., Lagranha, V.L., De Carvalho, T.G., Mayer, F.Q., Giugliani, R., 2011. Cell microencapsulation: a potential tool for the treatment of neuronopathic lysosomal storage diseases. Journal of inherited metabolic disease 34, 983–90.

Michalopoulos, G.K., 2007. Liver Regeneration. J Cell Physiol. 213, 286–300.

Ninomiya, M., Shirabe, K., Terashi, T., Ijichi, H., Yonemura, Y., Harada, N., Soejima, Y., Taketomi, a, Shimada, M., Maehara, Y., 2010. Deceleration of regenerative response improves the outcome of rat with massive hepatectomy. American journal of transplantation: official journal of the American Society of Transplantation and the American Society of Transplant Surgeons 10, 1580–7.

Orive, G., Gascón, A., Hernández, R., Igartua, M., Luis Pedraz, J., 2003. Cell microencapsulation technology for biomedical purposes: novel insights and challenges. Trends Pharmacol Sci 24, 207–10.

Riehle, A., Campbell, J.S., McMahan, R., Johnson, M., Beyer, Bammler, T., 2008. Regulation of liver regeneration and hepatocarcinogenesis by suppressor of cytokine signaling 3. Exp Med 205, 91–103.

Strauss, A., Grabhorn, E., Sornsakrin, M., Briem-Richter, A., Nashan, B., 2009. Liver transplantation for fulminant hepatic failure in infancy: a single center experience. Pediatr Transplant 13, 838–42.

Sudo, K., Yamada, Y., Saito, K., Shimizu, S., Ohashi, H., Kato, T., Moriwaki, H., Ito, H., □ M.S. a, 2008. TNF-α and IL-6 signals from the bone marrow derived cells are necessary for normal.pdf. Biochimica et biophysica acta 1782, 671–79.

Taub, R., 2004. Liver regeneration: from myth to mechanism. Nature reviews. Molecular cell biology 5, 836–47.

Tokai, H., Kawashita, Y., Ito, Y., Yamanouchi, K., Takatsuki, M., Eguchi, 2009. Efficacy and limitation of bone marrow transplantation in the treatment of acute and subacute liver failure in rats. Hepatol Res 39, 1137–43.

Vaquero, J., Campbell, J.S., Haque, J., McMahan, R.S., Riehle, K.J., Bauer, R.L., Fausto, N., 2011a. Toll-like receptor 4 and myeloid differentiation factor 88 provide mechanistic insights into the cause and effects of interleukin-6 activation in mouse liver regeneration. Hepatology (Baltimore, Md.) 54, 597–608.

Vaquero, J., Riehle, K.J., Fausto, N., Campbell, J.S., 2011b. Liver Regeneration after Partial Hepatectomy Is Not Impaired in Mice with Double Deficiency of Myd88 and IFNAR Genes. Gastroenterology research and practice 2011, 727403.

Wack, K., Ross, M., Zegarra, V., Sysko, L., Watkins, S., Stolz, D., 2001. Sinusoidal ultrastructure evaluated during the revascularization of regenerating rat liver. Hepatology 33, 363–78.

Wuestefeld, T., Klein, C., Streetz, K.L., Betz, U., Lauber, J., Buer, J., Manns, M.P., Müller, W., Trautwein, Christian, 2003. Interleukin-6/glycoprotein 130-dependent pathways are protective during liver regeneration. The Journal of biological chemistry 278, 11281–8.

Zhang, L., Ye, J.-S., Decot, V., Stoltz, J.-F., De Isla, N., 2012. Research on stem cells as candidates to be differentiated into hepatocytes. Bio-medical materials and engineering 22, 105–11.

Artigo Nº 2

Bone-marrow derived mononuclear cells differentiate into hepatocyte-like cells within few hours without fusion

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Bone marrow-derived mononuclear cells differentiate into hepatocyte-like cells within few hours without fusion

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Abstract

Background: Cell therapy using bone marrow mononuclear cells (BMMC) has been shown as a potential treatment for liver diseases. BMMC can act by fusion with the host's cells, differentiation into hepatocyte-like cells and/or secretion of paracrine factors. Here, we used encapsulated BMMC in a model of Carbon Tetrachloride (CCl_4)-induced acute liver injury to study *in vivo* and *in vitro* differentiation of BMMC.

Methods: Both *in vitro* and *in vivo* studies were conducted in Wistar rats submitted to CCl_4-induced acute liver injury. BMMC were isolated from Wistar rats and encapsulated in sodium alginate microcapsules. For *in vivo* experiments, animals received encapsulated BMMC 24 hours after CCl_4 administration and capsules were collected within 6, 24 and 48 hours ($tCCl_4$ group). For *in vitro* experiments, isolated hepatocytes from animals with CCl_4-induced liver injury were co-cultured with encapsulated BMMC for 6 h ($cCCl_4$ group). Control groups were not submitted to CCl_4 administration (tControl and cControl groups, respectively). The content of intracellular lipid droplets in hepatocytes was used to evaluate liver injury. BMMC differentiation was assessed by RT-PCR for hepatic genes and ability to produce and secrete urea.

Results: Liver damage was confirmed in CCl_4 treated animals by the presence of several intracellular lipid droplets in hepatocytes and the characteristic nutmeg aspect of the liver. Retrieved encapsulated BMMC from $tCCl_4$ group expressed hepatocyte markers, such as *Cytokeratin 18* and *Albumin* 48 hours after treatment. On the other hand, BMMC from $cCCl_4$ group showed *Albumin* expression 6 hours after co-culture. Urea production was increased in BMMC

from $cCCl_4$ group but not in cControl. BMMC from tControl or cControl groups did not express hepatocyte markers at any time point.

Conclusions: In this study we show that BMMC differentiate into hepatocyte-like cells in a short period of time both *in vivo* and *in vitro*. This differentiation is triggered by paracrine factors present only in injured liver.

Keywords: Bone marrow mononuclear cells, cell differentiation, paracrine effects, hepatocyte-like cells, acute liver failure

Abbreviations

Afp*,** *Alpha-fetoprotein*; ***Alb, *Albumin*; **BMMC,** Bone Marrow Mononuclear Cells; **$cCCl_4$**, Co-culture of BMMC with hepatocytes from CCl_4 treated rats; **CCl_4**, Carbon Tetrachloride; **cControl**, Co-culture of BMMC with hepatocytes from healthy rats; ***Ck8***, *Cytokeratin 8*; ***Ck18***, *Cytokeratin 18*; **DMEM**, Dulbecco's Modified Eagle Medium; **FBS**, Fetal Bovine Serum; ***Gapdh***, Glyceraldehyde 3-phosphate dehydrogenase; **HGF**, Hepatocyte Growth Factor; **MSC**, Mesenchymal Stem Cells; **ORO**, Oil Red O; **PEG**, Polyethylene Glycol; **PBS**, Phosphate Buffered Saline; **P/S**, Penicilin/Streptomicin; **RT-PCR**, Reverse transcription-polymerase chain reaction; **$tCCl_4$**, Transplantation of BMMC in rats with CCl_4-induced liver damaged; **tControl**, Transplantation of BMMC in healthy rats.

Introduction

Bone marrow mononuclear cells (BMMC) have emerged as potential candidates for cell therapy due to their ease of use. In models of acute liver failure, transplantation of these cells increase the survival rate [1, 2]. Furthermore, recent clinical trials have demonstrated that transplantation of these cells or their fractions improves the condition of patients with cirrhosis [3–5], as well as improve liver function in animal models of cirrhosis [6]. Moreover, BMMC are characterized by their ability to differentiate into several functional mature cell types both *in vivo* and *in vitro*, including cardiomyocytes [7, 8], endothelial cells [9], neurons [10, 11] and hepatocytes [12–18]. In addition, many groups have developed protocols for the *in vitro* differentiation of bone marrow-derived cells into hepatocyte-like cells [19–24]. Although bone marrow mononuclear fraction is used for *in vivo* transplantation, *in vitro* protocols usually work with mesenchymal stem cells (MSC) [19, 23–25].

Microencapsulation provides a vehicle for the discrete control of key parameters such as the diffusion of growth factors, metabolites, and wastes. It has been demonstrated that the alginate microenvironment maintains cell viability, is conducive to embryonic stem cell differentiation into hepatocytes, and maintains differentiated cellular function [26]. In addition, encapsulated bone marrow cells showed evidence of glycogen synthesis and expression of typical markers of hepatocytes, after transplantation in the 90% liver failure model [15, 27].

In the present work we used semi-permeable alginate microcapsules to isolate BMMC in a model of Carbon Tetrachloride (CCl_4)-induced acute liver injury. Alginate microcapsules isolate BMMC and allow the study of *in vivo* and *in*

vitro differentiation through paracrine factors. We also focused on investigating early reprogramming events that might take place during exposure to injury microenvironment.

Methods

Experimental design

Both *in vitro* and *in vivo* studies were conducted in Wistar rats submitted to CCl_4-induced acute liver injury. For *in vivo* studies, encapsulated BMMC were transplanted into animals with or without liver injury by CCl_4 and kept for 48 hours. For *in vitro* studies the encapsulated BMMC were co-cultured with hepatocytes isolated from rats with CCl_4-induced acute liver injury or without liver damage for 6 hours (figure 1).

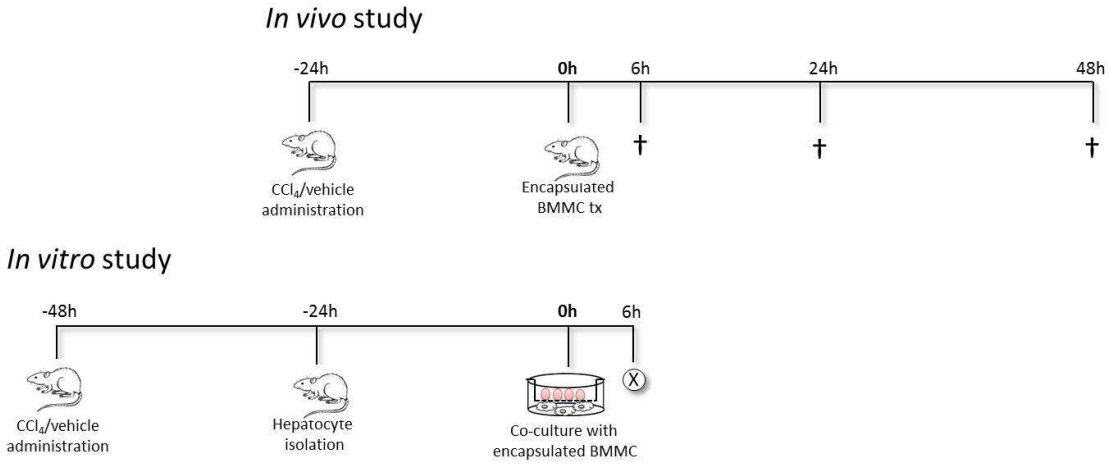

Figure 1: Experimental design of *in vivo* and *in vitro* studies. † Sacrifice of animals and retrieval of capsules; ⊗ Interruption of co-culture and recovery of encapsulated BMMC.

Animals

Adult male Wistar rats weighing 200 to 250gr were kept under 24 hour light/dark cycles and fed standard chow and water *ad libitum*. Animals were sacrificed in the CO_2 chamber either 6, 24 or 48 hours after CCl_4 administration for *in vivo* studies (n= 3/time point). For *in vitro* studies, animals (n=3) were submitted to hepatocyte isolation 24 hours after CCl_4 administration. Control animals (n=12, 9 and 3 for *in vivo* and *in vitro* studies, respectively) were submitted to the same procedures but receiving vehicle instead of CCl_4. Bone marrow donors (n=9, 6 and 3 for *in vivo* and *in vitro* studies, respectively) were rats not submitted to any other procedure. This study was approved by the Ethics Research Committee and national guidelines for animal care were followed.

Acute liver injury model

Acute liver injury was induced by a single CCl_4 (VETEC, Brazil) dose of 1.25 ml/kg diluted in olive oil [2]. A final volume of 1 ml was administered by gavage. Control animals received only olive oil by gavage.

Isolation of bone marrow mononuclear cells (BMMC)

BMMC were isolated from the femur and tibia of Wistar rats, as previously reported [1]. Briefly, bone marrow was flushed with Dulbecco's Modified Eagle Medium (DMEM-LGC, Brazil) supplemented with 10% Fetal Bovine Serum (FBS-Gibco, USA) and 1% Penicilin/Streptomicin (P/S-Gibco, USA) and BMMC were separated onto a Ficoll Histopaque (GE-Healthcare, USA) layer.

BMMC encapsulation

BMMC were encapsulated in sodium alginate microcapsules under sterile conditions, using the protocol described by our group [28, 29]. BMMC were mixed with 1.5% sodium alginate (Sigma-Aldrich, USA) in DMEM and extruded through an encapsulation unit, type J1 (Nisco, Zurich, Switzerland), attached to a syringe pump (JMS, Singapore). Droplets were sheared off with an air flow of 5 L/min delivered to the tip of a 27-G needle and the rate of infusion was 40 mL/h. The droplets fell into a bath of 125 mM $CaCl_2$ and ionically cross-linked with Ca_2^+ to form solid spherical hydrogel beads containing embedded cells. For *in vitro* experiment, in each well, beads were produced from a volume of 100 µL of alginate suspension, containing $1x10^5$ of BMMC. The resulting beads were maintained under normal tissue culture conditions: DMEM supplemented with 10% FBS and 1% P/S at 37ºC and 5% CO_2 for 24 hours prior to administration. For *in vivo* experiment, in each well, beads were produced from a volume of 2 mL of alginate suspension, containing $1x10^6$ BMMC/animal.

Capsules transplantation

For *in vivo* experiments, 24 hours after CCl_4 administration animals were anesthetized with inhaled isoflurane and a small incision was made in the abdomen. A total of $1x10^6$ encapsulated BMMC suspended in 2 mL saline buffer was placed in the peritoneal cavity ($tCCl_4$ group, n=3/time). The same procedure was performed in animals without liver injury (tControl group, n=3/time). Sacrifice was performed in CO_2 chambers 6, 24 or 48 hours after

capsules transplantation. Capsules were retrieved by washing the peritoneal cavity with Phosphate Buffered Saline (PBS).

Hepatocyte isolation

For *in vitro* studies hepatocytes were isolated 24 hours after CCl_4 using the modified 2-step perfusion method as previously described [30, 31]. Hepatocytes from animals without liver injury were isolated by the same method. Cells were cultured in DMEM medium, supplemented with 10% FBS and 1% P/S (Invitrogen, USA) in tissue culture flasks for 24 hours prior to co-culture experiments.

In vitro *co-culture*

Encapsulated BMMC ($1x10^5$) were co-cultured with hepatocytes ($3x10^6$) from rats with CCl_4-induced liver injury ($cCCl_4$ group, n=3) and controls (cControl group, n=3). In addition to the capsules, both cell types were separated by a semipermeable transwell membrane (70 □m filter; BD, USA) placing the hepatocytes in the lower chamber and the BMMC in the upper chamber. The cells were co-cultured in DMEM medium supplemented with 10% FBS and 1% P/S for 6 h.

Intracellular lipid droplets

To evaluate the liver injury, cultured hepatocytes were stained with Oil Red O (ORO, MP Biomedicals, USA) to identify intracellular lipid droplets (ILD). Hepatocytes were fixed with formalin. After washing, 1 mL of 100% PEG

(Ineos, Germany) was added for 2 min, and ORO /PEG (0.5 %) was added for 10 min. After that, cells were rinsed in 60% PEG for 1 min and washed. Hepatocytes were counter-stained with hematoxilin and observed under light microscope immediately [32].

Urea quantification

After co-culture, encapsulated BMMC were removed from hepatocyte contact, washed with PBS and placed in fresh medium for 2 h. The medium was collected and urea was quantified by QuantiChromTM Urea Assay Kit (DIUR-500, BioAssay Systems, USA) according to the manufacturer's instructions.

Reverse transcription-polymerase chain reaction (RT-PCR)

Encapsulated BMMC recovered from *in vivo* and *in vitro* experiments were dissolved using a 100-mM sodium citrate (Labsynth, Brazil) solution prior to RNA extraction. Total RNA was extracted using the RNeasy RNA isolation kit (Qiagen, Germany), and reverse transcription (RT) was carried out with 1 µg of RNA. Complementary DNA (cDNA) was synthesized using Superscript II RNA-reverse transcriptase (Invitrogen, USA).

For the RT-PCR, 2 µL cDNA-templates were mixed with 5 µL PCR-buffer, 1.5 mM µl $MgCl_2$, 10 mMol dNTPs, 20 pmol of each primer, and 2U Taq DNA polymerase in a total volume of 50 µL. All reagents were from Invitrogen (USA). PCR was carried out using primers and conditions showed in Table 1. Samples were analyzed on 1.5% agarose gels stained with ethidium bromide.

Table 1 – Primers and amplification conditions used to analyze gene expression of BMMC.

Primer name	Sequence	PCR condition	Fragment lenght
Alb	For: 5'GGTATGAATATGCAAGAAG3' Rev: 5'CACTCTTCCCAGGTTTCTTG3'	48ºC	350 bp
Ck-18	For: 5'GGACCTCAGCAAGATCATGGC3' Rev: 5'CCAGGATCTTACGGGTAGTTG3'	50ºC	518 bp
Afp	For: 5'CCCACCCTTCCACTTTCCAGA3' Rev: 5'GCTGGAACTGCCTTGTCATA3'	54ºC	164 bp
Gapdh	For: 5'GAGTTGCTGTTGAAGTCACAGG3' Rev: 5'CAGCAATGCATCCTGCAC3'	42ºC	429 bp

Alb, *Albumin*; *Ck-18*, *Cytokeratin 18*; *Afp*, *Alpha-fetoprotein*; *Gapdh*, *Glyceraldehyde 3-phosphate dehydrogenase*

Statistical analysis

Statistical comparison of urea levels of the cControl and cCCl4 groups was carried out using Student's t test, using the SPSS v.18. The level accepted for significance was $p<0.05$.

Results

In vivo *experiments*

In order to study BMMC differentiation, encapsulated cells were implanted in the peritoneal cavity of Wistar rats 24 hours after CCl4 or olive oil administration. In animals from tCCl4 group, liver injury was confirmed at the time of surgery by the characteristic nutmeg aspect of the liver. Animals from tControl group didn't

present this feature throughout the study. Animals were sacrificed in CO_2 chamber after 6, 24 or 48 h and capsules were retrieved from the peritoneum. Capsules were found freely disseminated in the peritoneal cavity and it was not observed liver tissue from the recipient attached to the capsules after explantation.

Gene expression pattern was markedly different in BMMC from tCCl4 retrieved after 48 hours, showing the expression of hepatocyte markers such as Albumin and Cytokeratin 18, but negative for Alfa-fetoprotein (figure 2). On the other hand, BMMC retrieved at 6 or 24 hours after injection were negative for all markers but Gapdh. Interestingly, BMMC from tControl group were also negative for hepatocyte markers at all time points, except for the internal control (Gapdh), showing an expression pattern similar to that of naïve BMMC.

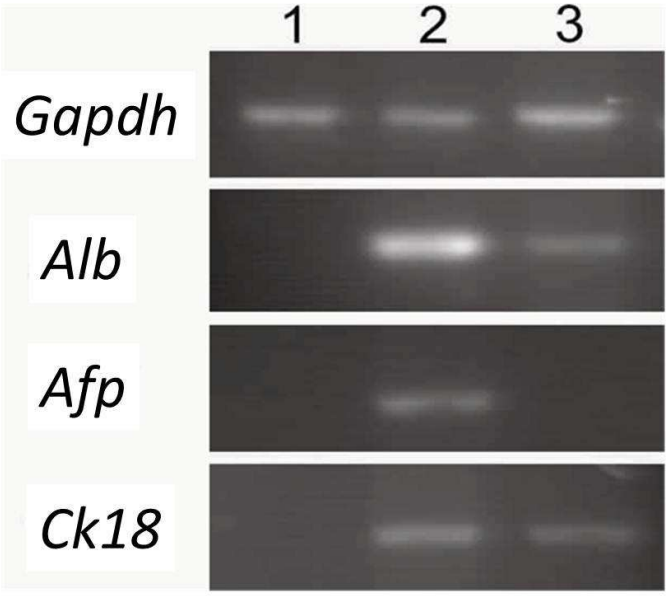

Figure 2: BMMC expression of hepatocyte markers 48 hours after implantation in animals with CCl_4-induced acute liver injury. RT-PCR analysis of gene expression in encapsulated BMMC from tControl (lane 1) or $tCCl_4$ (lane 3) retrieved 48 hours after implantation. Positive control (hepatocytes, lane 2).

In vitro *experiments*

Since encapsulated BMMC showed the expression of hepatocyte markers 48 h after implantation in animals with CCl_4-induced acute liver injury, we tried to replicate the same model *in vitro* in order to determine when this reprogramming takes place.

As a way to assess hepatocyte damage in this model, cells isolated from animals with or without liver injury were stained with oil red. In CCl_4 treated animals, esteatose could be observed with several intracellular lipid droplets, that were absent in control animals. Furthermore, we observed the macroscopic aspect of nutmeg liver by the time of hepatocyte isolation.

Encapsulated BMMC that were in co-culture with hepatocytes from CCl_4 treated animals for only six hours showed *Albumin* expression, whereas those in contact with control hepatocytes were negative (figure 3). No expression of *Ck18* or *Afp* was detected.

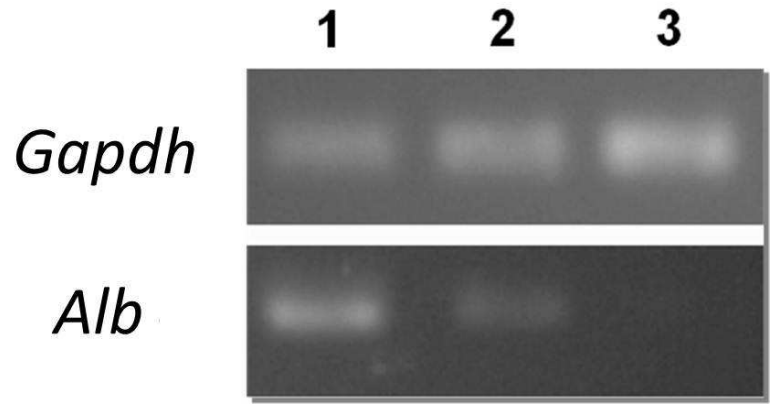

Figure 3: RT-PCR analysis of *Alb* gene expression in BMMC after 6 hour co-culture. Hepatocyte (positive control, lane 1). Encapsulated BMMC from $cCCl_4$ group (lane 2). Encapsulated BMMC from cControl group (lane 3). *Gapdh*: internal control.

In order to determine if this reprogramming also had functional repercussion in BMMC we tested urea production by these cells. After co-culture with

hepatocytes, encapsulated BMMC were kept for other 2 h in culture, with fresh medium. Urea production was greatly increased in BMMC from $cCCl_4$ group (figure 4) as compared to cControl group. However, it was lower than the amount produced by hepatocytes in culture (5.85 mg/dL).

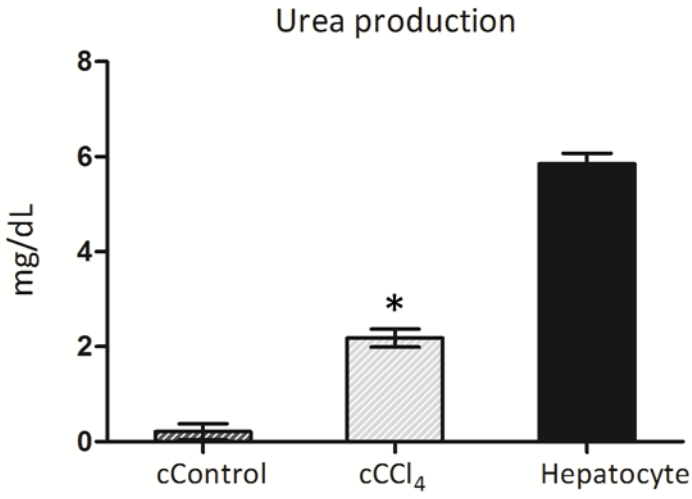

Figure 4: Quantification of urea production in the culture medium from encapsulated BMMC. Urea production was increased in BMMC from $cCCl_4$ group but not in cControl. Medium from hepatocyte culture was used as reference value. * p = 0.033 compared to cControl group (Student's t test).

Discussion

The ability of BMMC to differentiate into hepatocyte-like cells has been shown both *in vivo* and *in vitro* by different groups. In the present study we have shown that BMMC are able to express hepatocyte-specific genes after 48 hours of transplantation in CCl_4 treated animals. *In vitro*, these cells express *Albumin* and produce urea after only 6 hours of co-culture with injured hepatocytes.

Several studies have shown the ability of BMMC to differentiate into hepatocyte-like *in vivo*. Most of these studies show the expression of *Alb* and *Afp* [12–15, 17, 33], but also *Ck8* and *Ck18* [15]. As in the previous studies, this differentiation occurs only when BMMC are transplanted into injured animals,

suggesting that liver damage triggers the release of substances that modulate BMMC gene expression. However, unlike ours, other groups describe differentiation after 13 [34] and 60 days [12, 13].

There is still debate in the literature if these cells convert into hepatocyte-like by means of fusion or differentiation [18]. Our results favor the differentiation hypothesis, in accordance with other study that used microencapsulated cells [15]. Liu and Chang [15] have shown that BMMC encapsulated with the APA (Alginate-Polylysine-Alginate) method improve survival in a 90% partial hepatectomy rat model of acute liver failure. In addition, they found that some BMMC express ALB, AFP, CK 8, CK 18 and were able to store glycogen, after 2 weeks of transplantation. In our study, we used similar alginate microcapsules to isolate BMMC and we observed differentiation signs in a shorter period of time (48 h). It is important to highlight that we assessed differentiation by gene expression analysis, whereas the previous study by Liu and Chang used immunocytochemistry. It was not possible to prove the expression of ALB, AFP nor CK18 by immunocytochemistry due to difficulties in capsules' histology. It cannot be ruled out, however, that fusion mechanisms do exist or play a role in cell-mediated tissue recovery after injury. Yet, in this work alginate microcapsules prevented any type of fusion between BMMC and hepatocytes, although allowing for the interchange of soluble compounds between these two cell types.

