UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

PROGRAMA DE PÓS-GRADUÇÃO EM GENÉTICA E BIOLOGIA MOLECULAR

MECANISMO DE AÇÃO DAS PLAQUETAS NA INSUFICIÊNCIA HEPÁTICA AGUDA

Mónica Luján López

Orientadora: Profa. Dra. Ursula Matte

Porto Alegre, Maio de 2016

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Mónica Luján López

Tese submetida ao Programa de Pós-Graduação em Genética e Biologia Molecular da UFRGS como requisito parcial para a obtenção do grau de Doutor em Ciências (Genética e Biologia Molecular)

Orientadora: Profa. Dra. Ursula Matte

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Este trabalho foi realizado na Unidade de Experimentação Animal e no Centro de Terapia Gênica do Centro de Pesquisa Experimental do Hospital de Clínicas de Porto Alegre com recursos do CNPq, Fapergs e FIPE/HCPA. O uso dos animais foi autorizado pela Comissão de Ética no Uso de Animais do HCPA (projetos número 13-0097 e 14-0560).

"The absence of a neocortex does not appear to preclude an organism from experiencing affective states. Convergent evidence indicates that non-human animals have the neuroanatomical, neurochemical, and neurophysiological substrates of conscious states along with the capacity to exhibit intentional behaviors. Consequently, the weight of evidence indicates that humans are not unique in possessing the neurological substrates that generate consciousness. Nonhuman animals, including all mammals and birds, and many other creatures, including octopuses, also possess these neurological substrates".

The Cambridge Declaration on Consciousness, July 2012

AGRADECIMENTOS

A minha família pelo apoio incondicional e compreensão.

A Profa. Dra. Ursula Matte por ter orientado o trabalho e pelas oportunidades oferecidas.

Aos colegas e amigos do grupo do “fígado”, Graziella Rodrigues, Carolina Uribe, Virginia Angiolini e Alessandro Osvaldt que fizeram possível a realização do projeto.

Ao Centro de Terapia Gênica (CTG) pelo companheirismo, disponibilidade e auxílio.

A Unidade de Experimentação Animal pela colaboração durante os procedimentos experimentais.

Ao PPGBM pela oportunidade de realização deste trabalho. Principalmente, a Elmo Cardoso, por estar sempre disposto a ajudar. Ao CNPq, pela concessão de bolsa de doutorado.

A todas as pessoas que ajudaram direta ou indiretamente a execução deste projeto.

SUMÁRIO

LISTA DE ABREVIATURAS

5HT	Serotonina
APAP	Acetoaminofeno
CARS	Resposta anti-inflamatória compensatória
CK	Célula de Kupffer
CMO	Células da medula óssea
CTH	Células tronco hematopoiéticas
CTM	Células tronco mesenquimais
EGF	Fator de crescimento epidérmico
HGF	Fator de crescimento de hepatócitos
HMGB	Proteína com motivo de alta mobilidade
IGF-1	Fator de crescimento semelhante a insulina tipo 1
IHA	Insuficiência hepática aguda
IL-1	Interleucina 1
IL-6	Interleucina 6
IL-10	Interleucina 10
iPSC	Células tronco pluripotentes induzidas
LPS	Lipopolissacarideos
MAPK	Proteína quinase ativador mitogénico
MEC	Matriz extracelular
MyD88	Gene de resposta de diferenciação mielóide primária 88
NF-κB	Fator nuclear kappa B
NK	Célula *natural killer*
PDGF	Fator de crescimento derivado de plaquetas
PRP	Plasma rico em plaquetas
SIRS	Sindrome de resposta inflamatória sistémica
SOD	Superóxido dismustase
TGF-α	Fator de crescimento tumoral alfa
TGF-β	Fator de crescimento tumoral beta
TLR-4	Receptor tipo Toll 4
TNF-α	Fator de necrose tumoral alfa

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RESUMO

A insuficiência hepática aguda é caracterizada por morte celular maciça de hepatócitos. As primeiras células que se ativam após uma lesão hepática são as células de Kupffer. Estas células secretam citocinas e produzem espécies reativas de oxigénio, causando a apoptose de hepatócitos. Em um estudo anterior, mostramos que plaquetas encapsuladas aumentam a sobrevida de animais em um modelo de insuficiência hepática aguda em 10 dias. Aqui investigamos como as plaquetas exercem o seu efeito benéfico precoce nesse modelo. Para isso, plaquetas encapsuladas ou cápsulas vazias foram implantadas em ratos submetidos a hepatectomia parcial de 90%. Os animais foram eutanasiados 1, 3, 6, 12, 24, 48 e 72 horas (n=6/grupo/hora) após hepatectomia. O fígado remanescente foi coletado para avaliar o estresse oxidativo, a atividade da caspase 3 e a expressão de genes relacionados ao estresse oxidativo ou função hepática. Além disso, os níveis de lipopolissacarideos no soro e no tecido foram medidos. O número de células de Kupffer do fígado remanescente foi avaliado. A interação de plaquetas encapsuladas e células de Kupffer foi investigada utilizando um sistema de co-cultura. Foi observado que os níveis de lipopolissacarideos foram semelhantes em ambos os grupos, assim como a expressão do gene de *Tlr4* e *Myd88*, mas o *Lbp* foi maior no grupo plaquetas. O número de células de Kupffer no grupo plaquetas estava aumentado 1 hora após hepatectomia, voltando a níveis normais em seguida. No grupo controle estava aumentado às 6 até 72 horas. Além disso, as plaquetas modulam a expressão de interleucina-6 e interleucina-10 em células de Kupffer após 24 horas de co-cultura. Além disso, as plaquetas aumentam a atividade de superóxido dismutase e catalase e reduzem a peroxidação lipídica. Além disso, a atividade da caspase 3 também foi reduzida em animais que receberam plaquetas encapsuladas às 48 e 72 horas. A expressão da óxido nítrico sintase endotelial, do fator nuclear kappa B e interleucina-6 estavam elevados no grupo de plaquetas. A expressão do gene de albumina e do fator V também estavam aumentados no grupo plaquetas. Estes resultados indicam que as plaquetas interagem com as células de Kupffer e exercem o seu efeito benéfico através de redução do estresse oxidativo no fígado o que resulta em hepatócitos saudáveis e diminuição da apoptose. Além disso, estes efeitos são mediados por fatores parácrinos imediatamente após a lesão hepática

ABSTRACT

Acute liver failure is characterized by massive hepatocyte cell death. Kupffer cells are the first cells to be activated after liver injury. They secrete cytokines and produce reactive oxygen species, leading to apoptosis of hepatocytes. In a previous study, we showed that encapsulated platelets increase survival in a model of acute liver failure. Here we investigate how platelets exert their early beneficial effect in this model. For that, encapsulated platelets or empty capsules were implanted in rats submitted to 90% partial hepatectomy. Animals were euthanized at 1, 3, 6, 12, 24, 48 and 72 hours (n = 6/group/time) after hepatectomy. Liver was collected to assess oxidative stress, caspase activity, and gene expression related to oxidative stress or liver function. Also, lipopolysaccharide (LPS) levels in serum and tissue were assessed. The number of Kupffer cells in the remnant liver was evaluated. Interaction of encapsulated platelets and Kupffer cells was investigated using a co-culture system. It was observed that LPS levels were similar in both groups, as well as gene expression of Tlr4 and Myd88, but Lbp was higher in platelet group. The number of Kupffer cells in platelet group was increased at 1 hour and then returned to normal levels; in control group it was increased from 6 to 72 hours. Platelets modulate Interleukin-6 and Interleukinl-10 expression in Kupffer cells after 24 hours of co-culture. In addition, platelets increase superoxide dismutase and catalase activity and reduce lipid peroxidation. Moreover, caspase 3 activity was also reduced in animals receiving encapsulated platelets at 48 and 72 hours. Gene expression of endothelial nitric oxide synthase, nuclear factor kappa B and Interleukin-6 were elevated in platelet group. Gene expression of albumin and factor V were also increased in platelet group. These results indicate that platelets interact with Kupffer cells in this model and exert their beneficial effect through reduction of oxidative stress that results in healthier hepatocytes and decreased apoptosis. Furthermore, these effects are mediated by paracrine factors immediately after liver injury.

1 INTRODUÇÃO

1.1 Fígado

O fígado tem um papel central na homeostase metabólica, já que é responsável pelo metabolismo, síntese, armazenamento e redistribuição de nutrientes, carboidratos, gorduras e vitaminas; e produz grandes quantidades de proteínas séricas, incluindo as proteínas de fase aguda, albumina, enzimas e cofatores (Taub 2004). O fígado é o principal órgão detoxificante. Recebe toda a circulação do intestino, assim como do baço e do pâncreas, através da veia porta, e remove resíduos e xenobióticos por conversão metabólica e pela excreção biliar (Taub 2004; Michalopoulos 2007). Este órgão constitui a maior rede celular reticulo-endotelial no corpo e tem um papel importante na defesa contra microrganismos invasores (Ishibashi et al. 2009).

Os nutrientes que entram no fígado são transformados em proteínas que serão secretadas no sangue periférico, como albumina, a maioria dos fatores de coagulação, proteínas plasmáticas carreadoras, etc. A síntese de bile é essencial para absorção de gordura e nutrientes lipofílicos, e os lipídeos são enviados como lipoproteínas para outros tecidos. Os carboidratos são armazenados no fígado como glicogênio, o principal reservatório de glicose usado para estabilizar seus níveis de glicose no sangue. Sendo o regulador de glicose no plasma e dos níveis de amônia, o fígado é essencial para um ótimo funcionamento do cérebro (Michalopoulos 2007).