In vitro differentiation is usually obtained by the use of growth factors, especially Hepatocyte Growth Factor (HGF), although quite a number of differences exist between protocols [19, 23–25]. Some authors have shown differentiation also with hepatocyte conditioned-medium [21] or serum from

patients with hepatitis B virus-associated liver cirrhosis [35], co-culture with hepatocytes [36–38] or liver fragments [39] from healthy or injured animals. However, unlike the present study, the above mentioned groups use MSC and not BMMC. Even though MSC have a well known differentiation capacity, their isolation, culture and expansion are time consuming processes. On the other hand, BMMC are readily available, and do not need a culture step prior to administration.

Our results showed that BMMC co-cultured with injured hepatocytes for six hours showed expression of *Albumin* and urea production. Changes in gene expression at early times like this are not reported by studies with MSC that usually differentiate after 3 to 40 days in culture [22, 36]. In contrast, when BMMC were co-cultured with hepatocytes isolated from healthy animals, no *Albumin* expression or urea production was detected after 6 hours. Other reports have shown differentiation of MSC even after co-culture with healthy hepatocytes [36]. These differences may be reconciled by intrinsic characteristics of the cell types used in this study or by the short period of time analyzed. It is possible that injured hepatocytes secrete paracrine factors that lead to BMMC reprogramming sooner than healthy hepatocytes [40, 41].

Interestingly, *in vivo* differentiation occurred only after 48 hours. It is possible that *in vitro* hepatocytes secrete factors at higher concentrations, thus inducing differentiation in shorter times.

In summary, this work shows that BMMC are able to differentiate into hepatocyte-like cells in a short period of time both *in vivo* as *in vitro*. This differentiation occurs without fusion and is triggered by factors present only in

injured liver. The identification of such factors and of the cells in the mononuclear fraction that respond to them is under further investigation.

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References

1. Belardinelli MC, Pereira F, Baldo G, Vicente Tavares AM, Kieling CO, et al. (2008) Adult derived mononuclear bone marrow cells improve survival in a model of acetaminophen-induced acute liver failure in rats.Toxicology, 247:1–5.

2. Baldo G, Giugliani R, Uribe C, Belardinelli MC, Duarte MES, et al. (2010) Bone marrow mononuclear cell transplantation improves survival and induces hepatocyte proliferation in rats after CCl(4) acute liver damage. Dig Dis Sci, 55:3384–3392.

3. Couto B, Goldenberg R, da Fonseca L, Thomas J, Gutfilen B, et al. (2011) Bone marrow mononuclear cell therapy for patients with cirrhosis: a Phase 1 study. Liver Int 2011, 31:391–400.

4. Saito T, Okumoto K, Haga H, Nishise Y, Ishii R, et al. (2011) Potential therapeutic application of intravenous autologous bone marrow infusion in patients with alcoholic liver cirrhosis. Stem Cells Dev, 20:1503–1510.

5. Amer M, El-Sayed S, El-Kheir W, Gabr H, Gomaa A (2011) Clinical and laboratory evaluation of patients with end-stage liver cell failure injected with bone marrow-derived hepatocyte-like cells. Eur J Gastroenterol Hepatol, 23:936–941.

6. Baldo G, Kretzmann NA, Tieppo J, Filho GP, Cruz CU, et al. (2011) Bone Marrow Cells Reduce Collagen Deposition in the Rat Model of Common Bile DuctLigation. J Cell Sci Ther 2:112. doi:10.4172/2157-7013.1000112

7. Kawada H, Fujita J, Kinjo K, Matsuzaki Y, Tsuma M, et al. (2004) Nonhematopoietic mesenchymal stem cells can be mobilized and differentiate into cardiomyocytes after myocardial infarction. Blood, 104:3581–3587.

8. Hattan N, Kawaguchi H, Ando K, Kuwabara E, Fujita J, et al. (2005) Purified cardiomyocytes from bone marrow mesenchymal stem cells produce stable intracardiac grafts in mice. Cardiovasc Res, 65:334–344.

9. Oswald J, Boxberger S, Jorgensen B, Feldmann S, Ehninger G, et al. (2004) Mesenchymal Stem Cells Can Be Differentiated Into Endothelial Cells In Vitro. Stem Cells, 22:377-384.

10. Mezey E, Chandross KJ, Harta G, Maki R a, McKercher SR (2000) Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow. Science, 290:1779–1782.

11. Woodbury D, Schwarz EJ, Prockop DJ, Black IB (2000) Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res, 61:364–370.

12. Lagasse E, Connors H, Al-Dhalimy M, Reitsma M, Dohse M, et al. (2000) Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. Nat Med, 6:1229–1234.

13. Theise ND, Badve S, Saxena R, Henegariu O, Sell S, et al. (2000) Derivation of hepatocytes from bone marrow cells in mice after radiation-induced myeloablation. Hepatology, 31:235–240.

14. Sato Y, Araki H, Kato J, Nakamura K, Kawano Y, et al. (2005) Human mesenchymal stem cells xenografted directly to rat liver are differentiated into human hepatocytes without fusion. Blood, 106:756–763.

15. Liu ZC, Chang TM (2006) Transdifferentiation of bioencapsulated bone marrow cells into hepatocyte-like cells in the 90% hepatectomized rat model. Liver Transplant, 12:566–572.

16. Oh S-H, Witek RP, Bae S-H, Zheng D, Jung Y, et al. (2007) Bone marrow-derived hepatic oval cells differentiate into hepatocytes in 2-acetylaminofluorene/partial hepatectomy-induced liver regeneration. Gastroenterology, 132:1077–1087.

17. Choi D, Kim JH, Lim M, Song KW, Paik SS, et al. (2008) Hepatocyte-like cells from human mesenchymal stem cells engrafted in regenerating rat liver tracked with in vivo magnetic resonance imaging. Tissue Eng Part C Methods, 14:15–23.

18. Quintana-Bustamante O, Grueso E, Garcia-Escudero R, Arza E, Alvarez-Barrientos A, et al. (2012) Cell fusion reprogramming leads to a specific hepatic expression pattern during mouse bone marrow derived hepatocyte formation in vivo. PLoS One, 7:e33945.

19. Lee KD, Kuo TK, Whang-peng J, Chung YF, Lin CT, et al. (2004) In vitro hepatic differentiation of human mesenchymal stem cells. Hepatology, 40:1275-1284.

20. Cai Y, Zhen Z, Min J, Fang T, Chu Z, et al. (2004) Selection , proliferation and differentiation of bone marrow-derived liver stem cells with a culture system containing cholestatic serum in vitro. World J Gastroenterol, 10:3308–3312.

21. Chen Y, Dong X, Zhang G, Shao J, Xiang L (2007) In Vitro Differentiation of Mouse Bone Marrow Stromal Stem Cells Into Hepatocytes Induced by Conditioned Culture Medium of Hepatocytes. J Cell Biochem, 102:52–63.

22. Pournasr B, Mohamadnejad M, Bagheri M (2011) In Vitro Differentiation of Human Bone Marrow Mesenchymal Stem Cells into Hepatocyte-like Cells. Arch Iran Med, 14:244–249.

23. Ayatollahi M, Soleimani M, Tabei SZ, Kabir Salmani M (2011) Hepatogenic differentiation of mesenchymal stem cells induced by insulin like growth factor-I. World J Stem Cells, 3:113–121.

24. Dong X, Pan R, Zhang H, Yang C, Shao J, et al (2013) Modification of histone acetylation facilitates hepatic differentiation of human bone marrow mesenchymal stem cells. PLoS One, 8:e63405.

25. Oh SH, Miyazaki M, Kouchi H, Inoue Y, Sakaguchi M, et al. (2000) Hepatocyte growth factor induces differentiation of adult rat bone marrow cells into a hepatocyte lineage in vitro. Biochem Biophys Res Commun, 279:500–504.

26. Maguire T, Novik E, Schloss R, Yarmush M (2005) Alginate-PLL Microencapsulation: Effect on the Differentiation of Embryonic Stem Cells Into Hepatocytes. Biotechnology and Bioengineering, 93:281-291.

27. Liu ZC & Chang TMS (2010) Artificial Cell Microencapsulated Stem Cells in Regenerative Medicine, Tissue Engineering and Cell Therapy. Adv Exp Med Biol, 670:68-79.

28. Lagranha VL, Baldo G, Giacomet de Carvalho T, Burin M, Saraiva-Pereira ML, et al. (2008) In vitro correction of ARSA deficiency in human skin fibroblasts from Metachromatic Leukodystrophy patients after treatment with microencapsulated recombinant cells. Metab Brain Dis, 23:469–484.

29. Mayer FQ, Baldo G, de Carvalho TG, Lagranha VL, Giugliani R, et al. (2010) Effects of cryopreservation and hypothermic storage on cell viability and enzyme activity in recombinant encapsulated cells overexpressing alpha-L-iduronidase. Artif Organs, 34:434–439.

30. Seglen P (1976) Preparation of isolated rat liver cells. Methods Cell Biol, 13:29–83.

31. Rodrigues D, Reverbel T, Matte U, Maurer RL, Cruz CU, et al. (2012) Freshly isolated hepatocyte transplantation in acetaminophen-induced hepatotoxicity model in rats. ARQGA, 49:291–295.

32. Kinkel AD, Fernyhough ME, Helterline DL, Vierck JL, Oberg KS, et al. (2005) Oil red-O stains non-adipogenic cells: a precautionary note. Cytotechnology, 46(1):49–56.

33. Hwang S, Hong H-N, Kim H-S, Park S-R, Won Y-J, et al. (2012) Hepatogenic differentiation of mesenchymal stem cells in a rat model of thioacetamide-induced liver cirrhosis. Cell Biol Int, 36:279–88.

34. Petersen BE, Bowen WC, Patrene KD, Mars WM, Sullivan a K, et al. (1999) Bone marrow as a potential source of hepatic oval cells. Science, 284:1168–1170.

35. Yamazaki S, Miki K, Hasegawa K, Sata M, Takayama T, et al. (2003) Sera from liver failure patients and a demethylating agent stimulate transdifferentiation of murine bone marrow cells into hepatocytes in coculture with nonparenchymal liver cells. J Hepatol, 39:17-23.

36. Qihao Z, Xigu C, Guanghui C, Weiwei Z (2007) Spheroid formation and differentiation into hepatocyte-like cells of rat mesenchymal stem cell induced by co-culture with liver cells. DNA Cell Biol, 26:497–503.

37. Li L, Sharma N, Chippada U, Jiang X, Schloss R, Yarmush ML, et al. (2008) Functional modulation of ES-derived hepatocyte lineage cells via substrate compliance alteration. Ann Biomed Eng, 36:865–876.

38. Li TZ, Kim JH, Cho HH, Lee HS, Kim KS, et al. (2010) Therapeutic Potential of Bone-Marrow-Derived Mesenchymal Stem Cells Differentiated with Growth-Factor-Free Coculture Method. Tissue Eng Part A, 16:2649–2659.

39. Luk JM, Wang PP, Lee CK, Wang JH, Fan ST (2005) Hepatic potential of bone marrow stromal cells: Development of in vitro co-culture and intra-portal transplantation models. J Immunol Methods, 305:39-47.

40. Quesenberry PJ, Aliotta JM (2010) Cellular phenotype switching and microvesicles. Adv Drug Deliv Rev, 62:1141–1148.

41. Muraca M (2011) Evolving concepts in cell therapy of liver disease and current clinical perspectives. Dig Liver Dis, 43:180–187.

DISCUSSÃO

Discussão

O transplante hepático segue sendo o único tratamento definitivo para a IHA (Farmer et al., 2009; Miloh et al., 2010). Além da falta de doadores, que pode levar a uma mortalidade em lista de espera de até 40% dos pacientes (Polson and Lee, 2005) o transplante hepático é um procedimento de risco e apresenta várias implicações para os pacientes pelo resto da vida, como a necessidade de imunossupressão (P. a Lysy et al., 2008). Neste sentido, é importante ressaltar que a IHA se caracteriza por uma perda repentina das funções hepáticas em um indivíduo previamente saudável e que o fígado possui uma extrema capacidade de regeneração. Dessa forma, uma terapia capaz de promover essa regeneração de forma a sustentar as condições vitais do paciente poderia evitar a necessidade de transplante, permitindo a recuperação do seu organismo.

Dessa maneira, a terapia celular tem sido considerada uma alternativa promissora para o tratamento da IHA, porém antes que a mesma possa ser adotada na prática clínica, é preciso um conhecimento mais aprofundado sobre os seus mecanismos de ação. Para isso, estudos *in vitro* e *in vivo* são necessários e a maioria deles utiliza CMO, seja diretamente ou após cultivo, com ou sem purificação de suas frações. Em certo sentido, a profusão de tipos celulares e desenhos de estudo torna difícil comparar os dados disponíveis na literatura a respeito da terapia celular em IHA.

Vários estudos demonstraram o efeito benéfico do transplante das CMO em modelos animais de IHA (Petersen, 1999; Fujii et al., 2002; Wang et al., 2002; Dahlke et al., 2003; Sakaida et al., 2004; Liu and Chang, 2005; Liu and Chang, 2009). Os estudos de Lui & Chang (2006) são bastante similares ao

primeiro artigo apresentado nesse estudo. Esses autores avaliaram o efeito das CMO isoladas em microcápsulas em modelo murino de hepatectomia 90%. Os autores relataram que o implante de células da medula óssea contidas em microcápsulas de alginato de sódio revestidas de poli-L-lisina eliminou os óbitos em 14 dias após a hepatectomia de 90%. Nesse ponto, há a primeira discrepância com os nossos resultados, pois apesar de obtermos um aumento da sobrevida de 6,7 para 63% em 10 dias, nosso modelo não foi capaz de eliminar todos os óbitos. Provavelmente, a principal razão para esta diferença seja o número de animais avaliados em cada estudo (5 no trabalho de Liu & Chang e 11 no presente estudo).

Além disso, Liu & Chang (2006) avaliaram a expressão gênica em cápsulas retiradas dos animais e observam a expressão de ALB, CK8, CK18 e AFP duas semanas após transplante. Os autores sugerem que a imobilização e o isolamento proporcionado pelas microcápsulas protegeriam as células da reação imunológica e que aconteceria uma estimulação da regeneração hepática pelos fatores tróficos secretados pelas CMO e sua diferenciação em hepatócitos. No presente estudo não foi possível analisar a expressão gênica das células encapsuladas, pois após 10 dias havia formação de uma fibrose pericapsular que impediu extração de RNA. Neste caso, é provável que o tipo de alginato utilizado neste estudo, sem o revestimento de poli-L-lisina, tenha contribuído para a impossibilidade de recuperação das células encapsuladas após 10 dias.

No entanto, observamos outro resultado diferente do relatado por Liu & Chang (2006), que foi uma redução na taxa de regeneração hepática no grupo tratado com CMO em comparação com o grupo tratado com cápsulas vazias.

Além disso, ao analisar a curva da sobrevida observamos que a maior mortalidade no grupo tratado corria nas primeiras 72 horas, por isso decidimos avaliar os eventos precoces da regeneração hepática, com ênfase na expressão de alguns genes-chave nesse processo. Por isso, estudamos a expressão gênica de citocinas e fatores de crescimento em grupos de animais sacrificados às 6, 12, 24, 48 e 72 horas pós-hepatectomia.

Nossos resultados mostraram que a taxa de regeneração às 72 horas foi menor (44%) nos animais tratados com CMO encapsuladas do que nos animais tratados com cápsulas vazias (59%). Uma observação similar foi feita por outros autores, que sugeriram que uma proliferação hepatocitária inicial excessiva poderia ser prejudicial à função hepática, pois a capacidade funcional dos hepatócitos aglomerados estaria reduzida pelo desequilíbrio do aporte vascular (Arakawa et al., 2009; Ninomiya et al., 2010). A própria formação dos aglomerados poderia determinar compressão sinusoidal e redução do aporte sanguíneo (Ross et al., 2001; Wack et al., 2001; Ninomiya et al., 2010). A supressão da explosão inicial da resposta proliferativa hepatocitária determinaria um processo regenerativo mais equilibrado entre os hepatócitos e as células endoteliais, com aumento da capacidade funcional do tecido hepático remanescente. Esta resposta regenerativa mais cadenciada produziria menor formação de aglomerados hepatocitários e resultaria em maior sobrevida no modelo de hepatectomia de 90% (Ninomiya et al., 2010).

É importante ressaltar que essa comparação entre a taxa de regeneração foi realizada baseada no peso do fígado remanescente, que é um método bastante utilizado em estudos de regeneração hepática (Zhang et al., 2009). Entretanto, a análise histológica pode sugerir outras explicações para

variações no peso do órgão em estágios inicias pós-hepatectomia que não são diretamente relacionados à regeneração, entendida como proliferação celular. Nas primeiras 24 horas após a hepatectomia, a congestão sinusoidal foi o achado histológico hepático mais significativo e a esteatose hepatocitária passou a ser a característica histológica mais marcante. Um aspecto interessante observado foi a modificação do padrão da esteatose com o tempo decorrido após a hepatectomia. Nas primeiras 24 e 48 horas, a esteatose microgoticular predominava, enquanto que às 72 horas o padrão dominante foi o de esteatose macrogoticular. Esses achados podem ser a representação da dinâmica resposta adaptativa do fígado à situação de insuficiência. Tem sido sugerido que o acúmulo de gordura no citoplasma dos hepatócitos possui uma regulação específica e que deve ser essencial ao processo regenerativo (Shteyer et al., 2004). A esteatose microgoticular representaria uma resposta metabólica adaptativa do fígado em regeneração, como uma forma de reserva de energia ou de constituintes da membrana celulares prontamente disponíveis aos novos hepatócitos (Farrell, 2004; Shteyer et al., 2004). Em nosso material, a análise histológica sugere que a esteatose é mais intensa no grupo que recebeu as cápsulas vazias nas amostras de tecido hepático obtidas às 48 e 72 horas após a hepatectomia.

Por esse motivo, a análise de indicadores de proliferação celular é preferível. Em nosso caso, não houve diferença entre o número de mitoses em cada um dos grupos, nem em relação ao percentual de células marcadas com Bromodeoxiuridina (BrdU), (dados não apresentados no artigo, ver Anexo 1). Talvez um dos motivos para esta ausência de diferença seja a grande variabilidade entre os animais. Em parte isso se deve ao uso de animais não

isogênicos, mas também pelo fato de que os animais eram sacrificados em diferentes tempos pós-hepatectomia, independente do seu estado de saúde. Ou seja, um animal sacrificado às 12 horas pós-hepatectomia poderia ser um animal que teria sobrevivido por 10 dias ou que teria morrido naturalmente 13 horas após a cirurgia. De qualquer forma, existem poucos estudos que avaliaram o efeito da terapia com CMO sobre a atividade mitótica em modelos de IHA. Zhang e colaboradores (2009) também não identificaram maior índice de células marcadas por BrdU entre os animais que receberam infusão venosa de CMO 24 horas após a hepatectomia de 70% em ratos pré-tratados com retrorsina.

Avaliamos também a expressão de genes importantes para a regeneração hepática, como a IL-6 cuja produção é estimulada por TNF-α e NFκ-B nas células de Kupffer (Garcea and Maddern, 2009; Saliba and Samuel, 2013). Quando avaliamos estes genes na fase inicial da regeneração (6 horas pós-hepatectomia) observamos uma diminuição no grupo tratado com CMO encapsuladas quando comparado com o grupo cápsula vazia. Se por um lado a liberação de IL-6 constitui um passo crucial no processo regenerativo (Taub, 2004;Garcea and Maddern, 2009), sua ação também produz a ativação de genes de resposta de fase aguda e pode levar a uma situação conhecida como "tempestade de citocinas", que é prejudicial para o organismo (Yagi et al., 2009; Berry et al., 2010). Hipotetizamos que esses resultados poderiam dever-se, em parte, à diminuição da sinalização por parte das células de Kupffer. É sabido que após HP90% existe um aumento de lipopolissacarídeos (LPS) de origem intestinal (Garcea and Maddern, 2009; Vaquero et al., 2011c), os quais se unem aos TLR4 das células de Kupffer desencadeando o processo

regenerativo por meio da via de MYD 88. Não obstante não encontramos diferença entre os grupos na expressão de TLR4 e MYD88, não podendo descartar que o pico de expressão desses genes tivesse ocorrido em tempos anteriores ao estudado (antes de 6 horas).

Pelos dados obtidos seria possível que uma diminuição da expressão de genes importantes ao início do processo de regeneração (como TNF-α, NFκ-B e IL-6) levasse a uma diminuição na velocidade da regeneração. Por isso, estudamos genes envolvidos diretamente na regulação da proliferação hepatocitária como HGF e TGF-β. HGF tem várias funções no fígado sendo a principal delas ativar os hepatócitos para que passem da fase G0 a G1, ingressando no ciclo celular (Garcea and Maddern, 2009). Quando avaliamos a expressão de HGF entre as 12 e 48 horas observamos um aumento apenas no grupo tratado com CMO encapsuladas nas 24hs quando comparado com o grupo com cápsulas vazias. Por outro lado, a secreção de TGF-β pelo baço retarda a regeneração hepática via inibição do HGF (Arakawa et al., 2009). Nós observamos um forte aumento do TGF-α no grupo tratado com CMO encapsuladas entre as 12 e 48 horas. A diminuição da expressão do HGF e o significativo aumento de TGF-β poderiam evitar que os hepatócitos, após os sinais de lesão, entrem ou completem a divisão celular. Por fim, o tamanho do fígado é regulado e controlado pelas necessidades funcionais do organismo (Taub, 2004). SOCS3 é um importante regulador negativo de sinalização de citocina que impede a fosforilação de STAT3 terminando assim a sinalização de IL-6 para promover a regeneração (Alison et al., 2009; Riehle et al., 2011). No mesmo sentido, nossos dados mostraram que o tratamento com CMO encapsuladas aumenta a expressão de SOCS-3.

Nossos resultados demonstram que o tratamento com células derivadas da MOT produze uma redução na expressão dos genes que são fundamentais para dar inicio ao processo de regeneração (TNF-α, NFκ-B e IL-6 e HGF) e um aumento dos genes que são responsáveis por deter este processo (TGF-β e SOCS-3). Assim, o tratamento com CMO encapsuladas após HP 90% retarda o processo de regeneração, o que deve influenciar na sobrevida dos animais.

Como já mencionado, uma importante limitação deste estudo é que os animais foram mortos em momentos distintos, em lugar de serem analisados no momento de suas mortes naturais. Isto pode explicar a grande dispersão dos dados obtidos. No entanto, ter alguém para recolher os fígados dos animais em tempo hábil após a sua morte seria impraticável. Além disso, este estudo foi realizado em ratos Wistar não isogênicos, simulando uma situação real em que as CMO heterólogas poderiam ser usadas em pacientes com IHA. A imobilização e isolamento dessas células dentro das cápsulas é capaz de protegê-las do sistema imunitário do hospedeiro (Matte et al., 2011; Orive et al., 2003). Esta pode ser uma razão pela qual nos trabalhos de Liu & Chang (2005, 2006 e 2009), um efeito benéfico foi observado apenas com células encapsuladas. E também pode explicar a falta de eficácia relatada por Tokai et al. (2009) em um modelo de hepatectomia de 70%.

Inicialmente, tínhamos por objetivo avaliar também a expressão gênica das CMO encapsuladas nos animais sacrificados em menos tempo após a cirurgia. Porém, nesse caso, muitas das cápsulas aderiam ao fígado hepatectomizado ou ao peritônio (figura 6), de forma que o número de cápsulas recuperadas foi baixo e conseqüentemente o número de células obtidas não foi suficiente para extração de RNA. Por esse motivo, optamos por

utilizar um modelo químico de IHA para avaliar a diferenciação das células encapsuladas. Nesse caso utilizamos o modelo de CCl_4, uma droga hepatotóxica bastante conhecida e já utilizada previamente por nosso grupo (Goldani et al., 2007; Baldo et al., 2010)

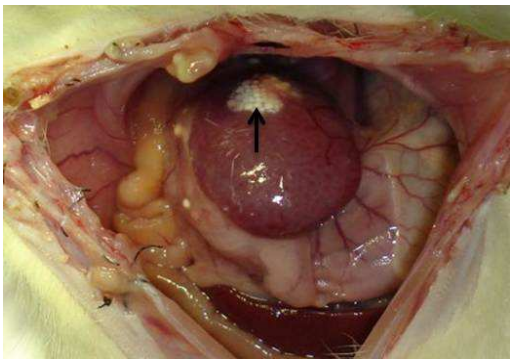

Figura 6. Recuperação de microcápsulas. Fotografia mostrando as microcápsulas (seta) aderidas ao fígado hepatectomizado.

No segundo trabalho apresentado nesse estudo, observamos o efeito do tecido lesado sobre células da fração mononuclear da medula óssea (CFMMO) encapsuladas, tanto *in vivo* como *in vitro*. Vários estudos têm demostrado a capacidade dessas células não só em aumentar a sobrevida em modelos de IHA como também sua capacidade de diferenciação em células tipo hepatócitos após o transplante. O efeito do transplante das CFMMO foi avaliado por nosso grupo em dois diferentes modelos animais de lesão hepática aguda. A sobrevida em 72 horas no modelo por paracetamol aumentou de 33,3% para 70,8% nos animais que receberam as células (Belardinelli et al., 2008a) e no modelo com CCl_4, a infusão da mesma quantidade de CFMMO ($1x10^6$ por rato) resultou em elevação da taxa de sobrevida de 48% para 100% dos animais. Além disso, vários autores tem demostrado que depois de transplantadas as CFMMO expressam marcadores

hepáticos como ALB e AFP (Lagasse et al., 2000; Theise et al., 2000; Sato et al., 2005; Choi et al., 2008; Hwang et al., 2012) e em alguns caso CK 8 e 18 também (Liu and Chang, 2006). Inicialmente avaliamos o tempo de diferenciação das CFMMO *in vivo* e verificamos que a expressão de marcadores típicos de hepatócitos como ALB e CK18 48 horas após o transplante em animais com lesão hepática. Quando transplantadas em animais sem lesão, a expressão de ALB e CK18 é negativa, indicando que é o ambiente hepático lesionado que secreta os fatores necessários para a diferenciação das CFMMO. Geralmente os estudos *in vivo* apresentam dados de diferenciação entre 13 (Petersen et al., 1999) e 60 dias (Lagasse et al., 2000; Theise et al., 2000), tempos bem superiores aos apresentados neste estudo. Entretanto, cabe destacar que a maioria dos outros estudos avalia também desfechos de sobrevida e por isso os animais são analisados em tempos maiores, enquanto que o presente trabalho avaliou apenas diferenciação.

A fim de estudar melhor os mecanismos envolvidos na diferenciação das CFMMO em células tipo hepatócitos, utilizamos um modelo *in vitro* que replicasse as características do modelo *in vivo*. Assim empregamos um sistema de co-cultivo, onde as CFMMO encapsuladas eram mantidas em contato com o meio de hepatócitos com ou sem lesão por CCl_4. Outros autores já demonstraram a capacidade das CFMMO de se diferenciar em células tipo hepatócitos *in vitro* (Oh et al., 2000; Jang et al., 2004; Luk et al., 2005; Ong et al., 2006; Chen et al., 2007; Qihao et al., 2007; Li et al., 2008; Cho et al., 2009 ; Ayatollahi et al., 2011; Dong et al., 2013). Estes estudos empregam varias técnicas que vão desde a utilização de fatores de crescimento, meio

condicionado derivado de hepatócitos ou de soro de pacientes até o co-cultivo com hepatócitos ou fragmentos de fígado.

O uso de HGF é o padrão ouro empregado nas técnicas de diferenciação *in vitro*. Entretanto é sabido que a sinalização de HGF contribui para a oncogêneses e progressão de tumores em vários cânceres humanos e promove a capacidade de invasão de células fortemente ligada à metástase tumoral (Oda et al., 2000; Peruzzi and Bottaro, 2006). Assim é recomendado evitar a utilização de fatores de crescimento como HGF na aplicação clinica (Li et al., 2010).

O co-cultivo tem se mostrado uma ferramenta amplamente utilizada. Nos sistemas de co-cultivo se colocam no mesmo ambiente dois tipos celulares (nesse caso CFMMO e hepatócitos ou fragmentos de fígado) separados por um inserto que permite o intercâmbio de meio de cultivo, mas não de células, entre eles. Assim, todas as substâncias secretadas pelos dois tipos celulares, incluindo citocinas e fatores de crescimento, transitam livremente entre os dois compartimentos. Para avaliação da diferenciação, as células são retiradas do sistema de co-cultivo e mantidas por um período isoladas.

Nossos resultados indicam primeiramente que hepatócitos isolados de animais que receberam CCl_4 apresentam características citológicas distintas das células normais, como esteatose, mesmo após 24 horas em cultura. Além disso, quando as CFMMO são colocadas em co-cultivo com esses hepatócitos em apenas 6 horas passam a expressar albumina, um marcador de hepatócito maduro, sem expressão de AFP ou CK18. Além disso, essas células passam a

produzir ureia, ainda que em níveis abaixo daqueles apresentados pelos hepatócitos. Finalmente, quando co-cultivadas com hepatócitos de ratos sem lesão por CCl_4 a expressão de albumina pelas CFMMO é negativa e a expressão de ureia é quase nula.

Quando comparamos nossos resultados com a literatura alguns pontos de divergência merecem ser ressaltados. Em primeiro lugar, a maioria dos trabalhos demonstra algum grau de diferenciação das células da medula óssea mesmo quando em contato com fragmentos de fígado ou hepatócitos de animais sem lesão (Luk et al., 2005; Qihao et al., 2007). Em nosso trabalho, utilizamos fragmentos de fígado de ratos controle e observamos diferenciação (dados não mostrados). No entanto, postulamos que um corte no tecido hepático representa, por si só, uma lesão e por isso optamos pelo uso de hepatócitos isolados. Mesmo assim, outros grupos trabalhando com hepatócitos isolados também mostraram algum grau de diferenciação, ainda que em menor proporção do que quando comparado com o hepatocitos lesado (Jang et al., 2004; Li et al., 2008). Além disso, a maioria dos estudos demonstra a expressão de outros marcadores, como AFP, CK8 e CK18, além de albumina (Cho et al., 2009).

Dois fatores podem contribuir para essas discrepâncias. Um deles é o tipo de célula utilizado, no nosso caso as CFMMO, enquanto a maioria dos estudos trabalha com as células tronco mesenquimais (Li et al., 2008) ou células tronco hematopoiéticas (Jang et al., 2004). Por trabalharmos com a microencapsulação celular pudemos utilizar as células da fração mononuclear como um todo *in vitro*. Outro ponto importante é relativo ao tempo de estudo. Poucos trabalhos demonstraram a expressão de genes hepáticos por células

derivadas da medula óssea em um tempo tão curto. Apenas Jang et al. (2004) avaliaram o efeito de do co-cultivo de células tronco hematopoiéticas com hepatócitos lesionados em 6 horas, mas não encontraram expressão positiva de nenhum marcador hepático. Os marcadores hepáticos como albumina, CK8 e CK18 começaram a se expressar a partir de 48 horas de co-cultivo. O restante dos estudos começam a avaliar o comportamento das células tronco derivadas da medula óssea a partir de 3 (Qihao et al., 2007) até 40 dias (BEHSHAD; Pournasr et al., 2011). É possível que, se estudássemos tempos maiores de co-cultivo, observaríamos também a expressão de genes hepáticos em células em contato com hepatócitos saudáveis, mas não se pode descartar que as CFMMO tenham uma resposta distinta dos demais tipos celulares estudados.

Quanto à expressão de marcadores como albumina, que é típico de hepatócitos maduros, sem a co-expressão de outros marcadores, como AFP e CK18, novamente não se pode descartar as diferenças entre os tipos celulares e, principalmente, o tempo de co-cultivo. É importante ressaltar que o principal achado deste trabalho é o tempo de co-cultivo necessário para a expressão de genes hepáticos, de 48 horas *in vivo* e 6 horas *in vitro*. Este é um período curto para reprogramação celular e é possível que distintos mecanismos estejam atuando nesse caso.

Recentemente, uma hipótese postulada por Quesenberry e colaboradores (Aliotta et al., 2007) sugere que exista um mecanismo de modulação da expressão gênica mediada por vesículas. Este grupo propõe que um tecido lesado libera vesículas contendo mRNA que poderiam ser captadas pelas células da medula, as quais utilizariam a sua maquinaria

celular para traduzir proteínas não próprias (Aliotta et al., 2007). No nosso caso, a expressão de mRNA de albumina que captamos nas CFMMO encapsuladas seria na verdade um mRNA produzido nos hepatócitos e não um indício de diferenciação das CFMMO. Esta hipótese é reforçada pelo conhecimento atual de que tecidos lesados liberam microRNAs em vesículas que podem inclusive ser detectadas na circulação (Herrera et al., 2010). O tamanho proposto para tais vesículas é bastante variável. Alguns autores sugerem entre 0,9 e 4 μm (Herrera et al., 2010; Xagorari et al., 2013) enquanto para os exossomas que transportam microRNAs o tamanho sugerido é de 30 a 100 nm (Valladi et al., 2007). O poro das microcápsulas usadas neste estudo possui de 1 a 1,5 μm (Lagranha et al., 2008), além disso, estas cápsulas estavam separadas dos hepatócitos por um inserto que é produzido com um filtro de 70 μm, dessa forma é possível que algumas vesículas tenham passado. Uma maneira de testar essa hipótese seria um sistema de co-cultivo com células de diferentes espécies, para detecção de mRNA com primers espécie-específicos.

Ainda que não possamos descartar a ideia de que mRNAs (ou mesmo miRNAs) possam estar passando de um tipo celular para outro, o fato de que a produção de ureia pelas CFMMO foi detectada 2 horas após o fim do co-cultivo sugere que realmente possa haver algum tipo de reprogramação nessas células. Análises mais detalhadas, como por exemplo com o uso de inibidores de remodelamento da cromatina, poderiam auxiliar a esclarecer esse fato.

De qualquer forma, nosso modelo com células microencapsuladas contribui para a confirmação da importância de mecanismos parácrinos de

ação da terapia celular, embora recentemente, vários estudos tenham demonstrado que a fusão celular é o mecanismo principal pelo qual se geram hepatócitos a partir de células tronco (Terada et al., 2002; Wang et al., 2003 ; Alvarez-Dolado et al., 2003; Vig et al., 2006; Quintana-Bustamante et al., 2012).

Assim a microencapsulação celular se mostrou uma ferramenta adequada para avaliar os processos que ocorrem na lesão hepática aguda de maneira bidirecional. Pudemos observar o efeito que as células da medula óssea (total ou fração mononuclear) exercem sobre o tecido hepático lesado, bem como o efeito que substâncias secretadas por este tecido têm sobre essas células.

CONCLUSÕES

Conclusões:

Pudemos observar o efeito que as células da medula óssea (total ou fração mononuclear) exercem sobre o tecido hepático lesado, bem como o efeito que substâncias secretadas por este tecido têm sobre essas células.

As células da medula óssea encapsuladas aumentaram a sobrevida em 10 dias de animais submtidos a hepatectomia de 90% através de mecanismos parácrinos.

As células da medula óssea encapsuladas alteraram a expressão de genes envolvidos na regeneração hepática, reduzindo a expressão dos que a favorecem e aumentando a expressão dos reguladores negativos e, como conseqüência, diminuíram a taxa de regeneração através de mecanismos parácrinos.

As células da fração mononuclear da medula óssea se diferenciaram em células tipo hepatócito tanto *in vivo* quanto *in vitro* em um modelo de lesão hepática aguda através de mecanismos parácrinos.

Assim a microencapsulação celular se mostrou uma ferramenta adequada para avaliar os processos que ocorrem na lesão hepática aguda de maneira bidirecional.

REFERÊNCIAS BIBLIOGRÁFICAS

Aliotta, J.M., Sanchez-Guijo, F.M., Dooner, G.J., Johnson, K.W., Dooner, M.S., Greer, K. a, Greer, D., Pimentel, J., Kolankiewicz, L.M., Puente, N., Faradyan, S., Ferland, P., Bearer, E.L., Passero, M. a, Adedi, M., Colvin, G. a, Quesenberry, P.J., 2007. Alteration of marrow cell gene expression, protein production, and engraftment into lung by lung-derived microvesicles: a novel mechanism for phenotype modulation. Stem cells (Dayton, Ohio) 25, 2245–56.

Almeida-Porada, G., Zanjani, E.D., Porada, C.D., 2010. Bone marrow stem cells and liver regeneration. Experimental hematology 38, 574–80.

Alvarez-Dolado, M., Pardal, R., Garcia-Verdugo, J.M., Fike, J.R., Lee, H.O., Pfeffer, K., Lois, C., Morrison, S.J., Alvarez-Buylla, A., 2003. Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. Nature 425, 968–73.

Amer, M., El-Sayed, S., El-Kheir, W., Gabr, H., Gomaa, A., 2011. Clinical and laboratory evaluation of patients with end-stage liver cell failure injected with bone marrow-derived hepatocyte-like cells. Eur J Gastroenterol Hepatol 23, 936–41.

Aoki, T., Jin, Z., Nishino, N., Kato, H., Shimizu, Y., Niiya, T., Murai, N., Enami, Y., Mitamura, K., Koizumi, T., Yasuda, D., Izumida, Y., Avital, I., Umehara, Y., Demetriou, A. a., Rozga, J., Kusano, M., 2005. Intrasplenic Transplantation of Encapsulated Hepatocytes Decreases Mortality and Improves Liver Functions in Fulminant Hepatic Failure from 90% Partial Hepatectomy in Rats. Transplantation 79, 783–790.

Arakawa, Y., Shimada, M., Uchiyama, H., Ikegami, T., Yoshizumi, T., Imura, S., 2009. Beneficial effects of splenectomy on massive hepatectomy model in rats. Hepatol Res 39, 391–7.

Ayatollahi, M., Soleimani, M., Tabei, S.Z., Kabir Salmani, M., 2011. Hepatogenic differentiation of mesenchymal stem cells induced by insulin like growth factor-I. World journal of stem cells 3, 113–21.

Baldo, G., Giugliani, R., Uribe, C., Belardinelli, M.C., Duarte, M.E.S., Meurer, Luíse, Da Silveira, T.R., Matte, Ursula, 2010. Bone marrow mononuclear cell transplantation improves survival and induces hepatocyte proliferation in rats after CCl(4) acute liver damage. Digestive diseases and sciences 55, 3384–92.

Baldo, G., Mayer, F.Q., Martinelli, B., Meyer, F.S., Burin, Maira, Meurer, Luise, Tavares, A.M.V., Giugliani, R., Matte, Ursula, 2012. Intraperitoneal implant of recombinant encapsulated cells overexpressing alpha-L-iduronidase partially corrects visceral pathology in mucopolysaccharidosis type I mice. Cytotherapy 14, 860–7.

Baldo, G., Frang, K., Mecham, R., Liu, Y., Wu, X., Atkinson, J., Ponder, K., 2011. Pathogenesis of aortic dilatation in mucopolysaccharidosis VII mice may involve complement activation. Mol Genet Metab 104, 608–619.

Banas, A., Teratani, T., Yamamoto, Y., Tokuhara, M., Takeshita, F., Quinn, G., Okochi, H., Ochiya, T., 2007a. Adipose tissue-derived mesenchymal stem cells as a source of human hepatocytes. Hepatology (Baltimore, Md.) 46, 219–28.

Banas, A., Yamamoto, Y., Teratani, T., Ochiya, T., 2007b. Stem Cell Plasticity: Learning From Hepatogenic Differentiation Strategies. Cancer 3228 –3241.

Bélanger, M., Butterworth, R.F., 2005. Acute liver failure: a critical appraisal of available animal models. Metabolic brain disease 20, 409–23.

Belardinelli, M.C., Pereira, F., Baldo, G., Tavares, M.V., Kieling, C.O., Meurer, Luise, Giugliani, R., Matte, Ursula, 2008a. Adult derived mononuclear bone marrow cells improve survival in a model of acetaminophen-induced acute liver failure in rats. Bone 247, 1–5.

Belardinelli, M.C., Pereira, F., Baldo, G., Vicente Tavares, A.M., Kieling, C.O., Da Silveira, T.R., Meurer, Luise, Soares Duarte, M.E., Giugliani, R., Matte, U., 2008b. Adult derived mononuclear bone marrow cells improve survival in a model of acetaminophen-induced acute liver failure in rats. Toxicology 247, 1–5.

Beltrami, A.P., Cesselli, D., Bergamin, N., Marcon, P., Rigo, S., Puppato, E., D'Aurizio, F., Verardo, R., Piazza, S., Pignatelli, A., Poz, A., Baccarani, U., Damiani, D., Fanin, R., Mariuzzi, L., Finato, N., Masolini, P., Burelli, S., Belluzzi, O., Schneider, C., Beltrami, C. a, 2007. Multipotent cells can be generated in vitro from several adult human organs (heart, liver, and bone marrow). Blood 110, 3438–46.

Berry, P. A, Antoniades, C.G., Hussain, M.J., McPhail, M.J.W., Bernal, W., Vergani, D., Wendon, J. A., 2010. Admission levels and early changes in serum interleukin-10 are predictive of poor outcome in acute liver failure and decompensated cirrhosis. Liver international□: official journal of the International Association for the Study of the Liver 30, 733–40.

Bilir BM, Guinette D, Karrer F, Kumpe DA, Krysl J, S.J., 2000. Hepatocyte transplantation in acute liver failure. Liver Transpl 6, 32–40.

Cai, Y., Zhen, Z., Min, J., Fang, T., Chu, Z., Chen, J., 2004. Selection , proliferation and differentiation of bone marrow-derived liver stem cells with a culture system containing cholestatic serum in vitro. World J Gastroenterol 10, 3308–3312.

Campbell, J. S, Prichard, L., Schaper, F., Schmitz, J., Stephenson-Famy, a, Rosenfeld, M.E., Argast, G.M., Heinrich, P.C., Fausto, N, 2001. Expression of suppressors of cytokine signaling during liver regeneration. The Journal of clinical investigation 107, 1285–92.

Campbell, J. S., Riehle, K.J., Brooling, J.T., Bauer, R.L., Mitchell, C., Fausto, Nelson, 2006. Proinflammatory cytokine production in liver regeneration is Myd88-dependent, but independent of Cd14, Tlr2, and Tlr4. Journal of immunology (Baltimore, Md.□: 1950) 176, 2522–8.

Canbay, A., Tacke, F., Hadem, J., Trautwein, C, Gerken, G., Manns, M., 2011. Acute liver failure: a lifethreatening disease. Dtsch Arztebl Int 108, 714–20.

Caplan AI, D.J., 2006. Mesenchymal stem cells as trophic mediators. J Cell Biochem. 98, 1076–68.

Chen, X.-W., Zhu, D.-J., Ju, Y.-L., Zhou, S.-F., 2012. Therapeutic effect of transplanting magnetically labeled bone marrow stromal stem cells in a liver injury rat model with 70%-hepatectomy. Medical science monitor□: international medical journal of experimental and clinical research 18, BR375–82.

Chen, Y., Dong, X., Zhang, G., Shao, Jian-zhong, Xiang, Li-xin, 2007. In Vitro Differentiation of Mouse Bone Marrow Stromal Stem Cells Into Hepatocytes Induced by Conditioned Culture Medium of Hepatocytes. Journal of Cellular Biochemistry 102, 52–63.

Cho, H., Ju, S.-Y., Cho, S.J., Jung, Y.-J., Woo, S.-Y., Seoh, J.-Y., Han, H.-S., Ryu, K.-H., 2009. Mesenchymal stem cells showed the highest potential for the regeneration of injured liver tissue compared with other subpopulations of the bone marrow. Cell biology international 33, 772–7.

Choi, K., Jung H., Lim, M., Song, K.W., Paik, S.S., Kim, S.J., Cheong, H.J., Jeon, J.S., Park, H.S., Song, Y.S., Khang, H., Won, J.-H., 2008. Hepatocyte-like cells from human mesenchymal stem cells engrafted in regenerating rat liver tracked with in vivo magnetic resonance imaging. Tissue engineering. Part C, Methods 14, 15–23.

Chung, R.T., Stravitz, R.T., Fontana, R.J., Schiodt, F. V, Mehal, W.Z., Reddy, K.R., Lee, W.M., 2012. Pathogenesis of liver injury in acute liver failure. Gastroenterology 143, e1–7.

Couto, B., Goldenberg, R., Da Fonseca, L., Thomas, J., Gutfilen, B., Resende, C., Azevedo, F., Mercante, D., AL, T., Coelho, H., Maiolino, A., Alves, A., Dias, J., Moreira, M., All, E., 2011. Bone marrow mononuclear cell therapy for patients with cirrhosis: a Phase 1 study. Liver International 31, 391–400.

Dahlke, M.H., Popp, F.C., Bahlmann, F.H., 2003. Liver regeneration in a retrorsine/CCl4-induced acute liver failure model: Do bone marrow-derived cells contribute. Journal of Hepatology 39, 365–373.

Dhawan, A., Mitry, R., Hughes, R., 2005. Hepatocyte transplantation for metabolic disorders, experience at King's College hospital and review of literature. Acta Gastroenterol Belg 68, 457–60.

Dong, H., Pan, R., Zhang, H., Yang, C., Shao, Jianzhong, Xiang, Lixin, 2013. Modification of histone acetylation facilitates hepatic differentiation of human bone marrow mesenchymal stem cells. PloS one 8, e63405.

Duncan, A.W., Dorrell, C., Grompe, M, 2009. Stem Cells and Liver Regeneration. YGAST 1–16.

Ellor S., Shupe T, P.B., 2008. Stem cell therapy for inherited metabolic disorders of the liver. Exp Hematol 36, 716–25.

Fainboim, L., Cherñavsky, A., Paladino, N., Flores, A.C., Arruvito, L., 2007. Cytokines and chronic liver disease. Cytokine & growth factor reviews 18, 143–57.

Farmer, D., Venick, R., McDiarmid, S., Duffy, J., Kattan, O., JC, H., 2009. Fulminant hepatic failure in children: superior and durable outcomes with liver transplantation over 25 years at a single center. Ann Surg 250, 484–93.

Farrell, G., 2004. Probing Prometheus: fat fueling the fire? Hepatology 40, 1252–5.

Fausto, N., 2005. Tweaking liver progenitor cells. Nature medicine 11, 1053–4.

Fausto, N., Campbell, J. S., Riehle, K.J., 2006. Liver regeneration. Hepatology (Baltimore, Md.) 43, S45–53.

Fausto, N., Riehle, H., 2005. Mechanisms of liver regeneration and their clinical implications. Journal of hepato-biliary-pancreatic surgery 12, 181–9.

Fouraschen, S.M.G., Pan, Q., De Ruiter, P.E., Farid, W.R.R., Kazemier, G., Kwekkeboom, J., Ijzermans, J.N.M., Metselaar, H.J., Tilanus, H.W., De Jonge, J., Van der Laan, L.J.W., 2012. Secreted factors of human liver-derived mesenchymal stem cells promote liver regeneration early after partial hepatectomy. Stem cells and development 21, 2410–9.

Friedman, S.L., 2008. Hepatic Stellate Cells□: Protean , Multifunctional and Enigmatic Cells of the Liver . Physiol Rev 88.125–172.

Fujii, H., Hirose, T., Oe, S., Yasuchika, K., Azuma, H., Fujikawa, T., Nagao, M., Yamaoka, Y., 2002. Contribution of bone marrow cells to liver regeneration after partial hepatectomy in mice. Journal of Hepatology 36.

Garcea, G., Maddern, G.J., 2009. Liver failure after major hepatic resection. Journal of hepato-biliary-pancreatic surgery 16, 145–55.

Gaub, J., Iversen, J., 1984. Rat liver regeneration after 90% partial hepatectomy. Hepatology 4, 902–4.

Ghanem, L.Y., Nosseir, M.M., Lotfi, A. a, Mohamed, A.S., Ibrahim, R. a, Hassanein, M.H., Mansour, E., Makhlouf, M.M., Fouad, Y.M., El-Khayat, H.R., 2012. Hematopoietic stem cell mobilization into the peripheral circulation in patients with chronic liver diseases. Journal of digestive diseases 13, 571–8.

Gibran, A., Muhammad S, M., 2012. Bone Marrow Cells Ameliorate Liver Fibrosis and Express Albumin after Transplantation in CCl 4 -Induced Fibrotic Liver 18, 263–267.

Goldani, H., Matte, US, Ramos, A., Costa, T., Winkelmann, L., Meurer, L, Vieira, S., Kieling, C., Silveira, T., 2007. The role of food restriction on CCl4-induced cirrhosis model in rats. Exp Toxicol Pathol. 58, 331–7.

Gruttadauria, S., Grosso, G., Pagano, D., Biondi, a, Echeverri, G.J., Seria, E., Pietrosi, G., Liotta, R., Basile, F., Gridelli, B., 2013. Marrow-derived mesenchymal stem cells restore biochemical markers of acute liver injury in experimental model. Transplantation proceedings 45, 480–6.

Habibullah, C., Syed, I., Qamar, A., Taher-Uz, Z., 1994. Human fetal hepatocyte transplantation in patients with fulminant hepatic failure. Transplantation 58, 951–2.

Hamazaki, K., Doi, Y., Koide, N., 2002. Microencapsulated multicellular spheroid of rat hepatocytes transplanted intraperitoneally after 90% hepatectomy. Hepatogastroenterology 49, 1514–6.

Hernández, R.M. Orive, G., Murua, A., Pedraz, J.L., 2010. Microcapsules and microcarriers for in situ cell delivery. Advanced drug delivery reviews 62, 711–30.

Herrera, M.B., Fonsato, V., Gatti, S., Deregibus, M.C., Sordi, a, Cantarella, D., Calogero, R., Bussolati, B., Tetta, C., Camussi, G., 2010. Human liver stem cell-derived microvesicles accelerate hepatic regeneration in hepatectomized rats. Journal of cellular and molecular medicine 14, 1605–18.

Herzog, E.L., Chai, L., Krause, Diane S, 2003. Plasticity of marrow-derived stem cells. Blood 102, 3483.

Higgins, G., Anderson, R., 1931. Experimental pathology of the liver – Restoration of the liver of the white rat following partial surgical removal. Arch Pathol 12, 186–202.

Holdcraft RW, Gazda LS, Circle L, Adkins H, Harbeck SG, Meyer EB, Bautista MA, Martis PC, Laramore MA, Vinerean HV, Hall RD, S.B., 2012. Enhancement of In Vitro and In Vivo Function of Agarose Encapsulated Porcine Islets by Changes in the Islet Microenvironment. Cell Transplant 29.

Houlihan, D.D., Newsome, P.N., 2008. Clinical advances in liver, pancreas, and biliary tract. Gastroenterology 438 – 450.

Hwang, S., Hong, H.-N., Kim, H.-S., Park, S.-R., Won, Y.-J., Choi, S.-T., Choi, D., Lee, S.-G., 2012. Hepatogenic differentiation of mesenchymal stem cells in a rat model of thioacetamide-induced liver cirrhosis. Cell biology international 36, 279–88.

Ikeda, H., Nagoshi, S., Ohno, a, Yanase, M., Maekawa, H., Fujiwara, K., 1998. Activated rat stellate cells express c-met and respond to hepatocyte growth factor to enhance transforming growth factor beta1 expression and DNA synthesis. Biochemical and biophysical research communications 250, 769–75.

Islam, S., Lim, S., 2009. Stem cells in liver regeneration , fibrosis and cancer□: the good , the bad and the ugly. Journal of Pathology 1, 282–298.

Jang, Y.Y., Collector, M.I., Baylin, S.B., Diehl, A.M., Sharkis, S.J., 2004. Hematopoietic stem cells convert into liver cells within days without fusion. Nat. Cell Biol. 6, 532–739.

Jensen, S.A., 2001. Liver gene regulation in rats following both 70 or 90 % hepatectomy and endotoxin treatment 525–530.