A subunidade funcional do fígado é o lóbulo hepático e está constituída por um arranjo hexagonal de placas de hepatócitos que se estendem formando placas de células hepáticas de uma célula de espessura e de 15-25 hepatócitos de comprimento. Entre duas placas de células, o sangue flui a partir do trato portal para a vênula hepática terminal, formando o assim chamado "sinusóide" (Ishibashi et al. 2009). O centro do lóbulo é a veia central, e na periferia se localiza a tríade portal (vênula porta, arteríola hepática e ducto biliar). Funcionalmente, o fígado pode ser divido em três zonas, baseados no fornecimento de oxigênio. A zona 1 (Z1) abarca os tratos portais, rica em sangue oxigenado das artérias hepáticas; a zona 3 (Z3) se localiza ao redor das veias centrais, onde a oxigenação é pobre; e a zona 2 (Z2) se encontra entre a Z1 e Z3. Existe diferença na expressão gênica de diversos genes entre os hepatócitos localizados nas diferentes zonas do lobo hepático, o que os leva a ter capacidades funcionais distintas (Ishibashi et al. 2009).

Aproximadamente 80% da massa hepática está composta de hepatócitos e se considera que o hepatócito é a menor unidade funcional do fígado (figura 1), já que são responsáveis pelas funções associadas ao órgão (Taub 2004). Os outros 20% correspondem às células do epitélio biliar, dos vasos sanguíneos, células de Kupffer (CK), e células estreladas (Hindley et al. 2014). As células endoteliais constituem a parede dos capilares e contêm pequenas fenestrações para permitir a livre difusão de substâncias entre o sangue e os hepatócitos. As células endoteliais dos sinusóides também possuem uma capacidade de endocitose pronunciada, constituindo parte importante do sistema reticuloendotelial. Além disso, são células ativas na secreção de fatores bioativos e de componentes da matriz extracelular no fígado e provêm uma extensa superfície para absorção de nutrientes pelas CK que residem nesse espaço. As CK são potentes mediadores da resposta anti-inflamatória através da secreção de citocinas e quimiocinas e tem um papel crucial na reposta imune. As células Pit, que são as *natural killers* específicas do fígado, tem a capacidade de eliminar células tumorais e parecem participar na defesa antiviral no fígado. As células hepáticas estreladas, também conhecidas como células de Ito, estão presentes no espaço de Disse, espaço perisinusoidal, e representam a principal fonte de matriz extracelular e de armazenamento de vitamina A. A heterogeneidade dos tipos celulares e sua cooperação entre si e com os hepatócitos fazem a funcionalidade do fígado (Bouwens et al. 1992; Taub 2004).

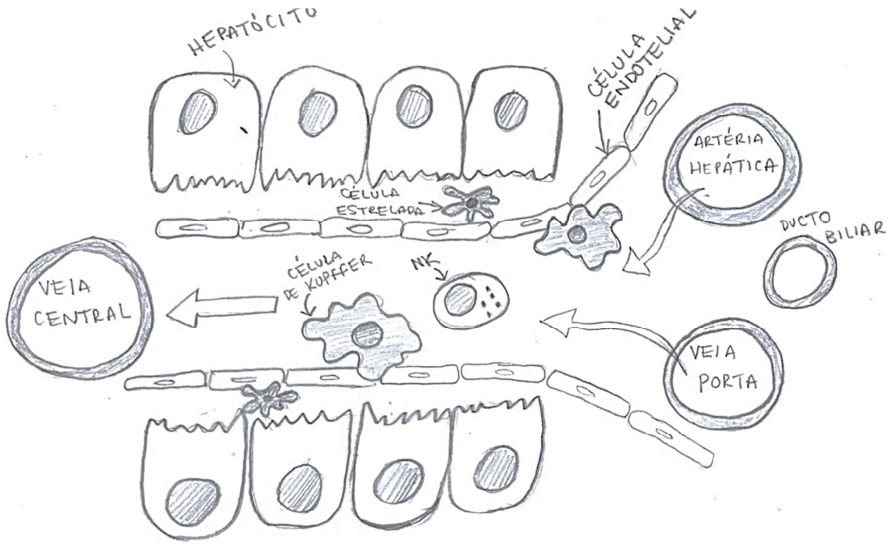

Figura 1. Arquitetura do fígado. As células endoteliais formam uma monocamada no endotélio fenestrado sinusoidal. As células estreladas se localizam no espaço de Disse. As

células de Kupffer residem dentro do espaço vascular sinusoidal do fígado, predominantemente na área periportal.

As substâncias químicas tóxicas e os agentes infecciosos que entram através dos alimentos no trato digestivo e na circulação são metabolizados pelos hepatócitos e captados pelas células não parenquimatosas (Michalopoulos 2007). As proteínas alheias derivadas dos nutrientes ou da microbiota residente, servem para apresentação de endotoxinas bacterianas e ativam a resposta imune (Heymann and Tacke 2016).

A ocorrência de infecção no tecido hepático ou a presença de substâncias tóxicas podem provocar lesão celular e a perda das funções do fígado. A imediata resposta regenerativa às injúrias hepáticas é um eficiente mecanismo de manutenção funcional do fígado, da viabilidade do organismo e de preservação da vida (Michalopoulos 2007). O fígado tem a capacidade única de regeneração e pode se recuperar completamente após várias rodadas de remoção de tecido (até 70%). Está demonstrado para todos os vertebrados que a hipertrofia e proliferação dos hepatócitos são a causa da regeneração que preserva o fígado após uma hepatectomia parcial ou dano por intoxicação (Hindley et al. 2014).

1.1.1 Regeneração hepática: citocinas e fatores de crescimentos.

A habilidade extraordinária do fígado de se regenerar após um dano ou ressecção tem fascinado cientistas e médicos desde sempre. A primeira alusão à capacidade do fígado de se regenerar foi descrita na Teogonia de Hesíodo (750-700 a.C.). Prometeu, um Titã, irritou Zeus por ter roubado o fogo e dado para os humanos. Como castigo, Prometeu foi acorrentado a uma pedra nas Montanhas do Cáucaso, onde Zeus o atormentava enviando uma águia todos os dias para devorar seu fígado. Durante a noite, seu fígado danificado se regenerava, deixando-o exposto novamente à tortura. Porém, evidências científicas da habilidade do fígado de se regenerar em humanos não tinham sido descritas até 1890 (Koniaris et al. 2003).

Existe uma notável semelhança entre os processos de regeneração hepática em roedores e humanos. Então, é razoável assumir que os mecanismos que regulam a regeneração são possivelmente muito similares entre espécies, e o conhecimento obtido de

estudos de regeneração hepática nesses animais se aplica para fígados humanos (Fausto 2001). Tanto é assim que a maioria dos dados sobre os mecanismos de controle da regeneração hepática foram obtidos a partir da utilização do modelo animal de hepatectomia parcial (HP) (Taub 2004; Michalopoulos 2010).

O hepatócito adulto se encontra em fase G0, sendo que a taxa de proliferação do fígado é entre 0,0012% a 0,01% a qualquer momento, embora, essa taxa possa mudar rapidamente sob estimulação (Koniaris et al. 2003). Após HP, os hepatócitos são as primeiras células a entrar em ciclo de divisão celular. O pico de replicação hepatocitária ocorre em 24 horas após a HP em ratos, e em 36 a 42 horas em camundongos (Fausto 2001; Taub 2004).

É bem aceito que há duas formas fisiológicas de regeneração como resposta a diferentes danos hepáticos para garantir o tamanho do fígado (Riehle et al. 2011; Michalopoulos 2013):

a) Substituição do tecido perdido com fidelidade fenotípica de tipos de células (cada tipo de célula do fígado entra em proliferação para substituir o seu próprio compartimento celular). Essa resposta ocorre principalmente no dano agudo, por drogas, toxinas, ressecção, ou doenças agudas virais.
b) Substituição de tecido por ativação de vias de transdiferenciação a partir de células-tronco extra-hepáticas. Este tipo de regeneração ocorre quando a lesão é grave, ou quando os hepatócitos maduros já não podem regenerar o fígado devido à senescência.

A regeneração acontece numa sequência de fases (figura 2): iniciação ou fase de sensibilização (*priming*), na qual os hepatócitos entram em um estado de competência replicativa; a fase de proliferação, onde ocorre a expansão celular; e a fase de terminação, onde a proliferação celular é suprimida para terminar a regeneração. A quarta fase envolve a remodelação dos lobos hepáticos durante a fase de expansão. Estas fases estão conectadas e compartilham muitos mecanismos (Zimmermann 2004). Esta sequência ordenada de eventos pode ser observada a partir dos primeiros 5 minutos após o dano ou HP e durará de 5 a 7 dias até a recuperação da massa hepática total (Michalopoulos 2010). Ao final do processo de síntese de DNA, pode ocorrer uma onda de apoptose dos hepatócitos, sugerindo a existência de um mecanismo para a correção de uma resposta regenerativa excessiva (Michalopoulos 2007).

A proliferação começa após um intervalo de 24 horas, refletindo o *priming* das células em G0 e a mudança de G0 ao ponto G1/S. A proliferação dos hepatócitos começa na zona periportal 1 (Z1) e estende-se até a Z3 nas 36-48 horas. A proliferação de células não-parenquimatosas ocorre depois, com um atraso de 48 horas aproximadamente para CK e células epiteliais, e de cerca de 96 horas para células endoteliais (Taub 2004; Zimmermann 2004).

O dano hepático provoca a liberação de espécies reativas de oxigênio (ROS) e lipopolissacarídeos (LPS), que desencadeiam a ativação do sistema do complemento, que por sua vez ativa as CK através do receptor da superfície celular *Toll like receptor* (TLR) 4 e receptores acoplados à proteína G e C3aR C5aR, o que provoca a ativação da via de sinalização de *nuclear factor kappa B* (NF-kB) e a produção de citocinas tais como fator de necroses tumoral alfa (TNF-α) e interleucina 6 (IL-6) (Riehle et al. 2011; Zheng et al. 2009; Tsutsui and Nishiguchi 2014). O TNF-α e a IL-6 são os responsáveis por sensibilizar os hepatócitos em estado de quiescência para entrarem no ciclo celular (de G0 a G1), e ativam as vias de sinalização de NF-κB, JAK/STAT3 (STAT -*Signal Transducer and Activator of Transcription*) e proteíno-quinases ativadas por mitógenos (MAPK- *Mitogen Activated Protein Kinases*); iniciando a transcrição de genes precoces imediatos; e preparação dos hepatócitos aos efeitos dos fatores de crescimento (Zheng et al. 2009; Michalopoulos 2014). A depleção das CK por lipossomas de diclorometileno difosfonado (CL2MDP) altera a expressão de citocinas e retarda a regeneração hepática após uma HP (Abshagen et al. 2007).

Muitos fatores de crescimentos encontram-se usualmente na sua forma inativa precursora ligados à matriz extracelular (MEC) ou integrados à membrana. Durante a regeneração hepática, proteases extracelulares como as metaloproteinases se ativam e degradam a MEC imediatamente após o dano hepático. Assim os fatores de crescimentos são liberados da MEC ou da membrana celular e ativados por proteases extracelulares (Michalopoulos 2007, 2013; Zheng et al. 2009).

A progressão dos hepatócitos competentes através do ciclo celular depende de dos fatores de crescimento mais importantes, o fator de crescimento tumoral alfa (TGF-α) e o fator de crescimento de hepatócitos (HGF), após os quais o processo de proliferação parece prosseguir sob o controle de ciclinas e quinases dependentes de ciclina (Taub 2004; Zimmermann 2004). O HGF é sintetizado particularmente pelas células estreladas e atinge

os hepatócitos de forma parácrina estimulando a proliferação. O HGF se liga e ativa o receptor tirosina quinase Met e outras vias de sinalização mitogênicas, como a fosfoinositol quinase (PI3K-siglas em inglês), ERK, S6 quinase e Akt (Taub 2004). O TGF-α é um ligante membro da família dos fatores de crescimento epidérmico (EGF). É o único fator de crescimento produzido pelos hepatócitos e atua como um agente autócrino ligando-se a receptores existentes nos próprios hepatócitos. Embora tenha efeitos sobre a motilidade celular e a vascularização tecidual, o principal efeito do TGF-alfa no fígado é a promoção da proliferação hepatocitária (Fausto and Riehle 2005).

Além disso, outros fatores de crescimentos como a insulina derivada do pâncreas, fator de crescimento epidérmico (EGF) do duodeno ou glândula salival, norepinefrina da glândula adrenal, serotonina das plaquetas, e postaglandinas das CK e hepatócitos, entre outros, também estão envolvidos no processo de regeneração hepática (Zimmermann 2004; Michalopoulos 2007).

Os principais fatores relacionados com a terminação da regeneração são o TGF-β e as ativinas. Ambos os fatores são anti-proliferativos e promovem a fibrose e apoptose. O TGF-β é produzido principalmente pelas células estreladas, e as ativinas pelos hepatócitos, atuando com efeito autócrino (Taub 2004; Zimmermann 2004). Quando o tamanho do fígado é restaurado a seu volume normal há um aumento da sinalização pela ativina A, apoptoses, e diminuição da expressão e ativação de fatores de proliferação devido à restauração da MEC, ocorrendo a terminação da regeneração hepática (Zheng et al. 2009).

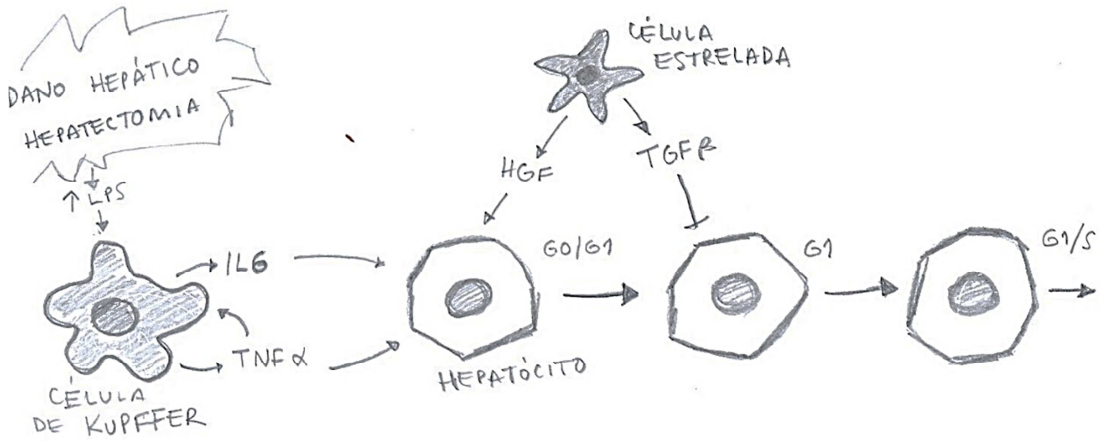

Figura 2. Regeneração hepática. Após um dano hepático ou hepatectomia fotores derivados do intestino como lipopolissacarídeso (LPS) aumentam e chegam ao fígado onde ativam as células de Kupffer. Estas células liberam o fator de necrose tumoral (TNFα) e interleucina

6 (IL6) que atingem o hepatótico preparando-os para responder aos fatores de crescimentos. As células estreladas liberam fator de crescimento de hepatócitos (HGF) promovendo a entrada no ciclo celular, uma vez que o tamanho do fígado é recuperado elas liberam o fator de transformador de crescimento (TGFβ).

1.1.2 Regeneração hepática: resposta metabólica.

Todo dano hepático grave altera o metabolismo, o qual também está envolvido na regeneração hepática (Huang et al. 2016). Depois de um dano hepático em modelos experimentais, o fígado continua a realizar funções metabólicas essenciais para a sobrevida (Huang and Rudnick 2014). A demanda metabólica e a oferta de nutrientes estimulam a proliferação hepatocitária na busca da homeostase sistêmica de nutrientes (Fausto et al. 2006).

Após uma HP, a maquinaria de gluconeogênese é ativada e há supressão da atividade glicolítica, para evitar a queda da glicose no sangue à custa da produção de ATP hepática derivada de glicose (Huang and Rudnick 2014). No entanto, roedores submetidos a HP desenvolvem hipoglicemia, seguido de catabolismo sistêmico e humoral, além da acumulação de metabólitos (Kieling et al. 2012; Huang et al. 2016).

Em paralelo, o fígado em regeneração acumula grandes quantidades de triglicerídeos, e a partir de 12 horas após HP se desenvolve uma marcada esteatose no fígado (Shteyer et al. 2004). Tem se sugerido que esta esteatose transitória resulta da captação de reservas de gordura derivadas de tecido adiposo pelo fígado em regeneração. A hipoglicemia induz a lipólise, o que leva, primeiramente a um aumento dos ácidos graxos livres no soro e depois a acumulação de gordura no fígado em regeneração (Huang and Rudnick 2014).

Além disso, o conteúdo de ATP hepático declina e o AMP aumenta no fígado remanescente. A β-oxidação dos ácidos graxos serve como a fonte predominante de nova produção de ATP na regeneração do fígado. Estas alterações metabólicas precedem o início da proliferação celular induzida por uma ressecção ou por toxinas, que subsequentemente irá promover a expressão de ciclinas e ativação do complexo ciclinas-CDK (Huang and Rudnick 2014).

1.2 Insuficiência hepática aguda

A insuficiência hepática aguda (IHA) é uma síndrome rara mas muito grave, e se caracteriza pela súbita e severa necrose celular e disfunção hepática, associada à icterícia, coagulopatia e encefalopatia hepática em indivíduos sem historia de doença hepática (Lee et al. 2008; Sundaram and Shaikh 2011). A maciça morte hepatocelular é o principal evento da IHA, embora a resposta inflamatória tenha um papel importante, acompanhada pela infiltração de células inflamatórias.

A insuficiência acontece quando a taxa e extensão da morte celular excede a capacidade regenerativa do fígado (Rutherford and Chung 2008; Xiaojing Wang and Ning 2014). Em consequência, há uma perturbação das funções hepáticas com redução na capacidade de eliminação de drogas, toxinas e bilirrubina, diminuição na síntese de fatores de coagulação, alteração na homeostase da glicose e aumento na produção de lactato. Como resultado, os pacientes tendem a desenvolver icterícia, coagulopatia e hemorragia gastrointestinal, hipoglicemia e acidose, encefalopatia e edema cerebral, infecções bacteriana e fúngicas, síndrome da resposta inflamatória sistêmica e falência de múltiplos órgãos, e frequentemente evoluem para o óbito (Kieling 2012a). Os sintomas de encefalopatia aparecem entre a primeira e segunda semana e se não são tratados, a doença pode levar a insuficiência renal e falência múltipla de órgãos, coma e até a morte (Lee 2012).

As causas da IHA são muitas e variam de país a país. Em países da Europa e na América do Norte a principal causa é a toxicidade por acetoaminofeno (acetil para-aminofenol, APAP) seguida de reações idiossincráticas a drogas (Nguyen and Vierling 2011; Lee 2012). Nos países do leste europeu, Ásia, África e América Central e do Sul, predominam hepatites virais, sendo a hepatites viral B responsável de 600000 mortes por ano (Ichai and Samuel 2011; Coppola et al. 2015). Nas crianças, as principais causas são as hepatites virais agudas e os erros metabólicos. No sul do Brasil, a hepatite viral A é a causa mais frequente de IHA nas crianças (Ferreira et al. 2008).

O transplante de fígado continua sendo o tratamento mais efetivo, aumentando a taxa de sobrevida em 80% (Sass and Shakil 2005), porém não é amplamente disponível, devido à falta de doadores de órgãos. Além disso, requer o uso de imunossupressores durante a vida toda (Polson and Lee 2005). Isto enfatiza a necessidade de desenvolver novas estratégias para o manejo e tratamento de IHA.