Jin S.Z., Meng X.W., Han M.Z., Sun X., Sun L.Y., L.B., 2009. Stromal cell derived factor-1 enhances bone marrow mononuclear cell migration in mice with acute liver failure. World J Gastroenterol 15, 2657–64.

Jung, YJ, Ryu, K., Cho, S., Woo, S., Seoh, J., Chun, C., 2006. Syngenic bone marrow cells restore hepatic function in carbon tetrachloride-induced mouse liver injury. Stem Cells Dev 15, 687–95.

Khan AA, N, P., Mahaboob VS, Rajendraprasad A, R.H., 2008. Safety and efficacy of autologous bone marrow stem cell transplantation through hepatic artery for the treatment of chronic liver failure: a preliminary study. Transplant Proc 40, 1140–4.

Khan AA, Parveen N, Mahaboob VS, Rajendraprasad A, R.H., 2008. Treatment of Crigler-Najjar Syndrome type 1 by hepatic progenitor cell transplantation: a simple procedure for management of hyperbilirubinemia. Transplant Proc 40, 1148–50.

Kieling, C.O., 2012. Terapia celular na insuficiência hepática aguda□: estudo experimental com células. Tese Doutorado, 1–187.

Kieling, C.O., Backes, N.A., MaurerLucyk, R., Uribe Cruz, C., Bersch, A.O., Reverbel, T., Matte, U., 2012. The effects of anesthetic regimen in 90 % hepatectomy in rats 1 Os efeitos do regime anestésico na hepatectomia de 90 % em ratos. Acta Cirúrgica Brasileira 27, 702–706.

Lagasse, Connors, H., Al-Dhalimy, M., Reitsma, M., Dohse, M., Osborne, L., Wang, X., Finegold, M, Weissman, I.L., Grompe, M, 2000. Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. Nature medicine 6, 1229–34.

Lagranha, V.L., Baldo, G., Giacomet de Carvalho, T., Burin, M., Saraiva-Pereira, M.L., Matte, U., Giugliani, R., 2008. In vitro correction of ARSA deficiency in human skin fibroblasts from Metachromatic Leukodystrophy patients after treatment with microencapsulated recombinant cells. Metab Brain Dis 23, 469–484.

Lee, Kuo, T.K., Whang-peng, J., Chung, Y.F., Lin, C.T., Chou, S.H., Chen, J.R., Chen, Y.P., Lee, O.K., 2004. In vitro hepatic differentiation of human mesenchymal stem cells. Hepatology 40, 1275–84.

Lee, W.M., Jr, R.H.S., Nyberg, S.L., Doo, E., Hoofnagle, J.H., 2008. Acute Liver Failure□: Summary of a Workshop 1401–1415.

Li, H., Kim, J., Cho, S., Lee, L., 2010. Therapeutic Potential of Bone-Marrow-Derived Mesenchymal Stem Cells Differentiated with Growth-Factor-Free Coculture Method. Tissue engineering. Part A 16, 2649–59.

Li, T.Z., Shin, S., Cho, H.H., Kim, Jae Hyung, Suh, H., 2008. Growth Factor-Free Cultured Rat Bone Marrow Derived Mesenchymal Stem Cells towards Hepatic Progenitor Cell Differentiation. Biotechnology and Bioprocess Engineering 2008, 13, 659–665.

Lin H, Xu R, Zhang Z, Chen L, Shi M, W.F., 2011. Implications of the immunoregulatory functions of mesenchymal stem cells in the treatment of human liver diseases. Cell Mol Immunol 8.

Liu, Z.C., Chang,T., 2006. Transdifferentiation of bioencapsulated bone marrow cells into hepatocyte-like cells in the 90% hepatectomized rat model. Liver Transplantation 12, 566–572.

Liu, Z.C., Chang, T., 2005. Transplantation of bioencapsulated bone marrow stem cells improves hepatic regeneration and survival of 90% hepatectomized rats: a preliminary report. Artif Cells Blood Substit Immobil Biotechnol 33, 405–10.

Liu, Z.C., Chang, T., 2009. Preliminary study on intrasplenic implantation of artificial cell bioencapsulated stem cells to increase the survival of 90% hepatectomized rats. Artificial cells, blood substitutes, and immobilization biotechnology 37, 53–5.

Luk, J.M., Wang, P.P., Lee, C.K., Wang, J.H., Fan, S.T., 2005. Hepatic potential of bone marrow stromal cells: Development of in vitro co-culture and intra-portal transplantation models. Journal of Immunological Methods 305, 39 – 47.

Lyra, A.C., Soares, M.B.P., Da Silva, L.F.M., Braga, E.L., Oliveira, S. a, Fortes, M.F., Silva, A.G.P., Brustolim, D., Genser, B., Dos Santos, R.R., Lyra, L.G.C., 2010. Infusion of autologous bone marrow mononuclear cells through hepatic artery results in a short-term improvement of liver function in patients with chronic liver disease: a pilot randomized controlled study. European journal of gastroenterology & hepatology 22, 33–42.

Lysy, P. A, Campard, D., Smets, F., Najimi, M., Sokal, E. M, 2008. Stem cells for liver tissue repair: current knowledge and perspectives. World journal of gastroenterology : WJG 14, 864–75.

Lysy, P.A., Campard, D, Smets, F, Najimi, M, Sokal, E M, 2008. Persistence of a chimerical phenotype after hepatocyte differentiation of human bone marrow mesenchymal stem cells. Cell Proliferation 36 – 58.

Mahadeb, P., Gras, J., Sokal, E., Otte, J., Lerut, J., Detaille, T., 2009. Liver transplantation in children with fulminant hepatic failure: The UCL experience. Pediatr Transplant 13, 414–20.

Mai, G., Nguyen, T.H., Huy, N.T., Morel, Philippe, Mei, J., Andres, A., Bosco, D., Baertschiger, Reto, Toso, C., Berney, T., Majno, P., Mentha, G., Trono, D., Buhler, L.H., 2005. Treatment of fulminant liver failure by transplantation of microencapsulated primary or immortalized xenogeneic hepatocytes. Xenotransplantation 12, 457–64.

Makino, H., Togo, S., Kubota, T., Morioka, D., Morita, T., Kobayashi, T., Tanaka, K., Shimizu, T., Matsuo, K., Nagashima, Y., Shimada, H., 2005. A good model of hepatic failure after excessive hepatectomy in mice. The Journal of surgical research 127, 171–6.

Martins, P.N. a, Theruvath, T.P., Neuhaus, P., 2008. Rodent models of partial hepatectomies. Liver international : official journal of the International Association for the Study of the Liver 28, 3–11.

Matte, U., Lagranha, V.L., De Carvalho, T.G., Mayer, F.Q., Giugliani, R., 2011. Cell microencapsulation: a potential tool for the treatment of neuronopathic lysosomal storage diseases. Journal of inherited metabolic disease 34, 983–90.

Mayer, F.Q., Baldo, G., De Carvalho, T.G., Lagranha, V.L., Giugliani, R., Matte, Ur., 2010. Effects of cryopreservation and hypothermic storage on cell viability and enzyme activity in recombinant encapsulated cells overexpressing alpha-L-iduronidase. Artificial organs 34, 434–9.

Meirelles, J., Fontes, M., Covas, A., Caplan, A., 2009. Mechanisms involved in the therapeutic properties of mesenchymal stem cells. Cytokine Growth Factor Rev 20, 419–27.

Meyburg J., Hoerster F., Weitz J., Hoffmann G.F., 2008. Use of the middle colic vein for liver cell transplantation in infants and small children. Transplant Proc 40, 936–7.

Michalopoulos, G.K., 1995. Hepatocyte Growth Factor (HGF) and its receptor (Met) in liver regeneration, neoplasia and disease. Liver Regeneration and Carcinogenesis 27–49.

Michalopoulos, G.K., 2007. Liver Regeneration. J Cell Physiol. 213, 286–300.

Miloh, T., Kerkar, N., Parkar, S., Emre, S., Annunziato, R., Mendez, C., 2010. Improved outcomes in pediatric liver transplantation for acute liver failure. Pediatr Transplant 14, 863.

Mukhopadhyay, A., 2013. Perspective on liver regeneration by bone marrow-derived stem cells-A scientific realization or a paradox. Cytotherapy. 0, 1-12

Muraca, M., 2011. Evolving concepts in cell therapy of liver disease and current clinical perspectives. Dig Liver Dis 43, 180–7.

Naito, M., Hasegawa, G., Ebe, Y., Yamamoto, T., 2004. Differentiation and function of Kupffer cells. Medical electron microscopy□: official journal of the Clinical Electron Microscopy Society of Japan 37, 16–28.

Ninomiya, M., Shirabe, K., Terashi, T., Ijichi, H., Yonemura, Y., Harada, N., Soejima, Y., Taketomi, a, Shimada, M, Maehara, Y., 2010. Deceleration of regenerative response improves the outcome of rat with massive hepatectomy. American journal of transplantation□: official journal of the American Society of Transplantation and the American Society of Transplant Surgeons 10, 1580–7.

Oberg, K.S., Vance, T.J., Hausman, G.J., V, R.A.H.M., 2005. Oil red-O stains non-adipogenic cells: a precautionary note. Cytotechnology 49–56.

Oda, Y., Sakamoto, a, Saito, T., Kinukawa, N., Iwamoto, Y., Tsuneyoshi, M., 2000. Expression of hepatocyte growth factor (HGF)/scatter factor and its receptor c-MET correlates with poor prognosis in synovial sarcoma. Human pathology 31, 185–92.

Oh, S.H., Miyazaki, M., Kouchi, H., Inoue, Y., Sakaguchi, M., Tsuji, T., Shima, N., Higashio, K., Namba, M., 2000. Hepatocyte growth factor induces differentiation of adult rat bone marrow cells into a hepatocyte lineage in vitro. Biochemical and Biophysical Research Communications 279, 500–504.

Oh, S.H., Witek, R.P., Bae, S.-H., Zheng, D., Piscaglia, A.C., Petersen, B.E., 2007. Bone marrow-derived hepatic oval cells differentiate into hepatocytes in 2-acetylaminofluorene/partial hepatectomy-induced liver regeneration. Gastroenterology 132, 1077–87.

Ong, S.Y., Dai, H., Leong, K.W., 2006. Hepatic differentiation potential of commercially available human mesenchymal stem cells. Tissue engineering 12, 3477–85.

Orive, G, Gascón, A., Hernández, R., Igartua, M., Luis Pedraz, J., 2003. Cell microencapsulation technology for biomedical purposes: novel insights and challenges. Trends Pharmacol Sci 24, 207–10.

Overturf, K., al-Dhalimy, M., Ou, C.N., Finegold, M, Grompe, M, 1997. Serial transplantation reveals the stem-cell-like regenerative potential of adult mouse hepatocytes. The American journal of pathology 151, 1273–80.

Panis, Y., McMullan, D.M., Emond, J.C., 1997. Progressive necrosis after hepatectomy and the pathophysiology of liver failure after massive resection. Surgery 121, 142–9.

Parekkadan, B, Poll, D. Van, Megeed, Z., Kobayashi, N., Tilles, A W, Berthiaume, F., Yarmush, M L, 2007. Immunomodulation of activated hepatic stellate cells by mesenchymal stem cells, Biochem. Biophys. Res. Commun. 363, 247–252.

Petersen, A., Bowen, W.C., Patrene, K.D., Mars, W.M., Sullivan, a K., Murase, N., Boggs, S.S., Greenberger, J.S., Goff, J.P., 1999. Bone marrow as a potential source of hepatic oval cells. Science (New York, N.Y.) 284, 1168–70.

Polson, J., Lee, W., 2005. AASLD position paper: the management of acute liver failure. Hepatology 41, 1179–97.

Pournasr, B., Mohamadnejad, M., Bagheri, M., 2011. In Vitro Differentiation of Human Bone Marrow Mesenchy- mal Stem Cells into Hepatocyte-like Cells. Archives of Iranian Medicine 14, 244–249.

Qihao, Z., Xigu, C., Guanghui, C., Weiwei, Z., 2007. Spheroid formation and differentiation into hepatocyte-like cells of rat mesenchymal stem cell induced by co-culture with liver cells. DNA and cell biology 26, 497–503.

Quintana-Bustamante, O., Grueso, E., Garcia-Escudero, R., Arza, E., Alvarez-Barrientos, A., Fabregat, I., Garcia-Bravo, M., Meza, N.W., Segovia, J.C., 2012. Cell fusion reprogramming leads to a specific hepatic expression pattern during mouse bone marrow derived hepatocyte formation in vivo. PloS one 7, e33945.

Rahman, T., Diakanov, I., Selden, C., Hodgson, H., 2005. Co-transplantation of encapsulated HepG2 and rat Sertoli cells improves outcome in a thioacetamide induced rat model of acute hepatic failureo Title. Transpl Int 18, 1001–9.

Rahman, T.M., Hodgson, H.J., 2000. Animal models of acute hepatic failure. International journal of experimental pathology 81, 145–57.

Recknagel R.O., Glende E.A. Jr., Dolak J.A. and Waller R.L. 1989. Mechanism of carbon tetrachloride toxicity. Pharmacol Ther. 43: 139-154.

Riehle, A., Campbell, JS, McMahan, R., Johnson, M., Beyer, Bammler, T., 2008. Regulation of liver regeneration and hepatocarcinogenesis by suppressor of cytokine signaling 3. Exp Med 205, 91–103.

Riehle, K.J., Dan, Y.Y., Campbell, J. S, Fausto, N., 2011. New concepts in liver regeneration. Journal of gastroenterology and hepatology 26 Suppl 1, 203–12.

Rodrigues, D., Reverbel, T., Matte, U., Group, T.S., Hepatology, E., 2012. Freshly isolated hepatocyte transplantation in acetaminophen-induced hepatotoxicity model in rats. ARQGA 291–295.

Roskams, T. a, Theise, Neil D, Balabaud, C., Bhagat, G., Bhathal, P.S., Bioulac-Sage, P., Brunt, E.M., Crawford, James M, Crosby, H. a, Desmet, V., Finegold, M.J., Geller, S. a, Gouw, A.S.H., Hytiroglou, P., Knisely, a S., Kojiro, M., Lefkowitch, J.H., Nakanuma, Y., Olynyk, J.K., Park, Y.N., Portmann, B., Saxena, Romil, Scheuer, P.J., Strain, A.J., Thung, S.N., Wanless, I.R., West, a B., 2004. Nomenclature of the finer

branches of the biliary tree: canals, ductules, and ductular reactions in human livers. Hepatology (Baltimore, Md.) 39, 1739–45.

Ross, M., Sander, C., Kleeb, T., Watkins, S., Stolz, D., 2001. Spatiotemporal expression of angiogenesis growth factor receptors during the revascularization of regenerating rat liver. Hepatology 34, 1135–48.

Saito, T., Okumoto, K., Haga, H., Nishise, Y., Ishii, R., Sato, C., Watanabe, H., 2011. Potential therapeutic application of intravenous autologous bone marrow infusion in patients with alcoholic liver cirrhosis. Stem Cells Dev. 20, 1503–10.

Sakaida, I., Terai, S., Yamamoto, N., Aoyama, K., Ishikawa, T., Nishina, H., Okita, K., 2004. Transplantation of bone marrow cells reduces CCl 4 -induced liver fibrosis. in mice, Hepatology 40, 1304–1311.

Saliba, F., Samuel, D., 2013. Acute liver failure: Current trends. Journal of hepatology.

Sass, D. a, Shakil, a O., 2005. Fulminant hepatic failure. Liver transplantation : official publication of the American Association for the Study of Liver Diseases and the International Liver Transplantation Society 11, 594–605.

Sato, A., H., Kato, J., Nakamura, K., Kawano, Y., Kobune, M., Sato, T., Miyanishi, K., Takayama, T., Takahashi, M., Takimoto, R., Iyama, S., Matsunaga, T., Ohtani, S., Matsuura, A., Hamada, H., Niitsu, Y., 2005. Human mesenchymal stem cells xenografted directly to rat liver are differentiated into human hepatocytes without fusion. Blood 106, 756–763.

Seglen, P., 1976. Preparation of isolated rat liver cells. Methods Cell Biol 13, 29–83.

Sgroi, A., Mai, G., Morel, P, Baertschiger, RM, Gonelle-Gispert, C., Serre-Beinier, V, 2011. Transplantation of encapsulated hepatocytes during acute liver failure improves survival without stimulating native liver regeneration. Cell Transplant.

Shinohara, H., Shimada, Mitsuo, Ogasawara, T., Morine, Y., Ikemoto, T., Imura, Satoru, Fujii, M., 2007. Pharmacokinetics of cyclosporine A after massive hepatectomy: a hint for small-for-size graft in living donor liver transplantation. Digestive diseases and sciences 52, 2490–6.

Shteyer, E., Liao, Y., Muglia, L., Hruz, P., Rudnick, D., 2004. Disruption of hepatic adipogenesis is associated with impaired liver regeneration in mice. Hepatology 40, 1322–32.

Sowa, J.-P., 2008. Extent of liver resection modulates the activation of transcription factors and the production of cytokines involved in liver regeneration. World Journal of Gastroenterology 14, 7093.

Strauss, A., Grabhorn, E., Sornsakrin, M., Briem-Richter, A., Nashan, B., 2009. Liver transplantation for fulminant hepatic failure in infancy: a single center experience. Pediatr Transplant 13, 838–42.

Strom SC, Fisher RA, Rubinstein WS, Barranger JA, Towbin RB, C.M., 1997. Transplantation of human hepatocytes. Transplant Proc.

Sudo, K., Yamada, Y., Saito, K., Shimizu, S., Ohashi, H., Kato, T., Moriwaki, H., Ito, H., □ M.S. a, 2008. TNF-α and IL-6 signals from the bone marrow derived cells are necessary for normal.pdf. Biochimica et biophysica acta 1782, 671–79.

Taub, R., 2004. Liver regeneration: from myth to mechanism. Nature reviews. Molecular cell biology 5, 836–47.

Terada, N., Hamazaki, T., Oka, M., Hoki, M., Mastalerz, D.M., Nakano, Y., Meyer, E.M., Morel, L., Petersen, B.E., Scott, E.W., 2002. Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature 416, 542–5.

Theise, N D, Badve, S., Saxena, R, Henegariu, O., Sell, S., Crawford, J M, Krause, D S, 2000a. Derivation of hepatocytes from bone marrow cells in mice after radiation-induced myeloablation. Hepatology (Baltimore, Md.) 31, 235–40.

Theise, N D, Nimmakayalu, M., Gardner, R., Illei, P.B., Morgan, G., Teperman, L., Henegariu, O., Krause, D S, 2000b. Liver from bone marrow in humans. Hepatology (Baltimore, Md.) 32, 11–6.

Tokai, H., Kawashita, Y., Ito, Y., Yamanouchi, K., Takatsuki, M., Eguchi, 2009. Efficacy and limitation of bone marrow transplantation in the treatment of acute and subacute liver failure in rats. Hepatol Res 39, 1137–43.

Tuñón, M.J., 2009. An overview of animal models for investigating the pathogenesis and therapeutic strategies in acute hepatic failure. World Journal of Gastroenterology 15, 3086.

Tygstrup, N., Jensen, S. a, Krog, B., Pietrangelo, a, Shafritz, D. a, 1996. Expression of messenger RNA for liver functions following 70% and 90% hepatectomy. Journal of hepatology 25, 72–8.

Ueno, S., Campbell, J., Fausto, N., 2006. Reactive oxygen species derived from NADPH oxidase system is not essential for liver regeneration after partial hepatectomy. The Journal of surgical research 136, 260–5.

Vaquero, J., Campbell, J. S, Haque, J., McMahan, R.S., Riehle, K.J., Bauer, R.L., Fausto, N., 2011a. Toll-like receptor 4 and myeloid differentiation factor 88 provide mechanistic insights into the cause and effects of interleukin-6 activation in mouse liver regeneration. Hepatology (Baltimore, Md.) 54, 597–608.

Vaquero, J., Campbell, J. S, Haque, J., McMahan, R.S., Riehle, K.J., Bauer, R.L., Fausto, N., 2011b. Toll-like receptor 4 and myeloid differentiation factor 88 provide mechanistic insights into the cause and effects of interleukin-6 activation in mouse liver regeneration. Hepatology (Baltimore, Md.) 54, 597–608.

Vaquero, J., Riehle, K.J., Fausto, N., Campbell, J. S, 2011c. Liver Regeneration after Partial Hepatectomy Is Not Impaired in Mice with Double Deficiency of Myd88 and IFNAR Genes. Gastroenterology research and practice 2011, 727403.

Viebahn, C.S., Yeoh, G.C.T., 2008. What fires prometheus? The link between inflammation and regeneration following chronic liver injury. The international journal of biochemistry & cell biology 40, 855–73.

Vig, P., Russo, F.P., Edwards, R.J., Tadrous, P.J., Wright, N.A., Thomas, H.C., Alison, M.R., Forbes, S.J., 2006. The Sources of Parenchymal Regeneration After Chronic Hepatocellular Liver Injury in Mice. Hepatology.

Vosough, M., Moslem, M., Pournasr, B, Baharvand, H., 2011. Cell-based therapeutics for liver disorders. Br Med Bull 100, 157–72.

Wack, K., Ross, M., Zegarra, V., Sysko, L., Watkins, S., Stolz, D., 2001. Sinusoidal ultrastructure evaluated during the revascularization of regenerating rat liver. Hepatology 33, 363–78.

Wang, Willenbring, H., Akkari, Y., Torimaru, Y., Foster, M., Al-dhalimy, Muhsen, Lagasse, Eric, Finegold, Milton, Olson, S., Grompe, Markus, 2003. Cell fusion is the principal source of bone-marrow-derived hepatocytes. letter to nature 422, 29–32.

Wang, X., Montini, E., Al-dhalimy, M, Lagasse, E, Finegold, M, Grompe, M, 2002. Kinetics of liver repopulation after bone marrow transplantation, Am. J. Pathol. 161, 565–574.

Wuestefeld, T., Klein, C., Streetz, K.L., Betz, U., Lauber, J., Buer, J., Manns, M.P., Müller, W., Trautwein, Christian, 2003. Interleukin-6/glycoprotein 130-dependent pathways are protective during liver regeneration. The Journal of biological chemistry 278, 11281–8.

Xagorari, A., Siotou, E., Yiangou, M., Tsolaki, E., Bougiouklis, D., Sakkas, L., Fassas, A., Anagnostopoulos, A., 2013. Protective effect of mesenchymal stem cell-conditioned medium on hepatic cell apoptosis after acute liver injury. International journal of clinical and experimental pathology 6, 831–40.

Yagi, H., Parekkadan, Biju, Suganuma, K., Soto-Gutierrez, A., Tompkins, R.G., Tilles, Arno W, Yarmush, Martin L, 2009. Long-term superior performance of a stem cell/hepatocyte device for the treatment of acute liver failure. Tissue engineering. Part A 15, 3377–88.

Yamazaki, S., Miki, K., Hasegawa, K., Sata, M., Takayama, T., Makuuchi, M., 2003. Sera from liver failure patients and a demethylating agent stimulate transdifferentiation of murine bone marrow cells into hepatocytes in coculture with nonparenchymal liver cells. J Hepatol. 3917.

Zhang, B., Inagaki, M., Jiang, B., Miyakoshi, M., Arikura, J., Ogawa, K., Kasai, S., 2009. Effects of bone marrow and hepatocyte transplantation on liver injury. The Journal of surgical research 157, 71–80.

Zhang, L., Ye, J.-S., Decot, V., Stoltz, J.-F., De Isla, N., 2012. Research on stem cells as candidates to be differentiated into hepatocytes. Bio-medical materials and engineering 22, 105–11.

Zhiyong, C., Kun, B., Jiqi, M., Chenghong, Y., 2013. Mesenchymal stem cell-conditioned medium reduces liver injury and enhances regeneration in reduced-size rat liver transplantation. The Journal of surgical research 1–9.

Zimmermann, H., Zimmermann, D., Reuss, R., Feilen, P.J., Manz, B., Katsen, a, Weber, M., Ihmig, F.R., Ehrhart, F., Gessner, P., Behringer, M., Steinbach, a, Wegner, L.H., Sukhorukov, V.L., Vásquez, J. a, Schneider, S., Weber, M.M., Volke, F., Wolf, R.,

Zimmermann, U., 2005. Towards a medically approved technology for alginate-based microcapsules allowing long-term immunoisolated transplantation. Journal of materials science. Materials in medicine 16, 491–501.