1.2.1 Processos envolvidos na IHA

Os mecanismos de lesão do fígado podem ser classificados em dois grupos; primeiro, patógenos ou substâncias tóxicas danificam as organelas celulares perturbando a homeostase intracelular e ativando as vias apoptóticas. Segundo, a resposta imune (inata e adaptativa) converge nas vias de sinalização de morte celular incluindo apoptose, necrose, e necroptose (necrose programada), levando a dano hepático mediado pelo sistema imune (Rutherford and Chung 2008; Z. Wu et al. 2010).

A resposta imune tem um papel essencial na patogêneses da IHA, pois há uma ativação das CKs, células dentríticas, células *natural killer* (NK), linfócitos T citotóxicos, linfócitos reguladores T (Treg), e produção de citocinas pró-inflamatórias que levam ao dano hepatocelular que ultrapassa a capacidade de regeneração hepática (Xiaojing Wang and Ning 2014).

Muitos estudos têm demonstrado que na IHA ocorre a ativação da síndrome de resposta inflamatória sistêmica (SIRS), a qual está associada a um agravamento da encefalopatia e mau prognóstico. A reação inflamatória que constitui SIRS está caracterizada pelo aumento dos níveis de citocinas pró - (TNF-α, IL-1, IL-6) e anti-inflamatórias (IL-10) circulantes (Antoniades et al. 2008; Z. Wu et al. 2010). Pacientes com insuficiência hepática aguda têm concentrações maiores de citocinas pró-inflamatórias (TNF-alfa e IL-6) e biomarcadores de morte celular como proteínas com motivos de alta mobilidade (HMGB) circulantes que os com hepatite aguda ou voluntários sadios (Mao et al. 2010; Cao et al. 2015).

Na evolução do paciente em IHA, o desenvolvimento de SIRS é contrabalanceado por uma forte e persistente resposta anti-inflamatória compensatória (CARS - *Compensatory anti-inflammatory response syndrome*), que envolve a liberação de IL-10 pelas CKs, caracterizando uma desregulação do sistema imune (Kolios et al. 2006; Antoniades et al. 2008). As CKs no fígado tem capacidade fagocitária e liberam uma ampla gama de citocinas que são críticas para determinar as respostas subsequentes por outras células imunes e de hepatócitos, assim como o grau do dano hepático. Após sinais inflamatórios, monócitos e macrófagos são rapidamente recrutados no fígado, onde adquirem perfis funcionais igual às CK (Kolios et al. 2006; Wu et al. 2010) . Entretanto, há estudos que indicam que as CK exercem uma regulação negativa na reparação do fígado

mediada pela liberação em grandes quantidades de Interleucina 1 (IL1), TGF-β e TNF-α (Boulton et al. 1998).

Ao mesmo tempo, os neutrófilos extravasam no parênquima hepático guiados por potentes promotores como o TNF-α, IL-1, fator de ativação das plaquetas, IL-18, HMGB e produtos de peroxidação lipídica liberados por células morrendo ou mortas, assim como por quimocinas como CXC (Jaeschke et al. 2012; Marques et al. 2012). Esta resposta ativa os neutrófilos, levando a aumento do estresse oxidativo dependente da adesão prolongada e degranulação. Além do mais, os produtos oxidantes difundem dentro dos hepatócitos acarretando estresse oxidativo intracelular. Por outro lado, os neutrófilos podem expressar o ligante Fas e induzir apoptose nos hepatócitos. Estes eventos podem causar mais dano hepático e contribuem à resposta inflamatória sistémica levando mais prejuízos a órgãos distantes e contribuindo para a falência múltipla de órgãos (Marques et al. 2012; Xu et al. 2014). A dissonância imunológica, com a perda do equilíbrio entre as atividades pró- e anti-inflamatórias, determina a predisposição para sepse, falência de múltiplos órgãos, edema cerebral, hipertensão intracraniana e morte, características do estado terminal do paciente em insuficiência hepática aguda (Antoniades et al. 2008).

1.2.2 Modelos animais de IHA

Os modelos animais são importantes instrumentos para a ampliação do entendimento da patogênese da insuficiência hepática aguda, da evolução da doença, do manejo das complicações e dos mecanismos envolvidos na regeneração hepática (Bélanger and Butterworth 2005).

Um modelo ideal deveria apresentar os critérios clínicos e bioquímicos definidos nos pacientes e ser capaz de fornecer um prognóstico preciso. No entanto, nenhum dos modelos que tem sido desenvolvido até o momento satisfazem esses requerimentos. Além disso, os critérios clínicos e bioquímicos usados para indicar a existência de IHA nos modelos animais tem, em geral, pouco em comum com aqueles usados na prática clínica (Tuñón et al. 2009). Apesar de suas limitações, o desenvolvimento de modelos experimentais adequados possibilita, além de maior compreensão da patofisiologia, a identificação de novas drogas alvos e os mecanismos de toxicidade e testar a eficácia de novas intervenções terapêuticas (Maes et al. 2016; Martins et al. 2008). Os padrões para modelos animais IHA estão resumidos na tabela 1 (Terblanche and Hickman 1991).

Tabela 1. Requisitos para modelo animal ideal de insuficiência hepática aguda.

Critério	**Comentário**
Reversibilidade	A IHA produzida precisa ser potencialmente reversível de modo que o animal possa responder e sobreviver ao tratamento utilizado.
Reprodutibilidade	Desfechos reprodutíveis são essências na padronização do modelo animal.
Morte por falência hepática	A lesão hepática deve resultar em morte.
Janela terapêutica	O tempo decorrente entre a injúria hepática e a morte deve ser suficiente para permitir o tratamento e a observação dos seus efeitos
Risco mínimo ao pessoal envolvido na pesquisa	As toxinas devem apresentar um risco mínimo para o pessoal de laboratório
Metabolismo/fisiologia apropriado	As espécies utilizadas deve ter propriedades metabólicas e fisiológicas semelhantes aos dos seres humanos

Existe uma variedade de modelos animais usados para entender o mecanismo de IHA, desde o uso de substâncias químicas (Concavalina A, tetracloreto de carbono, acetaminofeno, LPS, etc.) ou metabólicas (etanol, sulfatideos, etc), uso de patógenos infecciosos (vírus da hepatites) até os modelos cirúrgicos (hepatectomia) (Tuñón et al. 2009; Z. Wu et al. 2010).

O uso de agentes químicos como o acetaminofeno, tioacemida ou galactosamina podem reproduzir uma quantidade de características clínicas da IHA, tais como a hipoglicemia, encefalopatia e aumento dos níveis séricos de enzimas hepáticas. No entanto, a administração repetida ou uma terapia de suporte pode ser necessária em alguns modelos. Além disso, a hipertensão intracraniana, uma das principais características de IHA em humanos, está ausente em alguns modelos químicos (Tuñón et al. 2009).

O modelo químico experimental mais usado está baseado na administração de acetaminoafeno (APAP). Este modelo é frequentemente selecionado devido a sua relevância epidemiológica nos humanos (Bélanger and Butterworth 2005; Maes et al. 2016). Além disso, a patofisiologia nos camundongos reflete bastante o que acontece nos humanos, incluindo dano mitocondrial com estresse oxidativo, seguido de fragmentação do DNA e necrose (Maes et al. 2016). Por outro lado, o tetracloreto de carbono (CCl_4) tem sido amplamente utilizado para induzir lesão hepática crônica, especialmente como um modelo de cirrose hepática primária. No entanto, a sua utilização para induzir IHA tem sido muito limitada devido à baixa reprodutibilidade e grande variação entre as espécies. No Centro de Pesquisa Experimental do Hospital de Clínicas de Porto Alegre (HCPA) o APAP e o CCl_4 foram empregados em modelos animais de lesão hepática aguda (Baldo et al. 2010; Belardinelli et al. 2008; Matte et al. 2014). A grande limitação desses modelos tóxicos é a imprevisibilidade da extensão da lesão hepática devido à variabilidade metabólica individual e toxicidade extra-hepática (Rahman and Hodgson 2000; Bélanger and Butterworth 2005).

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

A desvacularização do fígado pode ser conseguida por anastomose porto-cava seguida pela oclusão da artéria hepática, e na maioria dos casos, também oclusão do ducto biliar comum e acessórios vasos hepáticos acessórios. Este modelo é mais útil para estudar o desenvolvimento e tratamento de IHA causada pela isquemia e os efeitos colaterais relacionados (Tuñón et al. 2009).

A hepatectomia parcial em ratos e camundongos tem sido utilizada como modelo experimental em estudos de regeneração hepática e de insuficiência hepática aguda (Martins et al. 2008). O modelo clássico de hepatectomia em ratos se baseia nos experimentos de Higgins e Anderson (Higgins and Anderson 1931). É bem reconhecido que a regeneração intensa e quase 100% de sobrevivência após uma hepatectomia parcial de 70% em ratos e porcos (Rahman and Hodgson 2000). Ressecções hepáticas mais

extensas (70 a 80%) induzem à insuficiência, e estão associadas à maior mortalidade (Panis et al. 1997). Hepatectomia de 90, 95 e 97% são utilizadas como modelo experimental de insuficiência hepática aguda (Martins et al. 2008; Kieling 2012b). Apesar de que o modelo de hepatectomia tem limitações em reproduzir algumas características da IHA, proporciona ferramentas indispensáveis ao estudo de muitos fenômenos relacionados à lesão hepática aguda, ao processo regenerativo e às repercussões sistêmicas da insuficiência hepática aguda (Rahman and Hodgson 2000; Kieling 2012b).

1.3 Terapia celular

As terapias baseadas no transplante celular são consideradas as alternativas terapêuticas mais adequadas para o tratamento de insuficiência hepática, já que as células transplantadas podem deter a deterioração da função hepática e promover a regeneração do fígado (Sun et al. 2014).

A terapia celular para IHA inclui desde suportes de fígado bioartificial, transplante de hepatócitos autólogos ou alogênicos, e células tronco capazes de se diferenciar a células tipo hepatócitos (Yarygin et al. 2015; Yu et al. 2012). O transplante celular é muito menos invasivo e mais barato comparado com o transplante hepático total ou parcial, além disso, a metodologia é relativamente simples, reversível e reprodutível (Forbes et al. 2015; Sun et al. 2014; Yarygin et al. 2015).

O transplante de hepatócitos seria uma abordagem alternativa ao transplante de fígado, pois os hepatócitos maduros são os principais contribuintes para reparação hepática e são, funcionalmente, as células mais robustas para a terapia celular do fígado (Tsolaki et al. 2015). De fato, muitos estudos pré-clínicos (Rodrigues et al. 2012) e clínicos foram realizados usando esta abordagem para curar doenças hepáticas metabólicas e em fase terminal (Gramignoli et al. 2015). No entanto, o transplante de hepatócitos se vê limitado pela indisponibilidade de órgãos e impacto negativo da cultura celular na viabilidade e função do enxerto (Gramignoli et al. 2015; Tsolaki et al. 2015).

Uma alternativa a este problema, são as células tipo hepatócitos que podem ser produzidas por reprogramação de células diferenciadas (iPSCs, -*induced pluripoten stem cells*) ou por diferenciação direta de células tronco embrionárias (Hu and Li 2015; Sanal 2015; Yarygin et al. 2015). As iPSC são uma fonte pluripotente de células e possuem um

potencial de diferenciação extenso (Ramanatham et al. 2015). A principal vantagem desta técnica é o uso de células autólogas, o que mitiga a escassez de células para transplante e os riscos do uso de imunossupressores e sensibilização imune (Ramanatham et al. 2015; Yarygin et al. 2015). Além disso, as iPSC passam por uma exaustiva e excelente caracterização *in vitro* do seu genoma e das suas propriedades bioquímicas, metabólicas, fisiológicas e microscópicas antes de serem transplantadas (Knoepfler 2012; Sanal 2015). No entanto, o transplante de iPSC diferenciadas teria sucesso apenas em situações sob extrema pressão seletiva como em alguns modelos animais geneticamente modificados, tais como, nos modelos murino imunodeficientes (Carpentier et al. 2014; Espejel et al. 2010). Além disso, nos modelos animais os hepatócitos hospedeiros são intencionalmente danificados, o que dá às iPSC diferenciadas uma vantagem. Assim o fígado danificado facilitaria o enxerto e repovoamento das células transplantadas (Sanal 2015).

Por outro lado, a reprogramação celular pode ser combinada com a engenharia de tecido e produzir órgãos bioartificiais (Shi et al. 2016). Por enquanto, esta abordagem está sendo testada unicamente em animais, desde a obtenção do esqueleto do órgão descelularizado, seguido do repovoamento com células diferenciadas a hepatócitos. Porém, o uso de células autólogas na clínica tem duas desvantagens, em primeiro lugar construir um órgão para cada paciente seria muito caro; em segundo lugar, levaria vários meses para fazer um fígado inteiro ou parte dele a partir de células autólogas, o que não seria adequado para pacientes com IHA (Yarygin et al. 2015).

Diversos estudos têm demonstrado o potencial da terapia celular com células derivadas de medula óssea (CMO) como alternativa terapêutica para doenças hepáticas (Baldo et al. 2010; Belardinelli et al. 2008; Theise et al. 2000; Uribe-cruz et al. 2015). A capacidade das CMO em se diferenciar em fígado, pâncreas e outros tecidos, levou a especular na década de 1990 que estas células seriam a fonte de todas as células tronco desses órgãos (Petersen 1999). Idilman et al., demonstrou que os hepatócitos autólogos repovoam um fígado transplantado. E estes hepatócitos derivam da medula óssea, em um evento que ocorre relativamente cedo após a lesão hepática do enxerto (Idilman et al. 2007).

A medula óssea está formada por diferentes tipos celulares, entre eles as células tronco hematopoiéticas e as células tronco mesenquimais (CTM). Estas últimas são auto-renováveis, são células progenitoras multipotentes com a capacidade de se diferenciar em

células de linhagem específica que formam o osso, cartilagem, gordura, tendão, e tecido muscular (Kisseleva et al. 2010). Além disso, as CTM também são capazes de se transdiferenciar em hepatócitos tanto *in vitro* como *in vivo* (Oh et al. 2015; Matte et al. 2014; Pournasr et al. 2011; Sato et al. 2005; Sgodda et al. 2007). Assim mesmo, as células tronco hematopoiéticas (CTH) tem ampla plasticidade e são capazes de transdiferenciar quando colocadas em ambientes ectópicos (Austin and Lagasse 2003).

As CMO, tanto CTM quanto CTH, também podem se fusionar com os hepatócitos danificados para recuperar sua função (Aurich et al. 2007; Hao et al. 2015; Xin Wang et al. 2003). Após a fusão, as CMO reprogramam seu genoma assemelhando-se aos perfis de expressão de hepatócitos. As células fusionadas formam hepatócitos normais, proliferam e melhoram o dano hepático (Vassilopoulos et al. 2003).

Outro mecanismo de ação das CMO é mediado por fatores parácrinos que atuam sobre a dinâmica celular local, estimulando a regeneração das células endógenas e a recuperação dos tecidos (Fouraschen et al. 2015). As CMO podem produzir uma série de citocinas e moléculas de sinalização que tem efeitos pleiotrópicos no sitio da lesão no fígado, incluindo efeitos anti-inflamatórios, anti-apoptóticos e proliferativos (Liu et al. 2015; Uribe-cruz et al. 2015).

As células liberam diversos tipos de vesículas ao ambiente extracelular, as quais representam um modo importante de comunicação intercelular já que servem de veículo para a transferência de proteínas de membrana e citosólicas, lipídeos e RNA entre células (Raposo and Stoorvogel 2013). Herrera et al., demonstraram que microvesículas derivadas de células tronco do fígado promovem a proliferação e suprimem a morte celular de hepatócitos em ratos submetidos a hepatectomia de 70% (Herrera et al. 2010). Por outro lado, as microvesículas são capazes de alterar a função celular e/ou reprogramar células alvos (Quesenberry et al. 2015). Simon et al., observaram que células derivadas da medula óssea adquiriram características de células tipo hepatócitos após 24 horas em co-cultivo com hepatócitos danificados com tetracloreto de carbono. As análises do sobrenadante revelaram presença de microvesículas com mRNAs típicos de células do fígado (albumina, fator de coagulação V, etc.), sugerindo um papel das miscrovesículas na indução de plasticidade celular (Simon et al. 2015).

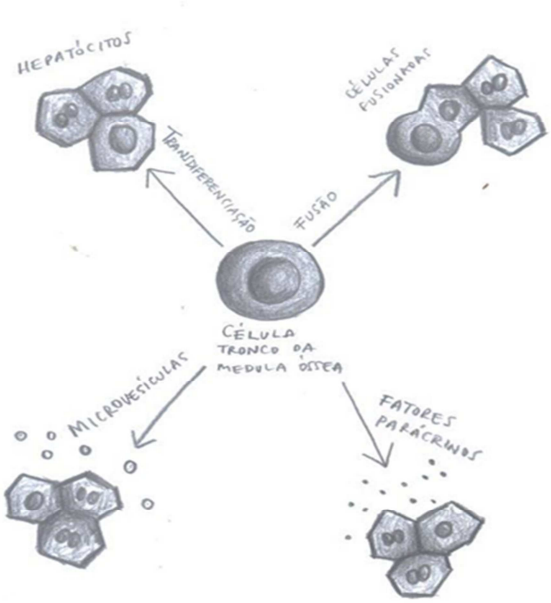

Figura 3. Mecanismo de ação das células tronco derivadas da medula óssea. As células tem a capacidade de transdiferenciar, se fusionar com outro tipo celular. Ao mesmo tempo, as células tronco liberam moléculas solúveis (fatores parácrinos) ou atrapados em microvesículas que logo se fusionar com outras células.

1.3.1 Plaquetas

As plaquetas são fragmentos citoplasmáticos anucleados originados pela fragmentação de megacariócitos (MK) na medula óssea que circulam no sangue e têm importantes papéis na hemostase, trombose, inflamação, e biologia vascular (Sim et al. 2016).

O plasma rico em plaqueta (PRP) tem sido utilizado desde 1950 em dermatologia e cirurgia maxilo-facial oral, mas o interesse para aplicações clínicas tem aumentado consideravelmente nos últimos anos. As plaquetas contém mais de 1100 proteínas, incluído enzimas, inibidores de enzimas, fatores de crescimento, mensageiros do sistema imune e outros componentes bioativos importantes na reparação e cicatrização do tecido (De Pascale et al. 2015; Knezevic et al. 2016).

As plaquetas possuem três tipos de organelas, chamadas α-grânulos, grânulos densos e grânulos lisossômicos. Os maiores compartimentos são os α-grânulos que contem grandes quantidades de fatores hemostáticos, mitógenos celulares tais como Fator de Crescimento Derivado de Plaquetas (PDGF), HGF, EGF, Fator de Crescimento do Tipo

Insulina 1 (IGF-1), TGFβ, e moléculas de adesão celular. Nos grânulos densos se acumulam adenosina difosfato (ADP), adenosina trifosfato (ATP), cálcio e serotonina, que estão envolvidos na ativação e recrutamento de plaquetas. Nos lisossomos, as plaquetas contem proteases e hidrolases. Através da liberação destas moléculas ou apresentando na sua superfície celular, as plaquetas interatuam com outros tipos celulares e contribuem à reparação do tecido, imuno modulação e proliferação celular (Nurden 2011; Martínez et al. 2015; Meyer et al. 2015).