ANEXO

TEMPO	rato	campo 1		campo 2		campo 3		campo 4		campo 5					
		POSITIVOS	NEGATIVOS	POSITIVOS	NEGATIVOS	POSITIVOS	NEGATIVOS	POSITIVOS	NEGATIVOS	POSITIVOS	NEGATIVOS	TOTAL +	TOTAL -	TOTAL	%CEL +
EC 24	34	17	104	17	78	19	103	8	92	10	118	71	495	566	12,54
EC 24	34	15	82	15	92	8	99	17	80	32	65	87	418	505	17,2
EC 24	137	1	109	1	113	0	128	0	115			2	465	467	0,43
EC 24	137	1	97	0	130	0	108	0	108	1	80	2	523	525	0,4
EC 24	138	27	98	30	104	39	113	36	77			132	392	524	25,19
EC 24	138	48	60	72	75	52	82	62	52	34	103	268	372	640	41,9
EC 48	32	15	62	17	91	9	83	21	84	10	69	72	389	461	15,62
EC 48	32	2	149	9	71	7	95	14	84	11	50	43	449	492	8,7
EC 48	181	11	129	5	114	18	92	10	118	16	99	60	552	612	9,80
EC 48	181	13	84	22	75	11	109	18	93	13	102	77	463	540	14,3
EC 48	182	8	113	8	118	5	107	6	102	6	116	33	556	589	5,60
EC 48	182	8	78	6	81	8	87	4	74	5	8	31	328	359	8,6
WBM24	18	12	92	4	103	8	81	3	86	24	66	51	428	479	10,65
WBM24	18	23	76	40	91	24	96	23	59	16	88	126	410	536	23,5
WBM24	129	2	80	1	125		76	2	103	1	67	6	451	457	1,31
WBM24	129	0	126	0	126	0	80	0	95	0	99	0	526	526	0,0
WBM24	130	0	70	2	82	1	97	0	82	0	81	3	412	415	0,72
WBM24	130	0	92	1	92	0	92	0	92	0	92	1	460	461	0,2
WBM48	49	15	99	11	95	11	80	18	89	14	112	69	475	544	12,68
WBM48	49	3	43	10	57	3	45	4	65		50	20	260	280	7,1
WBM48	185	5	52	9	53	1	71	4	59	11	66	30	301	331	9,1
WBM48	185	4	110	5	105	7	115	8	107	4	107	28	544	572	4,9
WBM48	186	29	70	16	72	23	74	17	79	19	64	104	359	463	22,5
WBM48	186	18	71	22	58	12	50	14	58	7	91	73	328	401	18,2

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ORIGINAL ARTICLE

Bone Marrow Mononuclear Cell Transplantation Improves Survival and Induces Hepatocyte Proliferation in Rats after CCl_4 Acute Liver Damage

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Abstract
Aim The aim of this research was to evaluate the effects of bone marrow mononuclear cell (BMC) transplantation in rats with toxic acute liver damage induced by carbon tetrachloride (CCl_4).
Methods Cells from male Wistar rats were obtained using Ficoll density gradient and 0.2 ml (1×10^6 cells) were injected into the portal vein of female rats ($n = 15$) 24 h after damage. Sham group ($n = 15$) was performed injecting only vehicle in CCl_4-treated animals. Survival, liver histology, number of mitosis and apoptosis, and identification of stained donor cells were observed 72 h after damage. ALT levels were measured at 0 h, 24 h, 48 h, and 72 h after injury.
Results Donor cells could be detected in recipient rats' livers by fluorescence staining and *Sry* PCR. The treated group revealed a significant improvement in survival rate after 72 h ($p = 0.003$). There was also a significant increase in the number of mitotic events in treated livers ($p = 0.029$). This result was confirmed using an in vitro cell proliferation assay in isolated hepatocytes treated with conditioned medium from BMC. ALT was reduced in the treated group after 72 h ($p = 0.034$).
Conclusions Results indicate that BMC transplantation has potential as a new therapeutic option for acute liver disease and suggest that these cells may contribute to hepatic recovery through release of mitotic cytokines.

Keywords Acute liver failure · Bone marrow stem cells · Carbon tetrachloride · Cell therapy · Conditioned medium · Hepatocyte proliferation

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Introduction

Pronounced hepatic damage may lead to acute liver failure (ALF), a syndrome characterized by rapid deterioration of liver function and high mortality [1]. The only established treatment is orthotopic liver transplantation (OLT). However, due to organ shortage, a significant percentage of patients die while waiting for transplant [2]. However, it is important to stress that liver failure is potentially reversible due to the liver's regenerative capacity. Thus, development of alternatives that could maintain or reestablish liver function after failure may help patients with ALF.

Cell-based therapies have emerged as an alternative to OLT [3]. Cell transplantation offers advantages, being a less-invasive procedure when compared to whole-organ transplantation and can be performed repeatedly if necessary. Bone marrow is the main source of cells used for therapy. Studies using purified hematopoietic [4] and mesenchymal [5] fractions or mononuclear cells [6] have been shown to improve liver function after transplant.

Despite advances in the area of bone marrow cell transplantation in the last few years, controversies remain about the efficacy and the mechanisms of organ recovery mediated by these cells [7]. Processes of cell fusion and differentiation in the liver following cell transplantation have been described [8–10]. However, these events occur at a very low frequency (lower than 0.05%), suggesting that such mechanisms are not the only ones involved in liver regeneration induced by cell transplantation [11].

This work evaluates the effects of bone marrow mononuclear cell transplantation in rats with toxic acute liver damage induced by carbon tetrachloride, and suggests that these cells may contribute to hepatic recovery through the release of mitotic cytokines.

Methods

Animals

Fifty-nine female Wistar rats weighing 200–240 g were used to perform the experiments. Male rats ($n = 10$) 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). Animals were kept at the Experimental Animal Unit of Research Center of Hospital de Clinicas de Porto Alegre (UEA-HCPA), under controlled temperature (between 18 and 22°C) in light–dark cycles of 12 h. Standard rat chow and water were given ad libitum.

Experimental Design

Rats were divided into two main groups. Sham group: rats ($n = 15$) were given 1.25 ml/kg of CCl_4 orally [12] and 24 h later received 0.2 ml of PBS solution via portal vein. BMC group: rats ($n = 15$) received the same dose of CCl_4 and 24 h later were injected with 1×10^6 ml of bone marrow mononuclear cells in phosphate buffer saline (PBS) suspension. Surviving animals were killed 72 h after CCl_4 damage induction. To better evaluate the early effects of BMC, a subset of four animals in the sham and BMC groups were killed 48 h after injury.

Two additional groups (lesion control and injection control) were performed and evaluated at 72 h. In the injection control group, four rats received just BMC via portal vein with no hepatic damage to ensure that the cell injection procedure was safe. The last group of ten animals (lesion control group) received only CCl_4 and no posterior intervention to evaluate the mortality rate in this animal model. Finally, one group receiving CCl_4 and BMC (survival group, $n = 7$) was kept for up to 15 days to discard a transient effect of BMC on survival.

Induction of Acute Liver Injury

Female Wistar rats were weighed just before the CCl_4 administration. The 1.25 ml/kg dose of CCl_4 was then diluted in olive oil to a final volume of 1 ml. The solution was administered through gavage as a single dosage [12].

Isolation of BMC

Male Wistar rats weighing 200–240 g were used as cell donors in a proportion of one donor to four recipients. In a sterile environment, the femurs and tibias were isolated and whole bone marrow from each bone was flushed with 3 ml DMEM (Gibco, USA) into a 15-ml tube. The cells were then placed onto a Ficoll Histopaque (GE-Healthcare, USA) layer and centrifuged at $800 \times g$ for 30 min. The interface was separated using a pipette, and cells were rinsed with 10 ml PBS three times. Cells were counted using the Neubauer chamber and Trypan Blue exclusion test to verify cell viability. DAPI [4′, 6-diamidino-2-phenylindole 2.7 mg/ml (Roche, Germany)] fluorescent staining was performed according to manufacturer's protocol. Again, cells were rinsed with PBS, counted using the Trypan Blue exclusion test in a Neubauer chamber, and adjusted to a final concentration of 5×10^6 cells/ml. The entire procedure took 2 h from isolation to transplantation, in the meanwhile, cells were kept at room temperature [6].

Cell Characterization

Three independent Ficoll isolation procedures were analyzed by simple size/scatter flow cytometry, aiming to characterize and count the mononuclear cell fraction (lymphocytes, monocytes, granulocytes), as well as detect any contamination of red blood cells and platelets. Results are expressed as percentage of each cell type from total cells.

In addition, three other isolation procedures were characterized regarding the presence of precursor cells by FACS using 2×10^6 mononuclear cells. Briefly, BMC were resuspended in 200 µl of PBS and incubated for 20 min at 4°C with FITC-anti-CD45 (CALTAG lab. CA,USA) and PE-anti-CD34 (Santa Cruz Biotechnology, Santa Cruz, CA) for characterization of hematopoietic stem cells through the ISHAGE protocol [13]. Anti-KDR (Abcam, Inc, CA, USA) and PE-anti-CD34 (Santa Cruz Biotechnology, Santa Cruz, CA) were used to quantify endothelial precursors cells [14]. The mesenchymal stem cell population was characterized by triple staining for FITC-anti-CD44 (Abcam Inc, CA, USA), PE-anti-CD71 (AbD Serotec, Munich, Germany) and PECy5-anti-CD29 (BioLegend, San Diego, CA) [15]. All assays were conducted using antibody concentrations as recommended by the manufactures. Phycoerythrin-PE and FITC mouse anti-rat IgG1, IgG2a and IgM were used as isotype controls. After antibody incubation, erythrocytes lysis solution (1 ml) was added for 15 min; then 1 ml of PBS was added for another 15 min to stop the hemolytic treatment. Cells were collected and washed with PBS by centrifugation and 500 µl of PBS was added to the cell suspension.

Analysis was carried out using the BD FACS-Calibur flow cytometry system with a one-laser system capable of detecting three fluorochromes excited by the 488-nm laser in a multiparameter manner. The samples were read with Cell Quest and PAINT-A-GATE software.

Cell Transplantation

Animals were anesthetized with 100 mg/kg of Ketamine (Eurofarma Lab, Brazil) 10% and 10 mg/kg of Xylazine (Sespo, Brazil) 2% intraperitoneally just before surgery. A 3-cm longitudinal incision was performed in the abdomen, the portal vein was exposed, and 0.2 ml of the cell suspension was injected in bolus. To avoid bleeding, a porcine collagen matrix (Mascia Brunelli, Italy) was placed over the vein. Animals were sutured in layers with polyglactin 4-0 (Ethicon, Brazil) and monofilament 4-0 (Ethicon, Brazil) and allowed to recover in the cage. The sham group was submitted to the same surgical procedure, although receiving 0.2 ml of vehicle with no cells [6].

Biochemistry

Blood was collected at time points 0, 24, 48, and 72 h after CCl_4 administration, through retrorbital puncture under anesthesia. Alanine aminotransferase (ALT) was measured using the enzyme kinetics method [16]. All animals had ALT levels at least threefold the normal reference values 24 h after CCl_4 administration.

Histological Evaluation

Rats that survived up to 72 or 48 h after CCl_4 administration were euthanized in CO_2 chambers. At the time of death, three lobes from the liver, whole lung, and spleen were collected and placed in a 10% buffered formalin solution for 24 h, and submitted to routine histology processing. Hematoxylin and eosin (H&E) stained sections were analyzed under optic microscopy by a trained pathologist, blind to the treatment groups. Necrosis was evaluated by counting the rows of necrotic hepatocytes from the perivenular zone (acinar zone 3). Bridging necrosis and hydropic degeneration were noted when present. Steatosis was graded as absent, mild, moderate, or severe. The mitotic and apoptotic indexes were counted in ten high-power fields (400× magnification). The inflammatory infiltrate was classified as either present or absent.

Donor Cell Tracking

To analyze the presence of DAPI-positive (fluorescent) cells, six unstained sections per organ were visualized under a fluorescence microscope (Olympus BH Series) using the appropriate filter set. Sections from both the lungs and spleen were also analyzed by H&E staining and for the presence of fluorescent cells.

In addition to the observation of DAPI-stained cells under fluorescent microscopy, recipient female liver samples were screened for the presence of the Y-chromosome gene *Sry* by PCR. Total DNA was extracted using TRIzol reagent (Invitrogen). DNA was quantified using Nanodrop (Thermo Scientific, USA) and 100 ng were used as template. The *Sry* gene was amplified using 20 pmol of each primer (SRY forward 5′- AAGCGCCCC ATGAATGCATT-3′ and SRY reverse 5′- CAGCTGCTT GCTGATCTCTG-3′), 5 µl of buffer (75 mM Tris–HCl—pH 9.0; 2 mM MgCl2; 50 mM KCl, 20 mM, 107 (NH4)2SO4; 0.001% BSA); 0.2 mM dNTP; 1.5 mM of $MgCl_2$ and 3 U of Taq polymerase. Annealing temperature was 62°C, and amplification was performed using 32 PCR cycles. A PCR product of 102 bp was visualized in 1.6% agarose gel.

Hepatocyte Isolation

Male Wistar rats ($n = 2$) 10 weeks old served as hepatocyte donors. Primary rat hepatocytes were isolated by the classical two-step collagenase perfusion technique according to standard procedure [17]. Determination of cell viability was performed by the Trypan Blue exclusion test using a Neubauer chamber. Cells were then resuspended in DMEM supplemented with 10% FBS and plated at a density of 1×10^4 cells in 96-well plates.

Cell Proliferation Test

Isolated hepatocytes were treated 24 h after being plated. Three groups ($n = 12$ wells/group) were tested: in the first group, medium was changed by fresh DMEM supplemented with 10% FBS. In a second group, hepatocytes were treated with conditioned medium from bone marrow mononuclear cells. The third group was treated with conditioned medium obtained from skin fibroblasts.

BMC-conditioned medium was obtained as follows: bone marrow cells were isolated by Ficoll gradient density and plated at 1×10^6 cells in six-well plates. Forty-eight hours afterwards, the medium was collected, centrifuged, and filtered using 0.22-μm filters to produce a conditioned medium. This medium was then added to hepatocytes in a proportion of 1:2 with normal medium supplemented with 10% FBS. The third group was treated exactly in the same way as the second group, but conditioned medium was obtained from human skin fibroblasts, as an additional control to verify if any effects observed would be specifically due to the bone marrow cells. Cell proliferation assay was performed by MTT tetrazolium [3-(4,5-dimethylthiazol-2-yl)-2,5 phenyltetrazolium bromide; Sigma Chemical, USA] test, as described previously [18] in hepatocytes 48 h after treatment. The color intensity observed in each well was correlated with the number of viable cells, considering that the control group had a 100% cell viability.

Data Analysis

Statistical analysis was performed using the SPSS (Statistical Package for the Social Sciences). Comparison between the sham and BMC groups for mitotic index was performed by the two-tailed Student's t test for independent samples. The results were expressed as mean ± standard deviation (SD). ALT levels and apoptotic index were evaluated by the Mann–Whitney U test and presented as median and interquartil range (IQR 25 and IQR 75). Survival rate was evaluated using the logrank (Mantel–Cox) test. Cell proliferation was analyzed by using ANOVA and Tukey's multiple comparison tests. A p value < 0.05 was considered statistically significant.

Ethics and Hazard Issues

This study was approved by the local ethics committee and all participants received adequate training concerning hazardous issues and animal handling.

Results

Cell Characterization

BMC cells were initially characterized by simple size/scatter flow cytometry. BMC population was mainly composed by lymphocytes (78.5 ± 4.2%) while monocytes presented 10.4 ± 0.6% of obtained cells. There was also a small percentage of granulocytes in BMC preparations (11.1 ± 3.7%). Red blood cells and platelets presented very low values (0.65 ± 0.57% and 0.45 ± 0.62, respectively).

To characterize the precursor cells in these preparations, we quantified endothelial precursor cells ($KDR^+/CD34^+$), mesenchymal stem cells ($CD44^+/CD71^+/CD29^+$) and hematopoietic stem cells (using ISHAGE protocol). Endothelial precursor cells corresponded to 1.39% ± 0.87 of the total cells injected, while mesenchymal and hematopoietic fractions corresponded to 0.48% ± 0.09 and 0.48% ± 0.37, respectively.

Animal Model

Carbon tetrachloride induced rapid histological changes in rat liver, including bridging necrosis, steatosis, and hydropic degeneration as soon as 24 h after damage, coinciding with ALT peak. Two days later, histology and ALT levels were almost normal (data not shown), indicating that the liver was already in its regenerative process. These results are in accordance with other animal models of acute liver injury described in the literature [6, 19, 20]. The mortality rate in animals that received only CCl_4 and no posterior intervention (lesion group) was 30%.

Survival Analysis

At the time of killing, the sham group presented a mortality rate of 47%. None of the bone-marrow-treated rats died during the experiment (100% survival), showing a marked increase in the survival rate (Fig. 1) when compared to the sham group ($p = 0.003$). Rats that received only cells and no CCl_4 damage also had a 100% survival, indicating that portal application of these cells was a safe procedure.

To verify if the observed effect was not only transient, we performed cell transplantation in an additional group of seven rats 24 h after CCl_4 administration. Again, survival

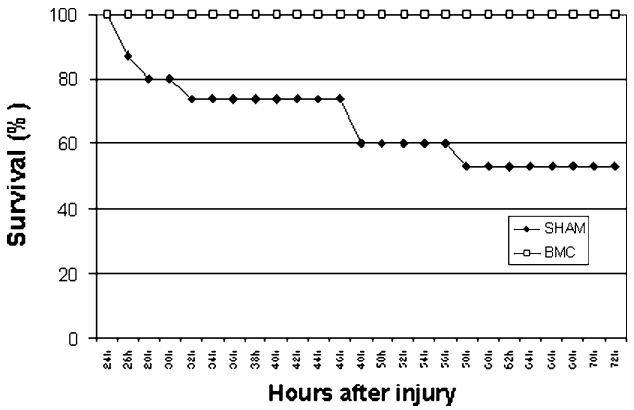

Fig. 1 Kaplan–Meier curve showing the survival rate from sham- and bone-marrow-treated animals 72 h after CCl_4 administration. The survival in the bone-marrow-treated group was statistically different when compared to the sham ($p = 0.003$) group using Chi-square test

was 100% after 15 days and liver histology showed a complete regeneration of the organ (data not shown).

Biochemistry

ALT levels reached mean values about 5–8 times higher than normal 24 h after injury in both groups, and diminished with time (Table 1). ALT levels were statistically reduced in the BMC group ($p = 0.034$), reaching normal values after 72 h. The group that received only bone marrow cells and no CCl_4 demonstrated no changes in ALT levels, indicating that the bone marrow cell injection per se did not cause any significant damage to the liver (data not shown).

Histological Evaluation

Since both groups appeared to be completing the liver regenerative process, differences in animals that survived for 72 h could not be observed (Fig. 2a, b). However, animals from the sham group that died before 72 h had an intense and bridging necrosis, presenting micro and macrovesicular steatosis and hydropic degeneration (Fig. 2c). Animals from both groups that survived for 72 h revealed the presence of an inflammatory infiltrate and macrophages reabsorbing the necrotic cells. Necrosis was reduced to almost null levels, but mild levels of steatosis and hydropic degeneration were still present (Fig. 2a, b).

Mitotic and Apoptotic Indexes

Mitotic and apoptotic indexes were calculated considering ten random high-power fields (400× magnification). The number of mitotic events (Fig. 3a, b) in the bone-marrow-treated group was approximately twice as high as in the sham group ($p = 0.029$). When analyzed in the fluorescence microscope, cells seen in mitosis were not stained blue with the fluorescent dye DAPI. This suggests that they were not donor-derived cells, but receptor's hepatocytes in proliferation. The apoptotic index was the same between the groups [median of 2.0 (0.0–3.0) for sham and 2.0 (1.0–3.0) for BMC]. Based on these results we killed four sham- and four BMC-treated rats 48 h after injury. None of the sham rats presented mitotic cells in the ten high-power fields, while BMC rats presented a mean value of 4.5 ± 3.5 mitotic cells in ten high-power fields.

Donor Cell Tracking

Fluorescent cells could be visualized in recipient livers from the BMC group. Cells were detected mostly as single cells placed around both portal and centrilobular tracts. Very rare aggregates of few cells were visualized. A visual analysis revealed that most fluorescent cells were near or within sinusoids, thus not consistent with features of hepatocytes (Fig. 4a). A few cells, however, adopted a hepatocyte-like phenotype (Fig. 4b), displaying a large rounded nucleus in the parenchyma. These results indicate rapid engraftment of these cells in the recipient liver. No fluorescent cells were visualized in the lungs or spleens.

In addition, we performed a PCR for the *Sry* gene. Results confirmed the presence of donor-derived (*Sry* positive) cells within the livers from treated rats (Fig. 4c).

Cell Proliferation Assay

To confirm data from mitotic index studies, the growth of isolated hepatocytes in contact with normal medium or conditioned medium from BMC was compared using the

Table 1 ALT levels (U/ml) after liver damage induced by CCl_4 in sham and bone marrow cells (BMC) groups

	0 h	24 h	48 h	72 h
BMC	42.0 (32.0–49.0)	195.0 (143.0–1098.0)	115.0 (90.0–232.0)	52.0 (48.0–70.0)*
	$n = 15$	$n = 15$	$n = 15$	$n = 15$
Sham	36.5 (31.3–42.0)	347.0 (131.5–520.3)	177.5 (81.3–586.3)	91 (56.3–146.3)
	$n = 15$	$n = 14$	$n = 8$	$n = 8$

Results are expressed as median and interquartil range (IQR 25 and IQR 75).* $p = 0.034$ compared to sham group, Mann–Whitney U test

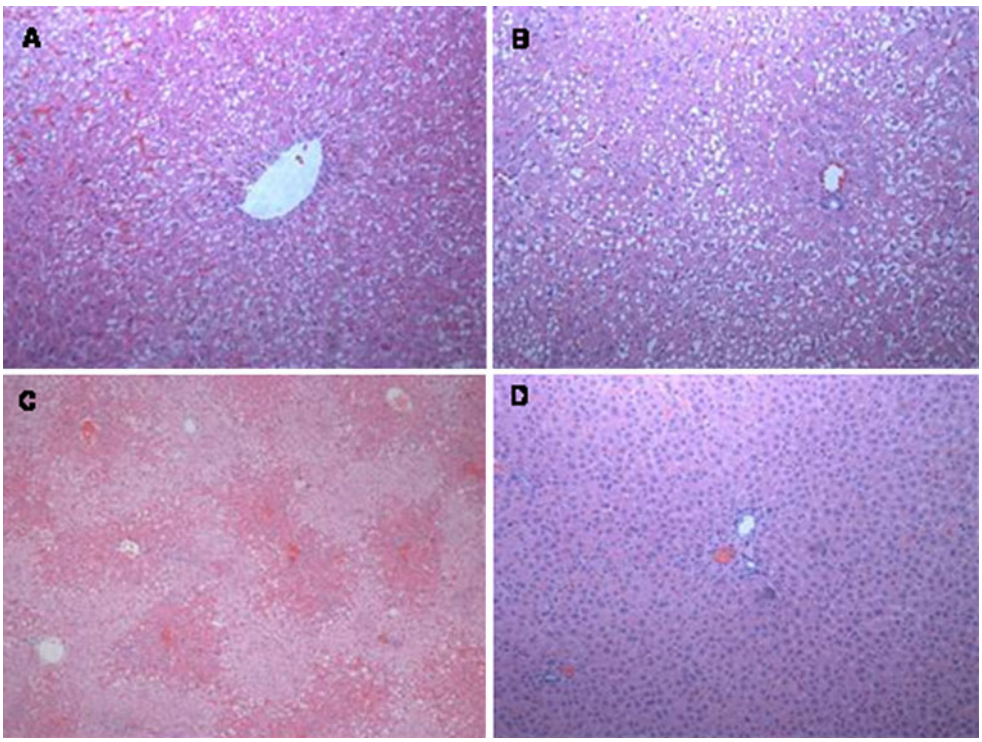

Fig. 2 H&E staining from liver sections. **a** BMC group, 72 h after injury. **b** Sham group, 72 h after injury. **c** Sham rat dead 48 h after injury, showing intense bridging necrosis. **d** Normal liver. Magnification 200×

MTT test. The number of hepatocytes in the group treated with BMC conditioned medium was significantly increased ($p < 0.05$) when compared to both the fibroblasts conditioned medium and control group (Fig. 5). This finding strengthens the hypothesis that some cytokines released from these cells could stimulate hepatocytes proliferation.

Discussion

In this study we verified the effects of BMC transplantation in rats with CCl_4-induced acute liver damage. A marked increase in the survival rate of BMC-treated animals was demonstrated, which could in the near future have important clinical applications. Other groups have also demonstrated a survival improvement of animals with ALF using other types of cell therapies, such as blood cord stem cells [21] and even hepatocytes derived from stem cell differentiation processes [9].

The benefits of using BMC as a source of cells for treating ALF are based mostly on their availability and feasibility of an autotransplantation. The use of mononuclear bone marrow cells as proposed in this study may offer other advantages when compared to stem cell purified populations. BMC contains both hematopoietic and mesenchymal stem cells, which are able to differentiate into hepatocytes [4, 22]. Recently the ability of monocytes [23] and other cell types [24] to differentiate into hepatocytes was also demonstrated, overcoming epigenetic mechanisms of gene expression control. Analyzing this context, mononuclear bone marrow cells seem to be a more adequate source of stem cells for acute liver damage. The main advantages of these cells are the possibility of an autotransplant and the rapid process involved in cell obtention. The last aspect is critical in the case of acute liver damage, since liver failure after an initial injury may occur within days, requiring a rapid intervention. Cell replacement must be done as fast as possible and cell differentiation protocols usually take at least 2–3 weeks. Evidence of the feasibility for using bone marrow cells in ALF was demonstrated in the work of Gasbarrini et al. [25]. In this first clinical use of bone marrow mononuclear cells for transplantation, the patient showed a rapid improvement in liver function but died 50 days after the procedure due to a bacterial infection.