O papel das plaquetas na fisiopatologia do fígado não tinha sido reconhecido até o final dos anos 90 (Clavien and Graf 2009). Estudos *in vitro* demonstraram que as plaquetas possuem fatores de crescimento estáveis, entre eles o HGF e IGF-1, que promovem a síntese de DNA em cultura primária de hepatócitos via Akt e ERK1/2 e a proliferação de células endoteliais dos sinusóides (Matsuo et al. 2008; Kawasaki et al. 2010). Por sua vez, estudos *in vivo* tem reportado que as plaquetas acumulam-se no fígado sob algumas condições patológicas, tais como isquemia/reperfussão, cirrose, colestase, hepatite viral, e no fígado remanescente após hepatectomia (Watanabe et al. 2009; Matsuo et al. 2011; Kurokawa et al. 2015).

A depleção de plaquetas ou inibição da função plaquetária reduzem a regeneração hepática em camundongos com HP 70% (Lesurtel et al. 2006). No entanto, animais com hepatectomia parcial submetidos à trombocitose exibem maior sobrevida e regeneração do fígado mediado pela ativação das vias Akt e STAT3 (S. Murata, Matsuo, et al. 2008; Myronovych et al. 2008; Shimabukuro et al. 2009). Matsuo et al. também demonstraram um aumento da proliferação hepática após administração de plaquetas pela veia porta em modelo de hepatectomia de 70% em ratos (Matsuo et al. 2011). Murata et al. demonstraram que após depleção de CKs, a regeneração hepática estava diminuída devido à baixa expressão de TNF-α, mas foi revertida pela trombocitose induzida com níveis de HGF, IGF-1 e Akt fosforilada maiores que os controles (S. Murata, Matsuo, et al. 2008). Além disso, tem sido demonstrado que a serotonina (5HT) derivada de plaquetas e seu receptor 5HT2B são potentes iniciadores da regeneração hepática após uma ressecção maciça (Lesurtel et al. 2006; Papadimas et al. 2012).

Imediatamente após HP, as plaquetas se translocam dos sinusóides ao espaço de Disse e liberam fatores de crescimento, tais como HGF, IGF-1 e VEGF, através de contato

direto com os hepatócitos. Estes fatores estimulam o início da divisão hepatocitária (Kurokawa et al. 2015; Matsuo et al. 2011).

Por outro lado, as plaquetas são capazes de ativar leucócitos e macrófagos através de interação direta ou pela liberação de citocinas e quimocinas (Ripoche 2011). Há evidencias que as plaquetas interagem com as CK após uma lesão hepática (Nakamura et al. 1998; Tamura et al. 2012; Ogawa et al. 2013; Takahashi et al. 2013). Em resposta à administração de LPS, IL-1 ou TNF, as plaquetas acumulam-se nos sinusóides hepáticos em poucos minutos (Endo and Nakamura 1992; Nakamura et al. 1998; Ohtaki et al. 2002). Porém, quando as CK são eliminadas, não há acúmulo de plaquetas no fígado após administração de LPS, sugerindo um papel essencial das CK neste processo (Nakamura et al. 1998).

Por sua vez, as CKs são estimuladas pelo contato direto com as plaquetas. Este efeito colaborativo entre as células parece acontecer após hepatectomia quando as CK ativadas induzem a ativação e acumulação das plaquetas no fígado. Em consequência, a regeneração hepática é promovida pelos fatores de crescimentos liberados pelas plaquetas e pelo efeito parácrino das CKs (Takahashi, et al. 2013; Kurokawa et al. 2015).

1.4 Fatores parácrinos

Nas últimas décadas tem se mostrado a importância dos mecanismos parácrinos das células tronco. Os fatores secretados pelas CTM possuem propriedades tróficas, imunomoduladoras, antiapoptóticas e seu perfil parácrino varia de acordo a sua ativação inicial por vários estímulos (Doorn et al. 2012; Fontaine et al. 2016). Além disso, podem promover a repopulação das células endógenas em tecidos necróticos (Liu et al. 2015).

Em um modelo de lesão hepática aguda por D-Galactosamina, a infusão de meio condicionado de CTM aumentou a sobrevida e reduziu os níveis de IL-6, IL-1β e TNF-α. Além disso, a terapia de meio condicionado resultou numa redução da apoptose hepatocelular e num aumento da proliferação celular e a melhora da função hepática (Parekkadan et al. 2007; van Poll et al. 2008).

De fato, há muita evidencia suportando o potencial dos fatores parácrinos secretados por células derivadas da medula óssea e que promovem a reparação do tecido lesionado (Liu et al. 2006; Kieling 2012b; López et al. 2014; Uribe-cruz et al. 2015b).

1.4.1 Encapsulação celular

A microencapsulação celular é uma metodologia que permite avaliar o efeito parácrino *in vivo*. A vantagem do uso de células encapsuladas é a possibilidade de imunoisolar as células que serão transplantadas e, ao mesmo tempo, mantê-las em contato com fatores solúveis no organismo, permitindo assim elucidar a interação entre as células transplantadas e o órgão lesado.

A tecnologia de microencapsulação celular utiliza membranas de polímeros semipermeáveis, com o fim de separar e proteger células xenobióticas do sistema imune do hospedeiro (Orive et al. 2004; Meier et al. 2015). Estas microcápsulas possibilitam, conforme o diâmetro dos poros, a entrada de nutrientes e oxigênio e saída de proteínas sintetizadas pelas células. Além disso, evita que macromoléculas, como anticorpos, entrem em contato com as células transplantadas (Matte et al. 2011; Meier et al. 2015). A microencapsulação também permite a fixação das células no local da implantação e evita sua migração para outras partes do corpo (Mai et al. 2005). Este procedimento é moderadamente invasivo, permite o uso de células geneticamente modificadas, e tem a perspectiva de baixa morbidade e alta segurança (Meier et al. 2015).

Grande variedade de substâncias naturais, como o alginato, colágeno e quitosana; ou sintéticas como celulose e silicone, tem sido utilizada na produção de microcápsulas (Orive et al. 2015). O alginato de sódio, por ser barato e não imunogênico, é uma das substâncias mais amplamente empregadas na encapsulação de diferentes tipos celulares (de Vos et al. 2014; Gimi and Nemani 2013). A microencapsulação celular foi utilizada preferencialmente no desenvolvimento de órgão bioartificiais e estudo de tratamentos de doenças genéticas com produção enzimática ou gênica deficiente e de doenças endócrinas, como o diabetes, o hipotireoidismo e o hipoparatireoidismo (Matte et al. 2011; Orive et al. 2004, 2015). No Centro de Terapia Gênica do HCPA, o alginato de sódio foi utilizado para o isolamento de fibroblastos (Lagranha et al. 2008) e células renais (Baldo et al. 2012; Mayer et al. 2010) para a terapia celular experimental de doenças genéticas.

A vantagem do uso de células encapsuladas é a possibilidade de manter as células transplantadas em contato com fatores solúveis no organismo, permitindo, assim, elucidar a sua interação com o órgão lesado.

Diversos estudos avaliaram os desfechos da microencapsulação de hepatócitos em modelos de insuficiência hepática aguda mostrando uma melhora na sobrevida dos animais (Aoki et al. 2005; Mei et al. 2009; Sgroi et al. 2011; Shi et al. 2009; Ham et al. 2015). O papel benéfico dos hepatócitos encapsulados estaria relacionado com o auxílio na manutenção das funções vitais hepáticas e na regulação inflamatória da regeneração hepática (J. Mei et al. 2009; Sgroi et al. 2011).

Por outro lado, há estudos que avaliam o potencial terapêutico de células da medula óssea encapsuladas na insuficiência hepática aguda (Kieling 2012b; Liu et al. 2006; Uribe-cruz et al. 2015). Células da medula óssea encapsuladas em alginato e implantadas no peritônio de ratos com hepatectomia parcial de 90% aumentaram a sobrevida em relação aos animais que receberam células livre ou cápsulas vazias (Liu et al. 2006; Uribe-cruz et al. 2015). Liu e Chang (Liu and Chang 2005; Liu et al. 2006) sugerem que os mecanismos envolvidos na melhora da IHA sejam por transdiferenciação das células da medula óssea em células tipo hepatócitos e pela secreção de HGF aumentada em comparação ao controle. Já, Uribe et al. sugerem que o aumento na sobrevida dos animais se deve a uma redução na expressão de genes relacionados à regeneração hepática e favorecendo a morte celular por apoptose e diminuído a necrose, consequentemente aumentando a sobrevida (Uribe-cruz et al. 2015).

Plaquetas encapsuladas em alginato de sódio e implantadas no peritônio de animais com HP de 90%, também tem a propriedade de aumentar a sobrevida, em comparação com células da medula óssea microencapsuladas ou cápsulas vazias (López 2012). Neste trabalho, foi observado que o efeito benéfico das plaquetas na sobrevida é independente do contato celular direto ou da localização no fígado. Além disso, foi avaliado o efeito protetivo das plaquetas durante três dias. Estes achados sugerem que fatores parácrinos derivados das plaquetas protegem o fígado após dano hepático (Anexo 1). Apesar disso, o exato mecanismo pelo qual as plaquetas exercem seus efeitos benéficos ainda não está bem esclarecido.

2 JUSTIFICATIVA

O modelo animal de IHA é uma ferramenta essencial para entender a patofisiologia da doença e assim avaliar novas terapias. A terapia celular tem sido uma alternativa promissora, no entanto, ainda não se conhecem todos os mecanismos pelos quais as células exercem sua função benéfica no fígado lesionado.

Neste contexto, é importante pesquisar e elucidar como os fatores parácrinos atuam sobre as células hepáticas, quais são as vias de sinalização e moléculas envolvidas na melhora da função hepática.

Tendo em vista as evidências acima descritas, neste estudo foi avaliado o papel das plaquetas na terapia celular no modelo de hepatectomia de 90%.

3 OBJETIVOS

3.1 Objetivo geral

Estudar o mecanismo de ação das plaquetas encapsuladas em animais submetidos à lesão hepática aguda induzida por hepatectomia de 90% (HP 90%).

3.2 Objetivos específicos

a) Avaliar a influência das plaquetas encapsuladas na resposta ao estresse oxidativo no tecido hepático após HP 90%.

b) Avaliar o efeito das plaquetas encapsuladas nas células de Kupffer em modelo *in vitro* e *in vivo* através da liberação e expressão de genes inflamatórios relacionados à regeneração hepática.

c) Avaliar o efeito parácrino precoce das plaquetas encapsuladas em modelo de IHA.

4 RESULTADOS

Os resultados deste trabalho serão apresentados em forma de artigos científicos:

1. Encapsulated platelets modulate Kupffer cell activation and reduce oxidative stress in a model of acute liver failure (submetido à revista *Liver Transplantation*)
2. Encapsulated platelets enhance an immediate response to Acute Liver Failure

4.1 Artigo 1

ENCAPSULATED PLATELETS MODULATE KUPFFER CELL ACTIVATION AND REDUCE OXIDATIVE STRESS IN A MODEL OF ACUTE LIVER FAILURE

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Key words: platelets, kupffer cells, acute liver failure, capsules, hepatectomy

List of abbreviations: ALF, acute liver failure; DPNH, dinitrophenylhydrazine; EC, empty capsules; eNOS, endothelial nitric oxide synthase; GPX, glutathione peroxidase; GST, glutathione transferase; GSH, reduced glutathione; HNE, hydroxynonenal; KC, Kupffer cell; LPS, lipopolysaccharides; NFκB, nuclear factor kappa B; PCOOH, phosphatidylcholine hydroperoxide; PH, partial hepatectomy; PLT, platelet; SOD, superoxide dismustase.

Conflict of interest: The authors declared no conflicts of interest.

Financial support: This work received financial support from FIPE/HCPA. López M. and Matte U. are recipients of CNPq scholarships.

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Abstract

Background & aims: Acute liver failure is characterized by massive hepatocyte cell death. Kupffer cells are the first cells to be activated after liver injury. They secrete cytokines and produce reactive oxygen species, leading to apoptosis of hepatocytes. In a previous study, we showed that encapsulated platelets increase survival in a model of acute liver failure. Here we investigate how platelets exert their beneficial effect. Methods: Wistar rats submitted to 90% hepatectomy were treated with platelets encapsulated in sodium alginate or empty capsules. Animals were euthanized at 6, 12, 24, 48 and 72 hours after hepatectomy and liver was collected to assess oxidative stress, caspase activity, and gene expression related to oxidative stress or liver function. The number of Kupffer cells in the remnant liver was evaluated. Interaction of encapsulated platelets and Kupffer cells was investigated using a co-culture system. Results: Platelets increase superoxide dismutase and catalase activity and reduce lipid peroxidation. In addition, caspase 3 activity was also reduced in animals receiving encapsulated platelets at 48 and 72 hours. Gene expression of endothelial nitric oxide synthase and nuclear factor kappa B were elevated in platelet group at each time point analyzed. Gene expression of albumin and factor V were also increased in platelet group. The number of Kupffer cells in platelet group returned to normal levels from 12 hours but remained elevated in control group until 72 hours. Finally, platelets modulate Interleukin-6 and Interleukinl-10 expression in Kupffer cells after 24 hours of co-culture. Conclusions: These results indicate that platelets interact with Kupffer cells, in this model and exert their beneficial effect through reduction of oxidative stress that results in healthier hepatocytes and decreased apoptosis.

Introduction

Acute liver failure (ALF) is a life-threatening condition characterized by the sudden loss of hepatic function without previous history of liver disease[1,2]. In ALF the extent of liver cell death exceeds the liver's impressive regenerative capacity and it shares striking similarities with septic shock with regard to the features of systemic inflammation, progression to multiple organ dysfunction and functional immunoparesis[1,3]. Oxidative stress is common in various types of liver injury and plays a critical role in the mechanism of ALF[4]. This is a condition in which the cellular levels of reactive oxygen species (ROS) surpass the neutralizing capacity of enzymatic and non-enzymatic antioxidants. Injurious oxidant stress therefore may result from the excessive production of ROS, a diminution in antioxidant levels, or a combination of both[5].

Kupffer cells (KC) are the first cells to be exposed to materials absorbed from the gastrointestinal tract. Their ability to eliminate and detoxify microorganisms, endotoxins, cells in apoptosis, immune complexes, and toxic agents (e.g. ethanol) is an important physiological function[6]. ROS are released by KCs after activation with cytokines, lipopolysaccharide (LPS) and prostaglandins as a defense against bacterial invasion. Platelets, also, respond to lipopolysaccharide administration, interleukin-1, or tumor necrosis factor by accumulating in the liver sinusoidal space within a few minutes, but only in the presence of KC[7]. It has been demonstrated that platelet activation is in close interaction with KC[7–9].

In a previous study of our group[10], animals submitted to 90% partial hepatectomy (PH) and treated with platelets entrapped in alginate microcapsules showed a significant improvement in survival that was directly related to a decrease in hepatocyte hydropic

degeneration. Therefore, we further investigated the mechanism of hepatoprotection played by platelets.

Materials and Methods

Animals and experimental procedure

Ninety-percent partial hepatectomy (90% PH), platelet isolation and encapsulation were performed as described previously by our group[10]. Briefly, 90% PH was performed on two-month-old male Wistar rats that received either microencapsulated platelets (PLT) or empty alginate microcapsules (EC). Platelets were isolated from healthy animals' bone marrow with acid dextrose citrate. A solution of $1.5x10^6$ cells were mixed with sodium alginate to generate the microcapsules and 2 mL of microcapsules diluted in PBS were injected in the intraperitoneal cavity of rats immediately after 90% PH. Animals were euthanized at 6, 12, 24, 48, and 72 hours after 90% PH (n=9-12/group/time point). For some assays, both experimental groups were also compared to healthy animals (not submitted to any experimental procedure), considered as normal values. Handling, care and processing of animals were carried out according to regulations approved by local Ethics Committee of the "Hospital de Clínicas de Porto Alegre, Brazil" (protocol number 10-0288) and complied with National Guidelines on Animal Care of Brazil. The livers were collected, flash frozen in liquid nitrogen and stored at -80^oC.

Quantitative Real-Time PCR

Total RNA was extracted from 50 mg of liver tissue using TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instruction. For cell pellets, RNAeasy kit (Qiagen, USA) was used according to manufacturer's instruction. RNAs were reverse-

transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Gene expression was measured using TaqMan assays (Applied Biosystems, USA) for Nuclear factor kappa B (*Nf-κB,* Rn01399583_m1), Endothelial Nitric Oxide Synthase (*eNos*, Rn02132634_s1), Interleukin 6 (*Il-6,* Rn01410330_m1*) and 10 (*Il10,* Rn00563409_m1*), Tumor necrosis factor alpha (*Tnf-α,* Rn00562055_m1*), Albumin (*Alb*, Rn00592480_m1), and Factor V (*FV*, Rn01483178_m1). Differences in gene expression were calculated using *β-actin* (Rn00667869_m1) as internal control[11].

Protein Quantification

Total proteins were obtained by disrupting 100 mg of frozen liver in 800 μL of PBS with protease inhibitor solution (Sigma, USA) (10 μL/mL). Homogenates were centrifuged at 13000 g, for 30 min at 4ºC. The supernatant was submitted to protein quantification using the Lowry method[12].

Catalase activity

Diluted samples were incubated with 10 μL of hydrogen peroxide 200 mM and immediately scanned in a spectrophotometer (Spectramax M5, Molecular Devices) at 240 nm every 16 sec for 5 min at 30°C. Bovine liver catalase (Sigma, USA), ranging from 0.2 to 0.6 units were used for the standard curve. Catalase activity was calculated based on the rate of decomposition of hydrogen peroxide and normalized to total protein[13].

Superoxide dismutase activity

In a 96-well microplate, glycine buffer 0.05 M and catalase 10 μM were pipetted in each well. Increasing quantities of SOD from bovine liver (Sigma, USA), ranging from 0.02 to 0.1 units, or liver homogenate were added. Epinephrine (Sigma, USA) 0.02 M, pH 2.0 was rapidly pipetted (5 μL) and the plate was immediately scanned at 480 nm every 16 sec for 7 min at 32°C. SOD activity was calculated based on the oxidation of epinephrine and normalized to total protein[14].

Glutathione transferase activity

Glutathione transferase (GST) activity was measured by mixing the samples with 0.1 M potassium phosphate buffer, pH 6.0 and 4-Hydroxynonenal (HNE) 0.1 mM. The reaction was started by adding reduced glutathione (GSH) 0.5 mM final concentration. The disappearance of HNE was accompanied spectrophotometrically at 224 nm. The specific activity was calculated using the 4-HNE extinction coefficient of 13,750 M^{-1} cm^{-1} [15].

Phosphatidylcholine hydroperoxide synthesis

Phosphatidylcholine hydroperoxide (PCOOH) was synthesized by hydroperoxidation of phosphatidylcholine using soybean lipoxidase type V (Sigma-Aldrich). Briefly, 3 mM sodium deoxycholate and 0.3 mM phosphatidylcholine were added to 10 mL of 0.2 M Tris–HCl, pH 8.8, with 200,000 U soybean lipoxidase type V. The reaction was performed in constantly stirring at 25 ºC for 1 hour, then loaded onto a Strata™-X reverse phase cartridge that had been activated with 9 mL methanol and 18 mL water. The sample mixture was applied into the cartridge and washed with 10 mL water. PCOOH was eluted with 1.5 mL methanol and it concentration was determined spectrophotometrically (100-

fold dilution) using ε234 = 25,000 M−1cm−1. The final concentration of PCOOH in methanol was typically 1 mM.