Even though bone marrow stem cells are already being used in clinical trials for many diseases, the actual mechanisms through which they improve organ regeneration are not completely understood. Processes of fusion with hepatocytes and differentiation had been described, but in most cases the frequency of these processes was lower than 1% [11], being insufficient to promote a full recovery of the organ and indicating that these are probably not the only mechanisms of regeneration mediated by cell transplantation. In this study, the increase in mitotic index

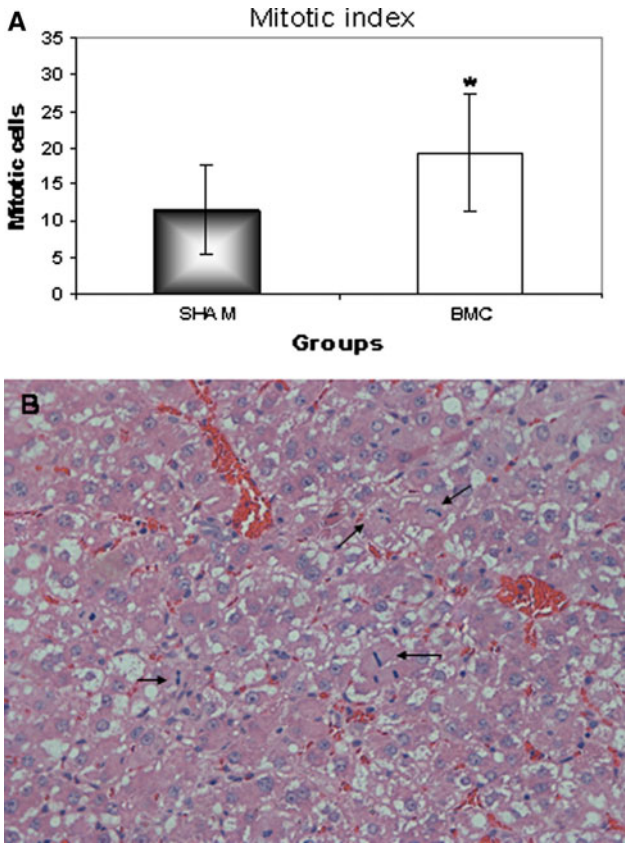

Fig. 3 Mitotic activity in sham- and bone-marrow-treated rats 72 h after CCl_4 administration. **a** Mitotic index counted in ten high-power fields, expressed as mean ± SD * $p = 0.029$ compared to the sham group (Student's *t* test). **b** H&E staining of a representative liver section of the bone-marrow-treated animal. *Arrows* indicates mitotic cells (magnification 400X)

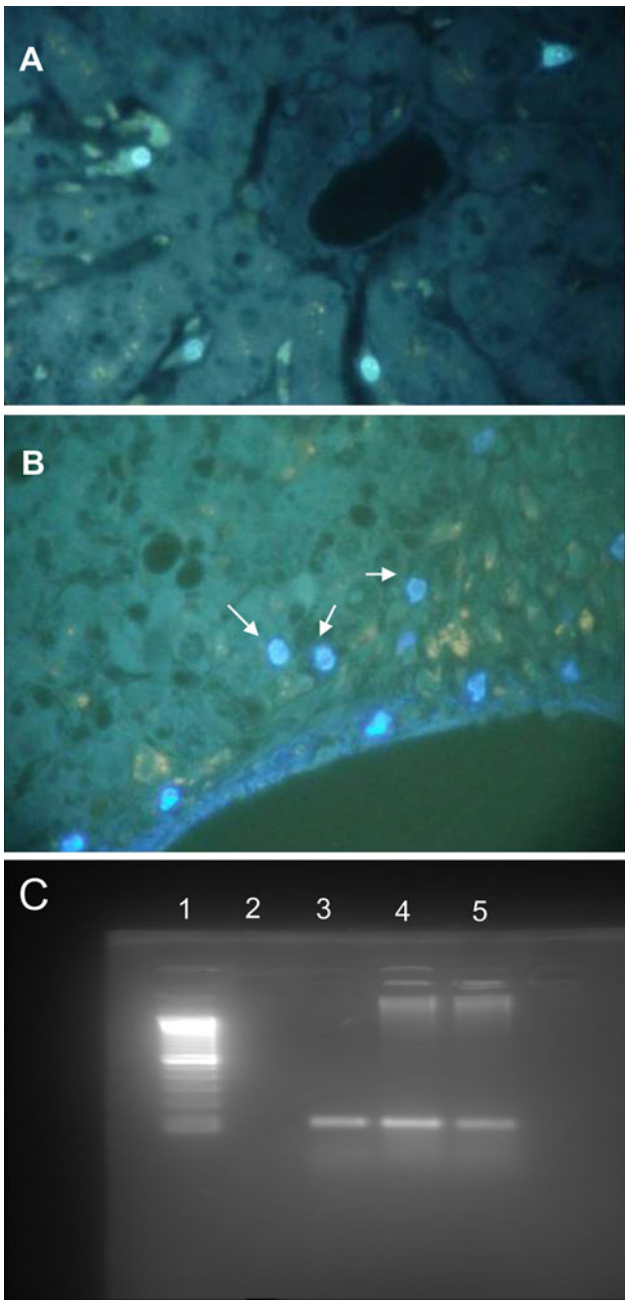

Fig. 4 Unstained liver sections of bone-marrow-treated animals analyzed under fluorescence microscopy revealing fluorescent engrafted cells with **a** undefined phenotype and **b** hepatocyte-like phenotype (*arrows*). Magnification 2,000×. **c** *Sry* PCR confirming presence of donor cells in recipients' livers. *Lanes: 1*—100-bp ladder, *2*—Blanc, *3*—Positive control (male DNA) *4* and *5*—BMC-treated rats

observed in the BMC group may provide a mechanism for BMC-based organ regeneration. The hypothesis is that the transplanted cells engraft into the liver (which could be observed by DAPI staining) and secrete mitotic cytokines in response to a specific damage. Cell proliferation was increased in isolated hepatocytes in vitro after treatment with conditioned medium from BMC cells, which strengthen our hypothesis. Other groups have recently proposed a similar paracrine effect from either whole mononuclear bone marrow cells in the reconstitution of injured myocardium [26], or purified mesenchymal stem cells in chronic liver damage [5]. However, neither group established a correlation with cell proliferation but directly with the regenerative process. Further experiments are needed to identify the possible cytokines responsible for this effect and to verify the hypothesis proposed by this study. Note that in undamaged animals injected with BMC, there was no indication of hepatocyte proliferation, suggesting that in vivo, this mechanism is injury-dependent. Microarray analysis of gene expression, after stem cell therapy for acute liver injury, showed an up-regulation of genes involved in cell proliferation [27]. The increase in the mitotic rate of hepatocytes observed in the present study could be a cellular response to a stem-cell-mediated up-regulation. It may also explain the differences in ALT levels observed at 72 h, reflecting a faster recovery of liver function.

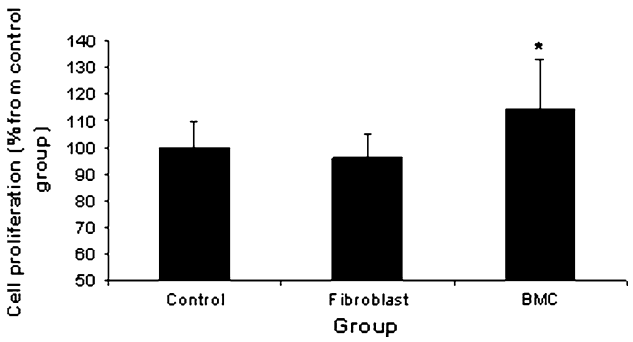

Fig. 5 In vitro cell proliferation MTT assay showing increased hepatocyte proliferation after treatment with conditioned medium from bone marrow mononuclear cells (BMC group), as described in "Methods" section. * $p < 0.05$ compared to other groups, ANOVA and Tukey test

It is important to emphasize that these findings do not exclude other mechanisms for liver repair induced by stem cells. Controversial studies demonstrated that differentiation is the main process underlying stem cell ability of regenerating tissues [10], however fusion, direct and indirect transdifferentiation have also been proposed [28]. We cannot precisely confirm whether the differentiation of stem cells into hepatocytes occurs in this animal model. However, we were able to visualize fluorescent cells with a hepatocyte phenotype, suggesting that differentiation or fusion processes may occur. It is likely that those listed mechanisms may occur simultaneously during the regenerative process.

The findings of this study indicate that bone marrow mononuclear cell transplantation may represent a good therapeutic alternative for treatment of ALF in drug-induced poisoning. However, adequate trials should be performed in other animal models prior to humans in order to verify the effects of transplantation of these cells. Bone-marrow-derived stem cells have yet to be clinically implemented for acute liver damage conditions, but BMC are relatively accessible, which makes them an appealing alternative.

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References

1. Onodera K, Sakata H, Yonekawa M, Kawamura A. Artificial liver support at present and in the future. *J Artif Organs*. 2006;9:17–28.
2. Sass DA, Shakil AO. Fulminant hepatic failure. *Liver Transpl*. 2005;11:594–605.
3. Nussler A, Konig S, Ott M, et al. Present status and perspectives of cell-based therapies for liver diseases. *Journal of Hepatology*. 2006;45:144–159.
4. Lagasse E, Connors H, Al-Dhalimy M, et al. Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. *Nature Medicine*. 2000;6:1229–1234.
5. Fang B, Shi M, Liao L, Yang S, Liu Y, Zhao RC. Systemic infusion of FLK1(+) mesenchymal stem cells ameliorate carbon tetrachloride-induced liver fibrosis in mice. *Transplantation*. 2004;78:83–88.
6. Belardinelli MC, Pereira FS, Baldo G, et al. Adult derived mononuclear bone marrow cells improve survival in a model of acetaminophen- induced hepatic failure. *Toxicology*. 2008;247:1–5.
7. Houlihan DD, Newsome PN. Critical review of clinical trials of bone marrow stem cells in liver disease. *Gastroenterology*. 2008;135:438–450.
8. Wang X, Willenbring H, Akkari Y, et al. Cell fusion is the principal source of bone-marrow derived hepatocytes. *Nature*. 2003;422:897–901.
9. Jang YY, Collector MI, Baylin SB, Diehl AM, Sharkis SJ. Hematopoietic stem cells convert into liver cells within days without fusion. *Nature Cell Biology*. 2004;6:532–539.
10. Shi XL, Qiu YD, Wu XY, et al. In vitro differentiation of mouse bone marrow mononuclear cells into hepatocyte-like cells. *Hepatol Res*. 2005;31:223–231.
11. Thorgeirsson SS, Grisham JW. Haematopoietic cells as hepatocyte stem cells: a critical review of the evidence. *Hepatology*. 2006;43:2–8.
12. Wong CK, Ooi VEC, Ang PO. Protective effects of seaweeds against liver injury caused by carbon tetrachloride in rats. *Chemosphere*. 2000;41:173–176.
13. Barnett D, Janossy G, Lubenko A, et al. Guideline for the flow cytometric enumeration of CD34 + haematopoietic stem cells. Prepared by the CD34 + haematopoietic stem cell working party. General haematology task force of the British committee for standards in haematology. *Clinical & Laboratory Haematology*. 1999;21:301–308.
14. Keymel S, Kalka C, Rassaf T, et al. Impaired endothelial progenitor cell function predicts age-dependent carotid intimal thickening. *Basic Research in Cardiology*. 2008;103:582–586.
15. da Silva ML, Caplan AI, Nardi NB. In search of the in vivo identity of mesenchymal stem cells. *Stem Cells*. 2008;26:2287–2299.
16. Henry RJ, Chiamori N, Golub O, Berkman S. Revised spectrophotometric methods for the determination of glutamic-oxalacetic transaminase, glutamic-pyruvic transaminase, and lactic acid dehydrogenase. *American Journal of Clinical Pathology*. 1960;34:381–398.
17. Seglen PO. Preparation of isolated rat liver cells. In: Prescott DM, ed. *Methods in Cell Biology*. New York: Academic Press; 1976:29–83.
18. Balestrin RC, Baldo G, Vieira MB, et al. Transient high-level expression of beta-galactosidase after transfection of fibroblasts from GM1 gangliosidosis patients with plasmid DNA. *Brazilian Journal of Medical and Biological Research*. 2008;41:283–288.
19. Nakatsuka R, Taniguchi M, Hirata M, Shiota G, Sato K. Transient expression of bone morphogenic protein-2 in acute liver injury by carbon tetrachloride. *Journal of Biochemistry*. 2007;141:113–119.
20. Wang T, Sun NL, Zhang WD, et al. Protective effects of dehydrocavidine on carbon tetrachloride-induced acute hepatotoxicity in rats. *Journal of Ethnopharmacology*. 2008;117:300–308.
21. Di Campli C, Piscaglia AC, Rutella S, et al. Improvement of mortality rate and decrease in histologic hepatic injury after

human cord blood stem cell infusion in a murine model of hepatotoxicity. *Transplant Proc*. 2005;37:2707–2710.
22. Kang XQ, Zang WJ, Song TS, et al. Rat bone marrow mesenchymal stem cells differentiate into hepatocytes in vitro. *World Journal of Gastroenterology*. 2005;11:3479–3484.
23. Ruhnke M, Ungefroren H, Nussler A, et al. Differentiation of in vitro-modified human peripheral blood monocytes into hepatocyte-like and pancreatic islet-like cells. *Gastroenterology*. 2005; 128:1774–1786.
24. Lysy PA, Smets F, Sibille C, Najimi M, Sokal EM. Human skin fibroblasts: from mesodermal to hepatocyte-like differentiation. *Hepatology*. 2007;46:1574–1585.
25. Gasbarrini A, Rapaccini GL, Rutella S, et al. Rescue therapy by portal infusion of autologous stem cells in a case of drug-induced hepatitis. *Digest Liver Dis*. 2007;39:878–882.
26. Braga LM, Rosa K, Rodrigues B, et al. Systemic delivery of adult stem cells improves cardiac function in spontaneously hypertensive rats. *Clinical and Experimental Pharmacology and Physiology*. 2008;35:113–119.
27. Piscaglia AC, Zocco MA, Di Campli C, et al. How does human stem cell therapy influence gene expression after liver injury? Microarray evaluation on a rat model. *Digest Liver Dis*. 2005; 37:952–963.
28. Herzog EL, Chai L, Krause DS. Plasticity of marrow-derived stem cells. *Blood*. 2003;102:3483–3493.

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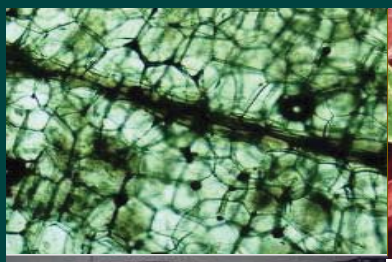

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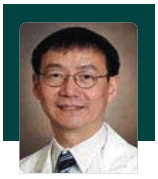
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Research Article Open Access

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 $1x10^6$ 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 22ºC) 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 $1x10^6$ 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 CO_2 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 $5x10^6$ 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 ($1x10^6$ 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 90ºC for 30 min after adding 500 µL of 0.67% thiobarbituric acid in 10% trichloroacetic acid, then centrifuged at 4ºC 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 30ºC [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 x10^5 cell/cm^2 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 µm 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 2x10^3 cells/cm^2 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

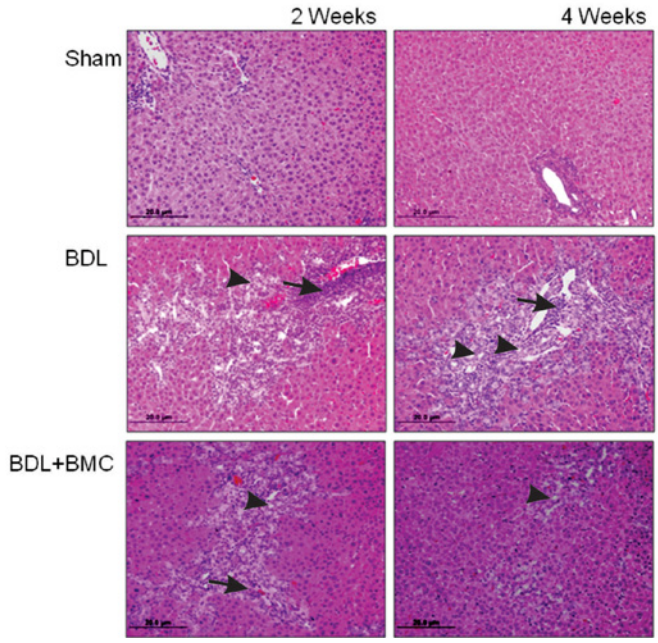

Figure 1: H&E staining. Liver sections from sham, BDL and BMC groups after two and four weeks of bile duct ligation, showing bile duct proliferation (arrow heads) and inflammatory infiltrate (arrows). Magnification 200X. BDL: Bile duct ligation. BMC: Bone Marrow Cell.

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.

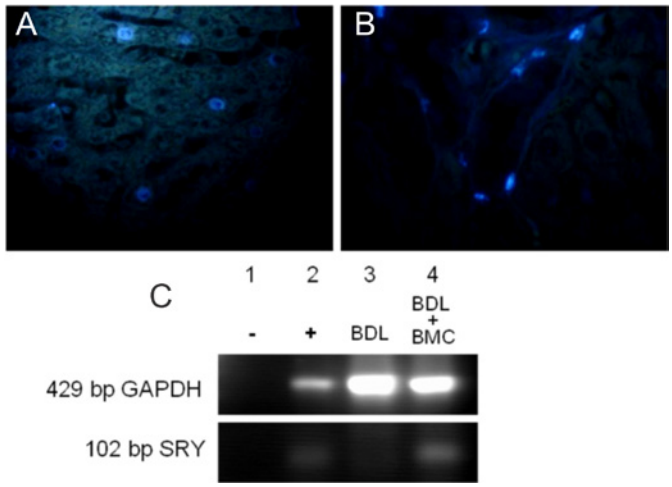

Figure 2: DAPI stained fluorescent nuclei in livers cells from bone marrow treated rats. A) Cells in liver parenchyma with hepatocyte-like phenotype. B) Cells placed around an area of ductular proliferation area. Magnification 200X. C) Visualization of PCR products for Sry (102 bp) gene in rat livers. Lane 1: Negative Control (blank); lane 2: Positive Control (male DNA); lane 3: BDL group; lane 4: BMC group; Gapdh (429bp) was used as internal control. BDL: Bile duct ligation. BMC: Bone Marrow Cell.

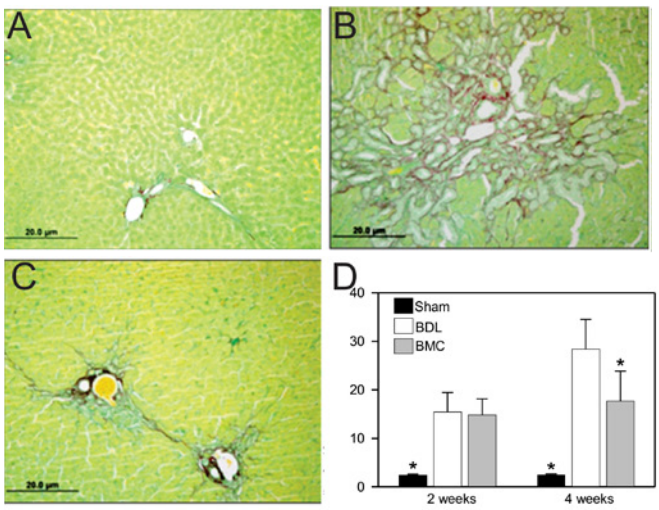

Figure 3: Extent of collagen deposition in Sham, BDL and BMC groups 4 weeks of BDL. A) Sirius Red staining from liver section from a Sham rat. B) LDB rat C) BMC-treated rat. Magnification 200X. D) Percentage of collagen area quantified as the red-stained area, measured in pixels at 2 and 4 weeks. (*P<0.05 compared with BDL and BMC, **P<0.05 compared with BDL). BDL: Bile duct ligation. BMC: Bone Marrow Cell.

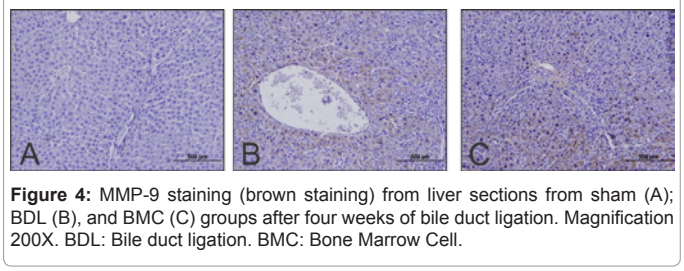

Figure 4: MMP-9 staining (brown staining) from liver sections from sham (A); BDL (B), and BMC (C) groups after four weeks of bile duct ligation. Magnification 200X. BDL: Bile duct ligation. BMC: Bone Marrow Cell.

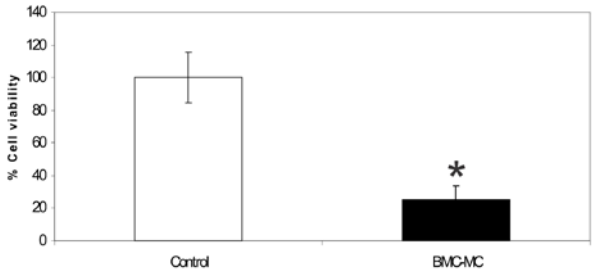

Figure 5: Cell viability analyzed by MTT test in GRX cells treated for 5 days with normal medium (Control) and conditioned-medium from bone marrow cells (BMC-CM). $p< 0.05$ compared with the control group. Student T test. BDL: Bile duct ligation. BMC: Bone Marrow Cell.

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 cholangyocytes in alpha-naphtylisothiocyanate-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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References

1. Parekkadan B, van Poll D, Megeed Z, Kobayashi N, Tilles AW, et al. (2007) Immunomodulation of activated hepatic stellate cells by mesenchymal stem cells. Biochem Biophys Res Commun 363: 247-252.
2. Baldo G, Ayala A, Melendez M, Nonnemache K, Lim L, et al. (2008) Prevalence of the serpin peptidase inhibitor (alpha-1-antitrypsin) PI*S and PI*Z alleles in Brazilian children with liver disease. Genet Mol Bio 31: 423-426.
3. Ferreira CT, Vieira SM, Kieling CO, Silveira TR (2008) Hepatitis A acute liver failure: follow-up of paediatric patients in southern Brazil. J Viral Hepat 15: 66-68.
4. Goldani HA, Matte US, Ramos AR, Costa TG, Winkelmann LV, et al. (2007) The role of food restriction on CCl4-induced cirrhosis model in rats. Exp Toxicol Pathol 58: 331-337.
5. Strassburg CP (2010) Autoimmune hepatitis. Best Pract Res Clin Gastroenterol 24: 667-682.
6. Penz-Österreicher M, Österreicher CH, Trauner M (2011) Fibrosis in autoimmune and cholestatic liver disease. Best Pract Res Clin Gastroenterol 25: 245-258.
7. Hohenester S, Oude-Elferink RP, Beuers U (2009) Primary biliary cirrhosis. Semin Immunopathol 31: 283-307.
8. Ye SL, Takayama T, Geschwind J, Marrero JA, Bronowicki JP (2010) Current approaches to the treatment of early hepatocellular carcinoma. Oncologist 15: 34-41.
9. Fang B, Shi M, Liao L, Yang S, Liu Y, et al. (2004) Systemic infusion of FLK1(+) mesenchymal stem cells ameliorate carbon tetrachloride-induced liver fibrosis in mice. Transplantation 78: 83-88.
10. Saito T, Okumoto K, Haga H, Nishise Y, Ishii R, et al. (2011) Potential therapeutic application of intravenous autologous bone marrow infusion in patients with alcoholic liver cirrhosis. Stem Cells Dev 20: 1503-1510.
11. Jang YY, Collector MI, Baylin SB, Diehl AM, Sharkis SJ (2004) Hematopoietic stem cells convert into liver cells within days without fusion. Nat Cell Biol 6: 532-539.
12. Alvarez-Dolado M, Martínez-Losa M (2011) Cell fusion and tissue regeneration. Adv Exp Med Biol 713: 161-175.
13. Thorgeirsson SS, Grisham JW (2006) Hematopoietic cells as hepatocyte stem cells: a critical review of the evidence. Hepatology 43: 2-8.
14. Santos JL, Choquette M, Bezerra JA (2010) Cholestatic liver disease in children. Curr Gastroenterol Rep 12: 30-39.
15. Tieppo J, Cuevas MJ, Vercelino R, Tuñón MJ, Marroni NP, et al. (2009) Quercetin administration ameliorates pulmonary complicatalase ions of cirrhosis in rats. J Nutr 139: 1339-1346.
16. Belardinelli MC, Pereira F, Baldo G, Vicente Tavares AM, Kieling CO, et al. (2008) Adult derived mononuclear bone marrow cells improve survival in a model of acetaminophen-induced acute liver failure in rats. Toxicology 247: 1-5.
17. Baldo G, Giugliani R, Uribe C, Belardinelli MC, Duarte ME, et al. (2010) Bone marrow mononuclear cell transplantation improves survival and induces hepatocyte proliferation in rats after CCl(4) acute liver damage. Dig Dis Sci 55: 3384-3399.
18. Masseroli M, Caballero T, O'Valle F, Del Moral RM, Pérez-Milena A, et al. (2000) Automatic quantification of liver fibrosis: design and validation of a new image analysis method: comparison with semi-quantitative indexes of fibrosis. J Hepatol 32: 453-464.
19. Vercelino R, Tieppo J, Dias AS, Marroni CA, Garcia E, et al. (2008)

N-acetylcysteine effects on genotoxic and oxidative stress parameters in cirrhotic rats with hepatopulmonary syndrome. Basic Clin Pharmacol Toxicol 102: 370-376.

20. Ohkawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95: 351-358.

21. Boveris A, Chance B (1973) The mitochondrial generation of hydrogen peroxide. General properties and effect of hiperbaric oxygen. Biochem J 134: 707-716.

22. Misra HP, Fridovich I (1972) The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem 247: 3170-3175.

23. Balestrin RC, Baldo G, Vieira MB, Sano R, Coelho JC, et al. (2008) Transient high-level expression of beta-galactosidase after transfection of fibroblasts from GM1 gangliosidosis patients with plasmid DNA. Braz J Med Biol Res 41: 283-288.

24. Couto BG, Goldenberg RC, da Fonseca LM, Thomas J, Gutfilen B, et al. (2008) Bone marrow mononuclear cell therapy for patients with cirrhosis: a Phase 1 study. Liver Int 31: 391-400.

25. Kisseleva T, Gigante E, Brenner DA (2010) Recent advances in live stem cell therapy. Curr Opin Gastroenterol 26: 395-402.

26. Moritoki Y, Ueno Y, Kanno N, Yamagiwa Y, Fukushima K, et al. (2010) Lack of evidence that bone marrow cells contribute to cholangiocyte repopulation during experimental cholestatic ductal hyperplasia. Liver Int 26: 457-466.

27. Sakaida I, Terai S, Yamamoto N, Aoyama K, Ishikawa T, et al. (2004) Transplantation of bone marrow cells reduces CCl4-induced liver fibrosis in mice. Hepatology 40: 1304-1311.

28. Higashiyama R, Inagaki Y, Hong YY, Kushida M, Nakao S, et al. (2007) Bone marrow-derived cells express matrix metalloproteinases and contribute to regression of liver fibrosis in mice. Hepatology 45: 213-222.

29. Kuo TK, Hung SP, Chuang CH, Chen CT, Shih YR, et al. (2008) Stem cell therapy for liver disease: parameters governing the success of using bone marrow mesenchymal stem cells. Gastroenterology 134: 2111-2121.

30. Tavares AM, da Rosa Araújo AS, Baldo G, Matte U, Khaper N, et al. (2010) Bone marrow derived cells decrease inflammation but not oxidative stress in an experimental model of acute myocardial infarction. Life Sci 87: 699-706.

31. Parekkadan B, van Poll D, Suganuma K, Carter EA, Berthiaume F, et al. (2007) Mesenchymal stem cell-derived molecules reverse fulminant hepatic failure. PLoS One 2: e941.

32. Friedman SL (2008) Hepatic fibrosis - overview. Toxicology 254: 120-129.

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FRESHLY ISOLATED HEPATOCYTE TRANSPLANTATION IN ACETAMINOPHEN-INDUCED HEPATOTOXICITY MODEL IN RATS

Daniela **RODRIGUES**[1, 2], Themis Reverbel da **SILVEIRA**[1, 3], Ursula **MATTE**[3, 4, 5]; The Study Group on Experimental Hepatology

ABSTRACT - ***Context*** - Hepatocyte transplantation is an attractive therapeutic modality for liver disease as an alternative for orthotopic liver transplantation. ***Objective*** - The aim of the current study was to investigate the feasibility of freshly isolated rat hepatocyte transplantation in acetaminophen-induced hepatotoxicity model. ***Methods*** - Hepatocytes were isolated from male Wistar rats and transplanted 24 hours after acetaminophen administration in female recipients. Female rats received either $1x10^7$ hepatocytes or phosphate buffered saline through the portal vein or into the spleen and were sacrificed after 48 hours. ***Results*** - Alanine aminotransferase levels measured within the experiment did not differ between groups at any time point. Molecular analysis and histology showed presence of hepatocytes in liver of transplanted animals injected either through portal vein or spleen. ***Conclusion*** - These data demonstrate the feasibility and efficacy of hepatocyte transplantation in the liver or spleen in a mild acetaminophen-induced hepatotoxicity model.

HEADINGS – Hepatocytes, transplantation. Acetaminophen. Drug-induced liver injury. Rats.

INTRODUCTION

Orthotopic liver transplantation (OLT) has emerged as an effective treatment for acute liver failure (ALF) and end-stage liver disease. However, wider application of this therapy is limited primarily by lack of donors. Therefore other liver support systems, such as extracorporeal systems and cellular therapy are under evaluation[18, 19, 24].

Hepatocyte transplantation has been used as a treatment for liver-based metabolic diseases such as Crigler-Najjar syndrome type I[7], glycogen storage disease type 1a[15], urea cycle defects[9, 14] and congenital deficiency of coagulation factor VII[5]. For ALF, hepatocyte transplantation can be used to bridge patients to whole-organ transplantation, decreasing mortality and avoiding the risks of undertaking a major surgery[6, 17]. Up to a maximum of 5% of normal liver mass can be transplanted into the splenic artery or the portal vein, leading to a reduction in ammonia, bilirubin levels and improvement in hepatic encephalopathy[27].

Acetaminophen (APAP) toxicity is the leading cause of ALF in the United States and United Kingdom, accounting for 46% and 70% of cases, respectively[12, 24]. The mechanism of liver toxicity is well established. The saturation of the normal metabolic pathway to cytochrome P450 metabolism leads to the excessive formation of highly reactive intermediate N-acetyl-p-benzoquinone imine (NAPQI). At therapeutic doses, NAPQI is efficiently detoxified by gluthatione (GSH). In overdose, conjugation of NAPQI with GSH leads to GSH depletion, and NAPQI covalently binds to cysteine residue in proteins to form APAP adducts. These protein adducts are formed from highly reactive species of superoxide and nitric oxide and that increases the permeability of mitochondrial membrane with release of superoxide of the mitochondria, a lethal event for the cell[11, 21].

In the present study, the feasibility of freshly isolated rat hepatocyte transplantation in APAP-induced hepatotoxicity model either injected through the portal vein or into the spleen was investigated.

METHODS

Animals

Male and female Wistar rats at least 8-week-old were kept at Experimental Animal Unit of Research

The present study was performed at Experimental Hepatology and Gastroenterology Laboratory and Gene Therapy Center, Hospital de Clínicas, Porto Alegre, RS, Brazil. [1] Experimental Hepatology and Gastroenterology Laboratory, Hospital de Clínicas de Porto Alegre; [2] Post Graduation Program: Sciences in Gastroenterology, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul (UFRGS); [3] Post Graduation Program in Adolescent and Child Health, UFRGS; [4] Post Graduation Program in Genetics and Molecular Biology, UFRGS; [5] Gene Therapy Center, Hospital de Clínicas de Porto Alegre, RS, Brazil.
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Center of Hospital de Clínicas de Porto Alegre (UEA-HCPA), Porto Alegre, RS, Brazil, under controlled temperature (between 18ºC and 22ºC) in 12-h light-dark cycle. Rats were given standard laboratory rodent chow and water ad libitum. Male rats were used as hepatocyte donors and female rats were used as recipients.

This study was approved by Research and Ethics Committee of HCPA and all procedures were in accordance with Brazilian Law 11794 and Act 6899 of July 15, 2009, that encompasses Brazilian regulation regarding the use of animals for scientific research.

APAP-induced hepatotoxicity model

The APAP-induced hepatotoxicity model was adapted from Salam et al.[24]. An oral solution of APAP at 200 mg/mL concentration (Medley, Brazil) was administered in a single dose of 1 g/kg via intraperitoneal injection in female rats weighing 200-350 g. Four days before the intraperitoneal injection, animals received phenobarbital (Fenocrisis 4%, Brazil) at 350 mg/L in drinking water to induce cytochrome P-450 enzymes to potentiate subsequent APAP-toxicity[2]. Phenobarbital was offered until the last day of the study (day 3). Animals were euthanized at day 3 after APAP administration.

Hepatocyte isolation

Hepatocytes were isolated from male rats weighing 350-500 g by perfusion of liver with modified two-step collagenase technique[25]. In the first step, portal vein was cannulated and inferior vena cava was opened to remove blood with HBSS medium without calcium, magnesium and phenol red (LGC Biotecnologia, Brazil) and EDTA (Nuclear, Brazil) with a 700 mL/h flow controlled by pump infusion. In the second step, superior vena cava was cannulated and inferior vena cava was clamped for perfusion with HBSS medium, 1 M calcium chloride (Vetec, Brazil) and type II collagenase 2% (Gibco, Invitrogen Corporation, USA). Penicillin/streptomycin 1% (Gibco, Invitrogen, USA) was added to all of the above solutions.

Liver fragments obtained were kept in HBSS medium and 10% antibiotics and taken to a laminar flow hood. The fragments were transferred to vials with HBSS medium and decreasing 5% to 2% antibiotics solution. Liver sections were minced and the cell suspension was passed through a gauze mesh, centrifuged 3 times at 800 rpm for 5 minutes and washed in HBSS medium 3 times. In the last washing, hepatocytes were resuspended in phosphate buffered saline (PBS), (Laborclin, Brazil) and cell viability was determined by trypan's blue exclusion.

In a subgroup of 11 randomly chosen animals, cells were stained prior to injection with DAPI (4', 6-diamidino-2-phenylindole, Roche Mannheim, Germany) 2.7 mg/mL to assess the migration pattern of donor hepatocytes according to the different sites of injection (portal vein or spleen)[3].

Hepatocyte transplantation

Hepatocytes ($1x10^7$) were administered 24 hours after APAP-induced hepatotoxicity in female rats. Animals were anesthetized with 50 mg/kg of ketamine (Dopalen, Vetbrands, Brazil) and 20 mg/kg of xilazine (Virbaxyl 2%, Virbac, Brazil) intraperitoneally.

Forty-four female rats were separated in four groups: group PV-H received hepatocyte transplantation through the portal vein (n = 8), group PV-PBS received PBS through the portal vein (n = 15), group SPL-H received hepatocyte transplantation into the spleen (n = 12), and group SPL-PBS received PBS into the spleen (n = 9). In animals that received the injection into the portal vein, a longitudinal 3 cm abdominal incision was performed, and a volume of 1 mL of PBS (containing or not $1x10^7$ hepatocytes) was slowly injected into the portal vein. Gelfoam (Cuntanplast, Mascia Brunelli Spa, Milano, Italy) was applied to the site of injection in order to avoid bleeding. In animals that received injection into the spleen, a vertical 2 cm abdominal incision below left costal edge was performed and the spleen was exposed. A volume of 1 mL of PBS (containing or not-hepatocytes) was slowly injected into the spleen pulp. Rats were sutured in layers with polyglactine 4-0 (Vycril Ethicon, São Paulo, Brazil) and in the skin with mononylon 4-0 (Monocryl Ethicon, São Paulo, Brazil). Animals recovered in the cage.

Biochemistry

Blood was collected at time point 0 (before APAP administration), and at days 1 and 3 after APAP administration through retrorbital punction under anesthesia. Samples for alanine aminotransferase (ALT) were centrifuged at 4000 rpm for 5 minutes, and stored at -20°C. ALT was measured using ultraviolet enzyme method at Roche/Hitachi ACN 685E equipment.

Histology

Animals that survived until day 3 were sacrificed in CO_2 chamber. Liver and spleen samples were collected and placed in 10% buffered formalin solution for 24 hours. Unstained paraffin embedded sections were observed under fluorescent microscopy and the number of DAPI-positive cells was counted in 100 high power fields. In addition, spleen slides were stained with H-E to visualize injected hepatocytes.

Molecular analysis

To detect the presence of donor cells, DNA was extracted with Trizol (Invitrogen, USA) from liver and spleen samples and PCR for *Sry* gene was performed using the following primers at 50ºC (for 5' AAGCGCCCCATGAATGCATT 3', rev 5' CAGCTGCTTGCTGATCTCTG 3'). PCR products were visualized in 1.5% agarose gels stained with ethidium bromide[1].

In a subgroup of eight animals from group SPL-H spleen samples were snap-frozen in liquid nitrogen and stored at -80ºC to perform reverse-transcriptase polymerase chain reaction (RT-PCR) for albumin expression. Total RNA was extracted using a commercial kit (Easy RNA, Qiagen, Germany) following the manufacturer's instructions. Conversion to cDNA was performed using Superscript II (Invitrogen, USA) from 3 g of RNA. RT-PCR was performed using the

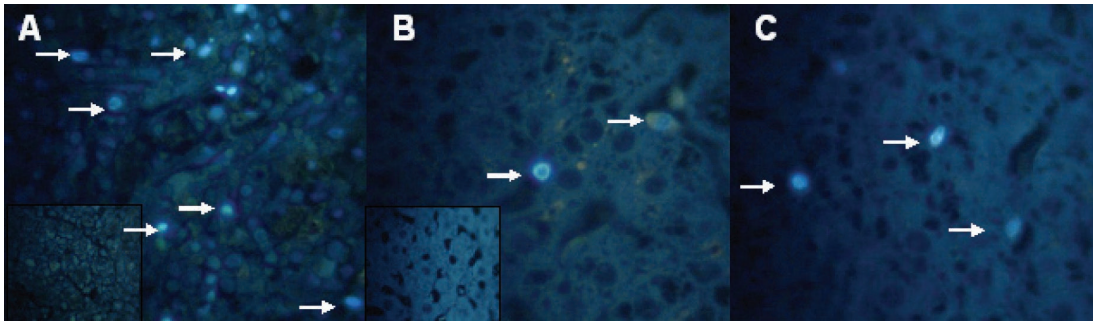

FIGURE 1. DAPI-positive cells (arrows) in spleen (A) and liver (B) of animals injected into the spleen or into the portal vein (C). Insets: DAPI negative images

following primers at 42°C for 40 cycles (for 5' TTGTATGAATATGCAAGAAG 3', rev 5' CACTCTTCCCAGGTTTCTTG 3'). PCR products were visualized in 1.5% agarose gel stained with ethidium bromide. RNA extracted from liver samples was used as positive control.

Gapdh was used as positive internal control both for RT-PCR and PCR reactions. Primers for *Gapdh* were: for 5' CAGCAATGCATCCTGCAC 3', rev 5' GAGTTGCTGTTGAAGTCACAGG 3').

Statistics

Statistics was performed using SPSS 12.0 (Statistical Package for Social Science). ALT levels were expressed as the median ± quartiles. Differences in ALT levels were assessed using Friedman's test.

RESULTS

APAP-induced hepatotoxicity model

At day 1, ALT levels increased with statistical significance in all 44 animals by Friedman's test (P<0.0001). At day 3, ALT levels returned to basal values in all groups, without difference among the groups (Table 1).

TABLE 1. Median and range of ALT levels in rats submitted to APAP-induced hepatotoxicity

Group (n)	Time (days)		
	0	1*	3
PV-H	42.0 (37.0-54.0)	267.0 (140-543.8)	43.5 (34.0-62.0)
PVPBS	36.0 (32.0-43.0)	130.0 (103.0-215.0)	39.0 (27.0-51.0)
SPL-H	57.5 (49.8-61.0)	197.5 (102.8-403.8)	38.5 (34.0-61.8)
SPL-PBS	43.5 (35.3-47.0)	158.0 (84.03-02.0)	31.0 (28.0-34.0)

*Values at day 1 are statistically different from days 0 and 3 for all groups (Friedman's test, P<0.0001) but not among groups at any time point

Hepatocyte transplantation and survival analysis

Hepatocytes were isolated from male Wistar rats' livers weighing from 15 g to 20 g. The number of isolated hepatocytes ranged between $2.54x10^7$ cells/mL and $2.22x10^8$ cells/mL. Viability of hepatocytes was >90% by trypan's blue exclusion test, and $1x10^7$ hepatocytes were transplanted.

Survival analyses showed that 39 animals survived until the end of experiment (day 3). All animals that received hepatocytes (groups PV and SPL) survived until day 3, however five rats that received PBS through the portal vein died, three on day 1 and two on day 2.

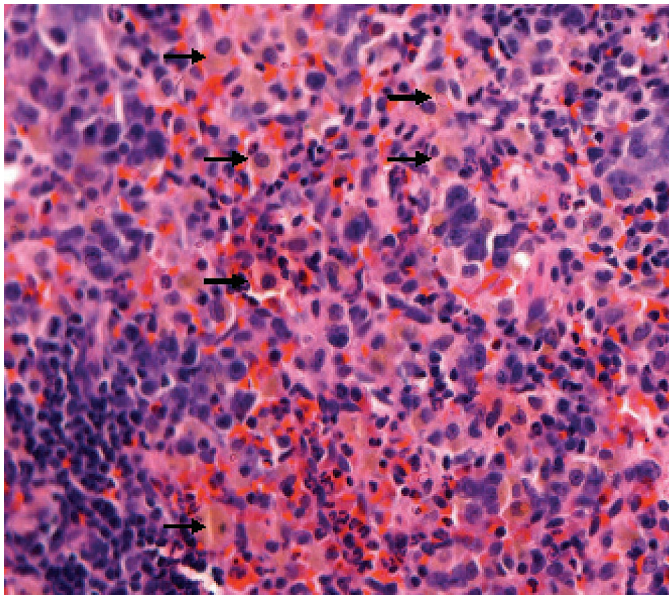

FIGURE 2. H-E staining of the spleen, showing scattered hepatocytes (arrows), magnification 40X

Histology

The presence of transplanted cells was determined by DAPI-staining prior to injection. The number of DAPI-positive hepatocytes in the liver of animals injected through the portal vein ranged from 12 to 130, with a mean of 61.2 ± 44.6 stained cells per animal. In animals injected through the spleen, the number of DAPI-positive hepatocytes in the liver ranged from 0 to 81, with a mean of 50.0 ± 31.8 stained cells per animal. In these animals, the number of DAPI-positive hepatocytes in the spleen ranged from 75 to 414, with a mean of 166.2 ± 127.3 stained cells per animal. The presence of hepatocytes in spleen was also visualized on H-E slides (Figures 1 and 2).

Molecular analysis

The engraftment of hepatocytes at day 3 in liver and spleen by PCR analysis for Y-chromosome gene (*Sry*) in

female recipient organs was investigated. *Sry* was positive in livers or spleens from animals injected through the portal vein or into the spleen.

In order to determine if spleen transplanted hepatocytes remained functional albumin expression was analyzed in the spleen of eight animals from group SPL-H. Albumin expression by RT-PCR was not detected in any animal, although all of them were positive for *Gapdh* and liver samples used for positive control were also positive.

DISCUSSION

Hepatocyte transplantation presents many advantages over OLT. For instance, cells from a single liver donor can be used by many recipients[8]. In our experience, a male donor rat could provide fresh hepatocytes for at least 10 female recipients. Freshly isolated hepatocytes have been shown to perform better than cryopreserved cells in many studies[4, 10], and hepatocytes are known to be very sensitive to freezing damage, decreasing their attachment efficiency and engraftment[28]. Therefore, this study aimed to test fresh hepatocyte transplantation in a rat model of APAP-induced hepatotoxicity.

In APAP-induced hepatotoxicity in humans, ALT levels start to increase from day 1, peak at day 3 and start to decrease at day 4[22]. In our study, animals had the ALT levels peak at day 1, started to decrease at day 2 and had returned to basal values at day 3. Lee et al.[12], analyzing 1,147 cases of APAP-induced ALF in humans demonstrated that ALT levels are very high (2,138 to 6,731). However, in APAP-induced hepatotoxicity in rats ALT levels are lower in comparison with humans, around of 256.5 ± 11.2[23]. That finding is similar to our results, 188.5 (116.7-330.7) at day 1, using a single dose of APAP 1g/kg i.p.. Even though, high mortality rates were not observed and no signs of extensive liver damage were seen by ALT or histology on day 3.

Hepatocytes can be transplanted directly into the liver or through the portal vein and in the spleen pulp. Although the optimal site for hepatocyte infusion and settlement has not yet been determined, the liver seems to be the natural home of transplanted hepatocytes[13]. The spleen is currently the most widely used recipient site for hepatocyte infusion because of its accessibility. Although separated from the portal blood flow, donor hepatocytes transplanted into the spleen have been shown to migrate to other organs, particularly to the host liver[26]. Whether the number of engrafted cells in the spleen and their metabolic capacity will be sufficient remains an open question[17].

In this study, the engraftment of hepatocytes in liver and spleen was investigated by PCR analysis for Y-chromosome gene (*Sry*) in female recipient organs and staining the transplanted cells with DAPI. In all hepatocyte transplantation groups, *Sry* was positive in liver tissue. This shows that hepatocytes transplanted into the spleen have migrated to the host liver, a finding that is also supported by DAPI-positive hepatocytes in the liver from six animals transplanted into the spleen.

No differences were found in the number of DAPI-positive hepatocytes in the liver of animals injected into the portal vein or into the spleen. This lack of difference in the number of DAPI-positive cells is not easily explained. One would assume that the liver of rats injected through the portal vein would present a greater number of hepatocytes, however some studies suggest that a space for cell engraftment is necessary in the recipient[26]. In this study partial hepatectomy or hepatic irradiation were not performed, and maybe the number of dead cells at the time of injection was not sufficient to create a permissive environment for hepatocyte engraftment. Nevertheless, these animals had some degree of lesion, as hepatocytes did remain in the spleen, and it has been suggested that this is required for the establishment of transplanted hepatocytes in rat spleens[20].

A second point to be considered for spleen hepatocyte injection is whether they remain functioning in the spleen. Shibata et al.[26] showed signals of Y-chromosome detectable in the spleen and spleen-resident hepatocytes expressing albumin 12 weeks after intrasplenic transplantation of freshly isolated hepatocytes in rats. In the data presented here, even though *Sry* was positive in spleen, albumin expression was not detected in a subgroup of eight animals injected into the spleen. One possible explanation for this finding is that, as fresh hepatocytes have a scattered pattern of distribution within the spleen[26], when spleen samples were collected for RT-PCR for albumin expression, hepatocytes could have been lost. On the other hand, the hypothesis that hepatocytes in the spleen underwent a dedifferentiation process caused by the loss of intercellular contact with neighbouring host cells, decreasing specific hepatocyte genes transcription[16], cannot be ruled out.

In conclusion, data presented here shows the feasibility and efficacy of hepatocyte transplantation in the liver or spleen in a mild APAP-induced hepatotoxicity model.

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Rodrigues D, Silveira TR, Matte U; Grupo de Estudos de Hepatologia Experimental. Transplante de hepatócitos recém-isolados em um modelo de hepatotoxicidade induzida por acetaminofeno em ratos. Arq Gastroenterol. 2012,49(4):291-5.

RESUMO - ***Contexto*** - O transplante de hepatócitos é uma modalidade terapêutica atrativa para doenças hepáticas como alternativa ao transplante hepático ortotópico. ***Objetivo*** - Investigar a factibilidade do uso de hepatócitos frescos isolados de ratos em um modelo de hepatotoxicidade induzida por paracetamol. ***Métodos*** - Hepatócitos foram isolados de ratos Wistar machos e transplantados 24 horas após a administração de paracetamol em receptores fêmeas. As ratas receberam $1x10^7$ hepatócitos ou tampão salina fosfato pela veia porta ou no baço e foram sacrificadas após 48 horas. ***Resultados*** - Os níveis de alanina aminotransferase medidos durante o experimento não diferiram entre os grupos em nenhum momento. Análises moleculares e histológicas demonstraram a presença de hepatócitos no fígado dos animais transplantados pelo baço ou pela veia porta. ***Conclusão*** - Os dados indicam a factibilidade e eficácia do transplante de hepatócitos no fígado ou baço em um modelo de hepatotoxicidade leve induzida por paracetamol.

DESCRITORES – Hepatócitos, transplante. Acetaminofeno. Doença hepática induzida por drogas. Ratos.

REFERENCES

1. Baldo G, Giugliani R, Uribe C, Belardinelli MC, Duarte ME, Meurer L, da Silveira TR, Matte U. Bone marrow mononuclear cell transplantation improves survival and induces hepatocyte proliferation in rats after CCl(4) acute liver damage. Dig Dis Sci. 2010;55:3384-92.
2. Belardinelli MC, Pereira F, Baldo G, Vicente Tavares AM, Kieling CO, da Silveira TR, Meurer L, Soares Duarte ME, Giugliani R, Matte U. Adult derived mononuclear bone marrow cells improve survival in a model of acetaminophen-induced acute liver failure in rats. Toxicology. 2008;247:1-5.
3. Chedrawy EG, Wang JS, Nguyen DM, Shum-Tim D, Chiu RC. Incorporation and integration of implanted myogenic and stem cells into native myocardial fibers: anatomic basis for functional improvements. J Thorac Cardiovasc Surg. 2002;124:584-90.
4. David P, Alexandre E, Audet M, Chenard-Neu MP, Wolf P, Jaeck D, Azimzadeh A, Richert L. Engraftment and albumin production of intrasplenically transplanted rat hepatocytes (Sprague-Dawley), freshly isolated versus cryopreserved, into Nagase analbuminemic rats (NAR). Cell Transplant. 2001;10:67-80.
5. Dhawan A, Mitry RR, Hughes RD, Lehec S, Terry C, Bansal S, Arya R, Wade JJ, Verma A, Heaton ND, Rela M, Mieli-Vergani G. Hepatocyte transplantation for inherited factor VII deficiency. Transplantation. 2004;78:1812-4.
6. Dhawan A, Mitry RR, Hughes RD. Hepatocyte transplantation for liver-based metabolic disorders. J Inherit Metab Dis. 2006;29:431-5.
7. Fox IJ, Chowdhury JR, Kaufman SS, Goertzen TC, Chowdhury NR, Warkentin PI, Dorko K, Sauter BV, Strom SC. Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation. N Engl J Med. 1998;338:1422-6.
8. Grompe M, Laconi E, Shafritz DA. Principles of therapeutic liver repopulation. Semin Liver Dis. 1999;19:7-14.
9. Horslen SP, McCowan TC, Goertzen TC, Warkentin PI, Cai HB, Strom SC, Fox IJ. Isolated hepatocyte transplantation in an infant with a severe urea cycle disorder. Pediatrics. 2003;111:1262-7.
10. Jamal HZ, Weglarz TC, Sandgren EP. Cryopreserved mouse hepatocyte retain regenerative capacity in vivo. Gastroenterology. 2000;118:390-4.
11. Kaplowitz N. Acetaminophen hepatoxicity: what do we know, what don't we know, and what do we do next? Hepatology. 2004;40:23-6.
12. Lee WM, Squires RH Jr, Nyberg SL, Doo E, Hoofnagle JH. Acute liver failure: summary of a workshop. Hepatology. 2008;47:1401-15.
13. Michalopoulos GK. Liver regeneration: molecular mechanisms of growth control. FASEB J. 1990;4:176-87.
14. Mitry RR, Dhawan A, Hughes RD, Bansal S, Lehec S, Terry C, Heaton ND, Karani JB, Mieli-Vergani G, Rela M. One liver, three recipients-segment. IV from split-liver procedures as a source of hepatocytes for cell transplantation. Transplantation. 2004;77:1614-6.
15. Muraca M, Gerunda G, Neri D, Vilei MT, Granato A, Feltracco P, Meroni M, Giron G, Burlina AB. Hepatocyte transplantation as a treatment for glycogen storage disease type 1a. Lancet. 2002;359:317-8.
16. Nicoluzzi JEL, Repka JCD, Rocha LCA, Campos ACL, Caron PE, Parc R. Assessment of bioartificial liver using human hepatocytes immunoprotected by macroencapsulation. Rev Col Bras Cir. 2004;31:311-7.
17. Nussler A, Konig S, Ott M, Sokal E, Christ B, Thasler W, Brulport M, Gabelein G, Schormann W, Schulze M, Ellis E, Kraemer M, Nocken F, Fleig W, Manns M, Strom SC, Hengstler JG. Present status and perspectives of cell-based therapies for liver disease. J Hepatol. 2006;45:144-59.
18. O'Grady JG. Acute liver failure. Postgrad Med J. 2005;81:148-54.
19. Polson J, Lee WM; American Association for the Study of Liver Disease. AASLD position paper: the management of acute liver failure. Hepatology. 2005;41:1179-97.
20. Ponder KP, Gupta S, Leland F, Darlington G, Finegold M, Demayo J, Ledley FD, Chowdhury JR, Woo SLC. Mouse hepatocytes migrate to liver parenchyma and function indefinitely after intransplenic transplantation. Proc Natl Acad Sci USA. 1991;88:1217-21.
21. Reid AB, Kurten RC, McCullough SS, Brock RW, Hinson JA. Mechanisms of acetaminophen-induced hepatotoxicity: role of oxidative stress and mitochondrial permeability transition in freshly isolated mouse hepatocytes. J Pharmacol Exp Ther. 2005;312:509-16.
22. Rumack BH. Acetaminophen misconceptions. Hepatology. 2004;40:10-5.
23. Salam OMEA, Baiuomy AR, El-Shenawy SM, Hassan NS. Effect of pentoxifylline on hepatic injury caused in the rat by the administration of carbon tetrachloride or acetaminophen. Pharmacol Res. 2005;57:596-603.
24. Sass DA, Shakil AO. Fulminant hepatic failure. Liver Transpl. 2005;11:594-605.
25. Seglen PO. Preparation of isolated rat liver cells. Methods Cell Biol. 1976;13:29-83.
26. Shibata C, Mizuguchi T, Kikkawa Y, Nobuoka T, Oshima H, Kawasaki H, Kawamoto M, Katsuramaki T, Mitaka T, Hirata K. Liver repopulation and long-term function of rat small hepatocyte transplantation as an alternative cell source for hepatocyte transplantation. Liver Transpl. 2006;12:78-87.
27. Smets F, Najimi M SF, Sokal EM. Cell transplantation in the treatment of liver diseases. Pediatr Transplant. 2008;12:6-13.
28. Terry C, Dhawan A, Mitry RR, Hughes RD. Cryopreservation of isolated human hepatocytes for transplantation: state of the art. Cryobiology. 2006;53:149-59.

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Cytotoxic effect of Amphotericin B in a myofibroblast cell line

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ABSTRACT

In this study we investigate whether Amphotericin B (AmB), a widely used antifungal agent, could decrease the proliferation of a myofibroblast cell line – GRX, a model of activated hepatic stellate cells (HSC). Three different hepatic cell lines (GRX, Hep G2 and ARL-6) were treated with two concentrations of AmB (1.25 μg/mL or 2.50 μg/mL). Cytotoxicity was assessed by MTT assay. The effects of AmB on GRX migration was evaluated by Wound-healing Assay. Cell cycle arrest was investigated by flow cytometry. Apoptosis and autophagy were analyzed by Caspase 3 and LC3 immunostaining, respectively. Treatment with AmB 1.25 or 2.50 μg/mL showed a decrease in viability of GRX cells. This decrease was not observed for Hep G2 or ARL-6 in any of the two AmB concentrations tested. GRX cells treated with 1.25 μg/mL AmB were unable to close the wound after 96 h. Cell cycle analysis showed an increase in sub-G1 population and a decrease in G2/M population in AmB-treated cells. In addition, AmB-treated GRX cells showed increased expression of LC-3 and Caspase-3 by immunohistochemistry, suggesting an increase in both autophagy and apoptosis. Here we show that AmB is cytotoxic for GRX cells, a model of activated HSC, but not for hepatic lineages HepG2 and ARL6.

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1. Introduction

Continuous liver injury can lead to changes in the extracellular matrix (ECM) resulting in a pathological state known as fibrosis. Hepatic fibrosis is characterized by an accumulation of type I collagen secreted by Hepatic Stellate Cells (HSC) (Friedman, 2008) and is mediated by a number of factors including viral infection, genetic disease and/or xenobiotic-induced damage. In the normal liver, HSC reside in a quiescent state, store vitamin A, modulate the microcirculation and regulate ECM production. Following injury, HSC transdifferentiate into activated myofibroblast-like cells characterized by loss of vitamin A droplets, increased alpha smooth muscle actin expression (α-SMA) and decreased sinusoidal blood flow. Continued HSC transdifferentiation leads to collagen deposition, persistent activation, increased proliferation and diminished responsiveness to apoptotic stimuli (Novo et al., 2006).

Reversal of hepatic fibrosis has been reported and is dependent, at least in part, on the induction of HSC apoptosis (Elsharkawy et al., 2005). Thus HSC seems to be an excellent therapeutic target for reduction of fibrosis in liver injury.

Amphotericin B (AmB) is a polyene macrolide antibiotic widely used for treating systemic fungal infections, visceral leishmaniasis (Chattopadhyay and Jafurulla, 2011) and to avoid fungal contamination in tissue culture (Mathieson et al., 2011). AmB interacts with sterols and, specifically in mammalian cell membrane, with cholesterol, forming trans-membrane channels that disturb the membrane barrier function resulting in the efflux of electrolytes from the cytosol. The disruption of membrane pumps and membrane potential induces apoptosis (Dentinger et al., 2001). At high doses, essential cytoplasmic components, especially proteins, are released to the extracellular milieu, which leads to further apoptotic destruction of the cell. Cell shrinkage and reduction of intracellular electrolytes, particularly potassium ions,

Abbreviations: AmB, Amphotericin B; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's Modified Eagle's Medium; ECM, extracellular matrix; HSC, Hepatic Stellate Cells; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltet-razoliumbromide; PBS, Phosphate Buffered Saline; α-SMA, alpha smooth muscle actin.

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are accompanied by DNA fragmentation and caspase 3–like activity (Chen et al., 2013).

AmB is also used in cell culture to treat or prevent fungal infections (Mathieson et al., 2011). An empiric observation made at our laboratory suggested that GRX cells were affected by AmB treatment. The purpose of this study was to further investigate the anti-proliferative effect of AmB on GRX cells, a cell line of activated HSC.

2. Materials and methods

2.1. Cell culture

GRX cell line (clone 123) was obtained from the Rio de Janeiro Cell Bank (Bio-Rio, Rio de Janeiro, Brazil). The cell line GRX are HSC derived from a granuloma of *Schistosoma mansoni* infected mice and are considered a model of activated myofibroblasts (Souza et al., 2008). The culture was maintained in Dulbecco's Modified Eagle's Medium (DMEM, LGC Biotecnologia, Brazil) supplemented with 5% fetal calf serum (GIBCO, USA) and 1% penicillin/streptomycin (GIBCO, USA), herein referred to as "culture medium", at 37 °C in the presence of 5% CO_2.

Hepatocarcinoma cell lines from human (HepG2, Bio-Rio, Rio de Janeiro, Brazil) and mice (ARL6, ATCC, USA) were used as controls to determine the effect of AmB on hepatocyte cell lines. Both cells were kept at the same conditions as described above, except for 10% fetal calf serum.

2.2. Cytotoxicity

Cells were seeded in 96-well culture plates at a concentration of 1×10^4/well in culture medium 24 h prior to treatment. This concentration was chosen to maintain the same cellular density used in all other experiments. In the treated group, the culture medium was removed and GRX, HepG2 and ARL-6 were treated with culture medium supplemented with AmB (GIBCO, UK) at 1.25 μg/mL or 2.50 μg/mL for additional 24 h. AmB was dissolved directly in the culture medium. The control group was maintained on medium without AmB. Four experimental replicates were made per group with 16 wells per treatment. Cytotoxycity was assessed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT, USB, USA), as described by (Fraga et al., 2008). Briefly, the culture medium was removed and MTT (0.5 mg/mL in DMEM) was added to each well and incubated for 4 h at 37 °C. After lysing cells with 100 μL DMSO, cytotoxicity was assessed by measuring absorbance at 570 nm using a microplate reader (Anthos2010, Anthos Labtec, Austria). The calculation of the cell viability was performed considering the control group as 100%.

2.3. Wound-healing assay

The GRX cells were seeded in 6-well culture plates at a concentration 1×10^5/well and grown to 80% confluence in culture medium for 24 h prior to treatment. Four experimental replicates were made per group with 6 wells per treatment. The culture medium was removed and the monolayer was wounded by scratching the plate with a sterile 10 μL micropipette tip, washed with sterile PBS (Phosphate Buffered Saline) and then treated with culture medium supplemented with 1.25 μg/mL AmB. The control group was maintained on medium without AmB. Cells were photographed under 10 × objective lens every 24 h for 96 h.

2.4. Cell cycle

For analysis of cell cycle phase distribution, 1×10^5 cells were plated onto a 6 well plate and after 24 h the medium was changed for medium supplemented with 1.25 μg/mL AmB for another 24 h. Four experimental replicates were made per group with 6 wells per treatment. The control group was maintained on medium without AmB. Afterwards, the cells were trypsinized, collected, and fixed with 70% ethanol for 30 min. The fixed cells were washed with cold PBS, resuspended in 1 mL of PBS containing 1 μg/mL RNase A (R4642, Sigma, USA) and 50 μg/mL propidium iodide (P4864, Sigma, USA), and then incubated in the dark for 30 min at room temperature. Finally, the cells were analyzed by flow cytometry (Attune, Life Technologies, USA). The percentage of cells in G1, S, and G2/M phase of cell cycle and the percentage of cells in sub-G1 (apoptosis) peak were calculated by eliminating the debris effect using Attune software (Life Technologies, USA).

2.5. LC-3 and Caspase 3 stain

In order to better understand the mechanism of action of AmB we investigated the expression of LC-3 (as autophagy marker) and Caspase 3 (to investigate apoptosis). For that, 1×10^5 cells were plated onto a 6 well plate and after 24 h the medium was changed for culture medium supplemented with 1.25 μg/mL AmB. The control group was maintained on medium without AmB. Twenty-four hours after treatment, the cells were fixed with 4% paraformaldehyde and permeabilized with methanol in ice for 15 min. After incubation for 1 h with primary antibodies (1:300) for LC3 and Caspase 3 (H-50 and H-277 respectively, Santa Cruz Biotechnology, USA) and washing with PBS, cells were incubated for 1 h with FITC-conjugated (1:400) or TexRed-conjugated (1:300) secondary antibody (F-0382, Sigma, USA), washed with PBS and mounted. Nuclei were stained by 4′,6-diamidino-2-phenylindole (DAPI) (1.5 μg/mL) included in the mounting medium (F6057, Sigma, USA and J2909 Santa Cruz Biotechnology, USA; respectively). Fluorescent labeling was studied under epifluorescence microscope (Olympus-BX 51, Tokyo, Japan) equipped with a digital camera using a plan-neofluar (Olympus-DP 71, Tokyo, Japan) objective (40× for overview images). Four experimental replicates were made per group with 6 wells per treatment.

2.6. Statistical analysis

Data were analyzed using SPSS (Statistical Package for Social Sciences, New York, USA) version 14.0. Comparison among groups was performed using ANOVA and Tukey as post hoc test for cell viability. For cell cycle analysis Student's t test was used. A $p < 0.05$ was considered statistically significant. The project was approved by our local ethics committee.

3. Results

3.1. Cytotoxicity

Treatment with AmB (1.25 μg/mL and 2.50 μg/mL) for 24 h showed a significant toxicity for GRX cells (Fig. 1) in a concentration-dependent manner, with viability levels decreasing to 67.2% and 30.8% ($p \leqslant 0.001$), compared to control. Interestingly, although HepG2 viability decreased to around 80% compared to control ($p > 0.05$), there was no difference in toxicity between the two concentrations of AmB. ARL6, on the other hand, seemed to be almost unaffected by AmB treatment.

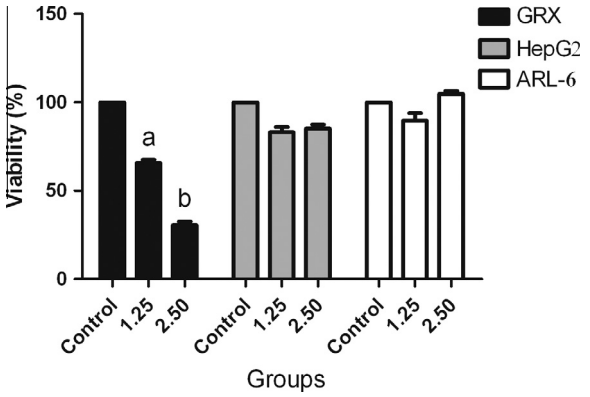

Fig. 1. MTT assay showing viability of GRX, HepG2 and ARL6 cells after treatment with AmB (1.25 μg/mL and 2.50 μg/mL) for 24 h. GRX cells decrease in cell viability significantly more than HepG2 or ARL6 cells compared to control ($a = p < 0.01$ compared to control, and $b = p < 0.01$ compared to 1.25 μg/mL). Bars correspond to standard deviations. ANOVA and Tukey test post hoc ($n = 4$/group).

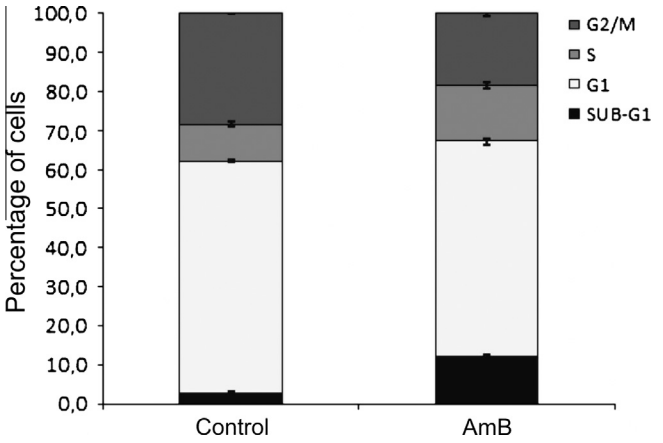

Fig. 3. Cell-cycle analysis on GRX cells treated with 1.25 μg/mL AmB for 24 h compared to control cells. Values show the percentage of total culture into distinct cell-cycle phases ($n = 4$/group). Statistically significant differences were found between sub-G1 (12% versus 2.8%, $p < 0.009$) and G2/M (18.5% versus 14%, $p < 0.03$) populations.

3.2. Wound-healing assay

Based on the results from MTT assay, all subsequent studies were performed only with GRX cells and AmB 1.25 μg/mL. At this concentration, AmB showed a suppressive effect on wound-healing of GRX cells (Fig. 2). After 96 h, control GRX cells were able to visibly reduce the wound width. AmB treated cells, however, despite some migration and/or proliferation were unable to close the wound.

3.3. Cell cycle

The effect of 1.25 μg/mL AmB on GRX cells was also assessed by flow cytometry. Cell cycle analysis showed an increase in sub-G1 population (12% versus 2.8%, $p < 0.009$), and a decrease in G2/M population (18.5% versus 14%, $p < 0.03$) in AmB-treated cells when compared with control (Fig. 3).

3.4. LC-3 and Caspase 3 stain

Twenty-four hours after treatment with 1.25 μg/mL AmB, GRX cells showed increased expression of LC-3 and Caspase-3 by immunohistochemistry when compared with control GRX cells (Fig. 4).

4. Discussion

HSC activation is the main step that leads to hepatic fibrosis. This activation involves changes in the liver cells, described as proliferation, contractility, chemotaxis, matrix degradation, and cytokine release (Friedman, 2008). The GRX cell line, when maintained under standard culture conditions, presents a myofibroblast-like phenotype, similar to activated HSC, (Guimarães et al., 2006; Guma et al., 2001). Thus it can be used as a tool for studying therapies targeting activated HSC.

In this study we observed the effect of AmB on GRX cell viability. AmB is widely used in tissue culture as antifungal agent at 2.50 μg/mL (Mathieson et al., 2011). However, at this concentration, AmB resulted in high mortality of GRX cells, with only 30.8% viability, making it difficult to use this concentration for subsequent assays. When the concentration of AmB was reduced to half (1.25 μg/mL), cell viability was increased to 67.2%. It is interesting to note that AmB was cytotoxic only for GRX cells but not for HepG2 or ARL6, two hepatic cell lines. This contrast may be due to differences in the composition of cell membranes, which is important to the formation of AmB/cholesterol complexes. It has been suggested that the distribution of cholesterol and lipid composition in certain regions of the plasma membrane facilitate

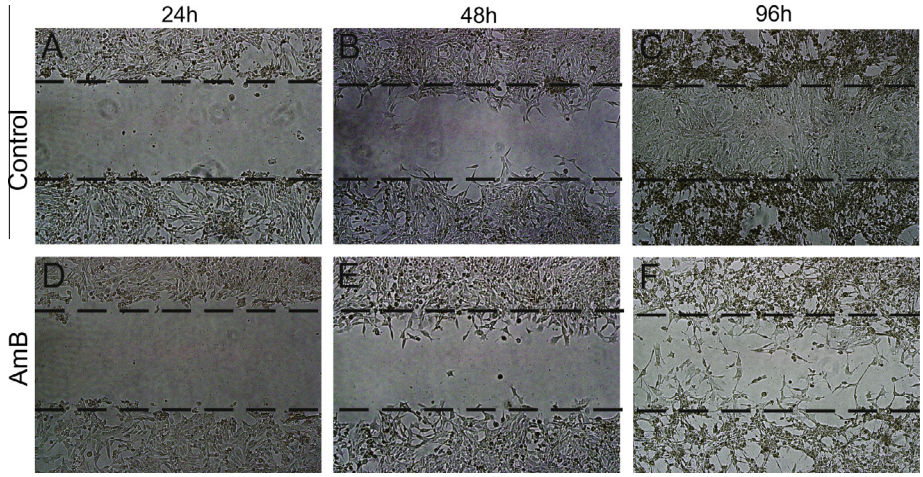

Fig. 2. Effect of 1.25 μg/mL AmB on GRX cell wound-healing assay. Cells were assessed at 24, 48 and 96 h after treatment (10× magnification), ($n = 4$/group).

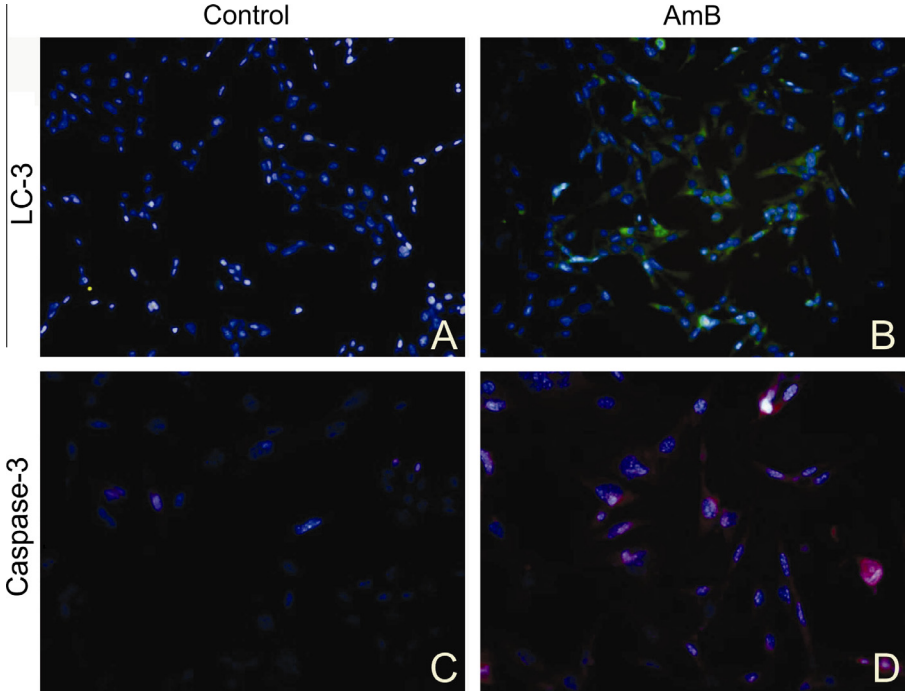

Fig. 4. Immunofluorescence for LC-3 and Caspase-3. GRX treated with 1.25 μg/mL exhibit increased expression of LC-3 (B) and Caspase 3 (D) when compared to control cells (A and C). LC-3 (FITC, Green), 20×. Caspase-3 (TexRed, red), 40× GRX cells nuclei (DAPI, Blue), (n = 4/group). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the insertion of AmB molecules in ordered saturated membrane domains (Czub and Baginski, 2006).

Other studies have also demonstrated that some mammal cells are more susceptible than others to this drug (Andersson et al., 2006; Romero et al., 2009). Binet and Bolard (1988) showed that AmB strongly increases hepatocytes' plasma membrane permeability to monovalent cations, with the major part of the intracellular potassium leaking from the intracellular space within in a few seconds. The loss of K^+ is only partially compensated by the entry of Na^+ ions. At the same time, no modification of internal free Ca^{++}, as well as total Calcium content, is detected in the hepatocytes. These results suggest that the permeabilization of the hepatic plasma membrane by AmB is specific for the monovalent cations, and especially for Potassium. This event is generally preceded by an increase in cytosolic Ca^{++} which stimulates intracellular proteases. Another mechanism of action is mediated by the formation of two types of ionic channels: nonaqueous channels and aqueous pores, differing in permeability properties (Romero et al., 2009).

We then investigated the effects of AmB in cell migration using wound healing assay. Hepatic stellate cells are activated in response to liver injury promoting tissue repair. This response involves cell mobilization and migration towards the lesion (da Silva et al., 2003). Our data show that AmB decreased migration of GRX cells, as even after 96 h treated cells were not able to fully close the wound. This result is interesting because inhibition of HSC migration could indicate a decrease in the activation of these cells.

Together with the results of the wound healing, the evaluation of cell cycle, apoptosis and autophagy confirmed that Amb has an effect on GRX cell line. Thus our results also showed that AmB induced changes in cell cycle through an increase in the sub-G1 phase and diminution in the G2 / M phase compared whit control. As cells in sub-G1 were regarded as apoptotic, the ability of AmB to modulate apoptosis was studied by the expression of Caspase 3. After treatment, AmB induced an increase in the number of GRX expressing Caspase 3indicating stimulation of apoptosis. Autophagy was also induced by AmB as seen by the increased expression LC3 (autophagy).

In brief, this study showed that, *in vitro*, AmB is able to selectively induce cytotoxicity of GRX cells, a model of HSC, without toxicity to liver parenchymal cells. The specific mechanism involved in this selectivity must be elucidated in further studies, as well as whether this effect can be replicated *in vivo* despite the known toxic effects of AmB.

Conflict of interest

There are no conflicts or competing interests to declare.

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References

Andersson, B., Janson, V., Behnam-Motlagh, P., Henriksson, R., Grankvist, K., 2006. Induction of apoptosis by intracellular potassium ion depletion: using the fluorescent dye PBFI in a 96-well plate method in cultured lung cancer cells. Toxicol. In Vitro 20 (6), 986–994.

Binet, A., Bolard, J., 1988. Recovery of hepatocytes from attack by the pore former amphotericin B. Biochem. J. 253 (2), 435–440.

Chattopadhyay, A., Jafurulla, M., 2011. A novel mechanism for an old drug: amphotericin B in the treatment of visceral leishmaniasis. Biochem. Biophys. Res. Commun. 416 (1–2), 7–12.

Chen, L.Y., Sheu, M.T., Liao, C.K., Tsai, F.C., Kao, W.Y., Su, C.H., 2013. Taiwanofungus camphoratus (Syn Antrodia camphorata) extract and Amphotericin B exert adjuvant effects via mitochondrial apoptotic pathway. Integr. Cancer Ther. 12 (2), 153–164.

Czub, J., Baginski, M., 2006. Modulation of amphotericin B membrane interaction by cholesterol and ergosterol–a molecular dynamics study. J. Phys. Chem. B 110 (33), 16743–16753.

da Silva, F.M., Guimarães, E.L., Grivicich, I., Trindade, V.M., Guaragna, R.M., Borojevic, R., Guma, F.C., 2003. Hepatic stellate cell activation in vitro: cell

cycle arrest at G2/M and modification of cell motility. J Cell Biochem. 90 (2), 387–396.
Dentinger, P.J., Swenson, C.F., Anaizi, N.H., 2001. Stability of amphotericin B in an extemporaneously compounded oral suspension. Am. J. Health Syst. Pharm. 58 (11), 1021–1024.
Elsharkawy, A.M., Oakley, F., Mann, D.A., 2005. The role and regulation of hepatic stellate cell apoptosis in reversal of liver fibrosis. Apoptosis 10 (5), 927–939.
Fraga, M., Laux, M., Dos Santos, G.R., Zandoná, B., Dos Santos Giuberti, C., de Oliveira, M.C., da Silveira, M.U., Teixeira, H.F., 2008. Evaluation of the toxicity of oligonucleotide/cationic nanoemulsion complexes on Hep G2 cells through MTT assay. Pharmazie 63 (9), 667–670.
Friedman, S.L., 2008. Mechanisms of hepatic fibrogenesis. Gastroenterology 134 (6), 1655–1669.
Guimarães, E.L., Franceschi, M.F., Grivicich, I., Dal-Pizzol, F., Moreira, J.C., Guaragna, R.M., Borojevic, R., Margis, R., Guma, F.C., 2006. Relationship between oxidative stress levels and activation state on a hepatic stellate cell line. Liver Int. 26 (4), 477–485.
Guma, F.C.R., Mello, T.G., Mermelstein, C.S., Fortuna, V.A., Wofchuk, S.T., Gottfried, C., Guaragna, R.M., Costa, M.L., Borojevic, R., 2001. Intermediate filaments modulation in an in vitro model of the hepatic stellate cell activation or conversion into the lipocyte phenotype. Biochem. Cell Biol. 79 (4), 409–417.
Mathieson, W., Kirkland, S., Leonard, R., Thomas, G.A., 2011. Antimicrobials and in vitro systems: antibiotics and antimycotics alter the proteome of MCF-7 cells in culture. J. Cell Biochem. 112 (8), 2170–2178.
Novo, E., Marra, F., Zamara, E., Valfrè di Bonzo, L., Monitillo, L., Cannito, S., Petrai, I., Mazzocca, A., Bonacchi, A., De Franco, R.S., Colombatto, S., Autelli, R., Pinzani, M., Parola, M., 2006. Overexpression of Bcl-2 by activated human hepatic stellate cells: resistance to apoptosis as a mechanism of progressive hepatic fibrogenesis in humans. Gut 55 (8), 1174–1182.
Romero, E.A., Valdivieso, E., Cohen, B.E., 2009. Formation of two different types of ion channels by amphotericin B in human erythrocyte membranes. J. Membr. Biol. 230 (2), 69–81.
Souza, I.C., Martins, L.A., Coelho, B.P., Grivicich, I., Guaragna, R.M., Gottfried, C., Borojevic, R., Guma, F.C., 2008. Resveratrol inhibits cell growth by inducing cell cycle arrest in activated hepatic stellate cells. Mol. Cell Biochem. 315 (1–2), 1–7.