Glutathione peroxidase 4 (GPx-4) activity

Liver samples were mixed in assay medium containing 1.5 mM potassium cyanide, 7 mM reduced glutathione, 1 U/mL of gluthatione reductase, and 0.1 M potassium phosphate buffer, pH 7.0. The reaction started by adding of PCOOH (100 μM) into the assay medium, and was accompanied by the disappearance of NADPH at 340nm. The activity was calculated using the NADPH molar coefficient 25,000 $M^{-1}cm^{-1}$ [16].

Determination of total sulfhydryl groups

The reaction started with the addition of Tris/EDTA 3 mM pH 8.0 (5:1). After, the basal absorbance was measured at 412 nm, the Ellmans's reagent (5,5'-dithiobis-(2-nitrobenzoic acid))[17] was added in each well and incubated for one hour in the dark. The plate was scanned at 412 nm. Total sulfhydryl groups was normalized to total protein.

Quantification of carbonyl groups

Protein (5mg) was mixed with 0.5 mL of a 0.1% solution of 2,4-DNPH (in 2 M hydrochloric acid), and 2 M hydrochloric acid was added until 1 mL of solution. For reaction blank, the procedure was the same except for adding 2,4-DNPH. The samples were incubated for 30 min vortexing every 10 min. After that, 250 μL of 50% ice-cold trichloroacetic acid were added in each tube and incubated for 5 min in ice. The samples were centrifuged at 11000 g for 3 min and the pellet was washed with 1 mL of ethanol-

ethyl acetate (1:1; v/v) to remove free DNPH reagent 3 times. The pellet was resuspended in 1 mL of 6 M guanidine with 2 mM potassium phosphate buffer (pH 2.3). The samples were vortexed and incubated at 60ºC for 10 minutes until the dissolution of the pellet. The samples were transfer to a microplate and scanned at 380 nm[18].

Thiobarbituric acid reactive substance (TBARs) assay

Liver homogenate (0.25 mg of protein) with TCA 20% (1:1, final volume 300 µL) was vortexed vigorously and centrifuged at 3000 g, 4°C for 10 min. The supernatant (100 µL) was removed and 100 µL of thiobarbituric acid (TBA) 0.67% was added and vortexed. For the standard curve tetramethoxypropane was hydrolyzed in H_2SO_4 1% and TBA (100 µL) was added and vortexed. All microtubes were incubated at ºC for 15 min, 300 µL of butanol was added in each tube and centrifuged at 1000g, 4° C for 10 min. The samples were transfer to a microplate and scanned was measured at 532 nm.

Caspase 3 activity

Caspase 3 activity was measured using a fluorimetric substrate assay (Sigma-Aldrich, USA), according to manufacturer's instruction. Briefly, 100 µg of liver was placed in an opaque 96-well plate and 200 µL of mixture reaction solution (containing Acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin) was added in each well. The plate was incubated in the dark at 25ºC and every 10 min the fluorescence was read at 360 nm of excitation and 460 nm of emission. Caspase 3 activity was normalized by total protein.

Immunohistochemistry

Kupffer cells were quantified by immunostaining for CD68. For that, paraffin-embedded liver specimens were cut in 4 μm sections and immunohistochemistry was performed using the primary antibody rabbit IgG-CD68 (1:800, Abcam, USA) incubated over night at 4 ºC, after washing with PBS-tween 20 biotinylated link universal and streptavidin-HRP were added (Dako, USA) and revealed with DAB kit (Dako, USA). The slides were counterstained with hematoxylin. The number of $CD68^+$ cells was counted in 5 randomly selected HPF (x400) per slide.

Kupffer cell isolation

For KCs isolation, healthy donor animals (n=2) were anesthetized by inhalation of isofluorane and placed in a supine position. The abdomen was opened and the portal vein was cannulated with a 22G catheter. Immediately, the inferior venous cava was cut to perfuse the liver *in situ* with PBS at a rate of 10 ml/min for 5 min. The liver was then excised and washed twice with PBS. The organ was minced to small pieces and digested using 30 ml of 1 mg/mL collagenase type IV (Sigma, USA) at 37ºC for 30 min. Then, liver homogenates were filtered through a gauze to remove undigested tissue fragments. The filtrate was washed by centrifuging twice at 300g, at 4ºC for 5 min. The pellet was resuspended and differential centrifugation at 50g (4ºC) for 3 min was performed to separate non-parenchymal from parenchymal cells. Finally, the supernatant was centrifuged at 300g for 5 min. The cell pellet was seeded on six-well culture plates in complete culture medium Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum, 100 mg/mL streptomycin and 100 U/mL penicillin[19]. Following incubation for 2 h in a humidified atmosphere of 5% carbon dioxide (CO2) at 37ºC, the

cells were gently washed with fresh culture medium without fetal calf serum and submitted to the co-culture system.

Co-culture system

To analyze the interaction between platelets and Kuppfer cells through paracrine factors we have performed a two-chamber co-culture system. For that, Kupffer cells ($4x10^6$) were seeded in the lower chamber and encapsulated platelet ($1.5x10^6$) were placed in the upper chamber isolated using a 6-well cell culture insert with 0.4 μm pore sizes. Three groups were performed, KC-PLT, KC-EC and KC alone and maintained for 6 or 24 h. After that, KCs were collected to analyze cytokine expression.

Statistical analysis

Quantitative data was expressed as mean ±SD or median ± minimum to maximum values. Comparisons between groups were made using an unpaired Student's t-test or Mann-Whitney in each time point according to variable distribution. In some cases when compared to normal values, the test used was One Way ANOVA.

Results

Antioxidant enzymes activities

In order to investigate if encapsulated platelets could modify oxidative stress in remnant liver tissue the activities of SOD and catalase were analyzed (figure 1A). SOD activity in both groups did not change at 6 h after 90% PH in comparison to normal range; however, in PLT group it began to increase after 12 h compared to EC group ($P \leq .01$) and normal

values until 72 h ($P \leq .02$). At 24 h PLT group still presented higher levels, compared to normal animals ($P < .01$), but not different form EC group ($P = .20$). After 48 and 72 h both groups had significantly increased SOD activity ($P < .001$) when compared to normal values.

Catalase activity was reduced in experimental groups at 6 h compared to normal values ($P < .001$). For EC group, catalase activities were lower compared to PLT group at 24 and 48 h after 90% PH ($P < .01$). Moreover, in PLT group catalase activity was increased at 48 h compared to normal values ($P \leq .01$, figure 1B).

On the other hand, when we assessed the enzymes responsible for the clearance of peroxidation by-products there were no differences between groups for both GST and GPX-4 activities ($P > .05$, supplementary figure 1). These results suggest that encapsulated platelets may promote the activity of enzymes that prevent ROS formation in the liver, but have no role in counteracting their effects.

Protein damage, lipid peroxidation, and antioxidant capacity

Regarding the production of carbonylated proteins, a measure of protein damage, no differences were observed between groups over time (supplementary figure 1). These results suggest that the protein damage caused by hepatectomy was similar in both groups and not reverted by encapsulated platelets.

On the other hand, lipid peroxidation evaluated by TBARS assay showed that encapsulated platelets protect the liver from lipid damage, as EC group showed increased lipid peroxidation at 6 ($P < .01$), 24 and 72 h after 90% PH ($P < .001$; figure 1C) compared to normal values and PLT group.

The antioxidant capacity was determined by measuring the content of reduced thiols. An equal decrease in sulfhydril content was observed in both experimental groups, mainly in the first 6 h after 90% PH ($P < .001$ vs normal values; supplementary figure 1). Liver sulfhydryl levels increased over time similarly in both groups, albeit at 24 h the groups showed a decrease compared to normal values ($P < .01$). At 72 h both groups reached normal levels.

Liver function and apoptosis

The ultimate consequence of lipid peroxidation is apoptosis; so we assessed Caspase 3 activity in liver homogenates. At 6 h after PH, caspase activity was highly increased mainly in EC group ($P <.001$). PLT group showed more activity at 12 h compared to EC group, it was not different to normal values. In addition, PLT group showed less caspase 3 activity from 48 h until 72 h after 90% PH compared to EC group ($P < .05$, figure 2A). This result suggests that encapsulated platelets may prevent cell death in this model.

The expression of *Nf-κB* and *eNos*, genes involved in mechanisms related to oxidative stress, were analyzed by qPCR in remnant livers. Levels of *eNos* and *Nf-κB* expression were higher at all time points in PLT group compared to EC group ($P \leq .04$; $P \leq .05$, respectively, figure 2B-C). In order to assess liver function, gene expression of *Alb* and *FV* were performed. The expressions of these genes were higher in PLT group at 24 h ($P = .02$ and $P < .01$, respectively), at 48 h ($P = .01$ and $P = .03$, respectively) and at 72 h ($P \leq .01$, figure 2D-E) compared to EC group. These results suggest that encapsulated platelets improve liver synthesis.

Number of Kupffer cells

As KC have been implicated in oxidative stress and are known to interact with platelets, the number of KC was determined by counting the number of $CD68^+$ cells (figure 3). Both groups presented an elevated number of $CD68^+$cells at 6 h compared to normal liver. Nevertheless, PLT group showed a decrease to normal number of $CD68^+$ cell from 12h to 72h. On the other hand, in EC group the number of $CD68^+$ cells remained elevated until 72h ($P < .01$, figure 3B). These data indicate that encapsulated platelets may regulate KC recruitment and persistence in liver.

Platelet and Kupffer cells interactions *in vitro*

In order to identify an interaction between KC and encapsulated platelet, a two-chamber co-culture system was performed. After 6 h or 24 h of co-culture cytokines expression of KCs were analyzed. At 24 h, *Il-6* expression was higher and *Il-10* expression was lower in EC-KC group compared to PLT-KC group ($P = .03$ and $P = .01$ respectively, figure 4A-B). Interestingly, this modulation occurs in a similar manner in the liver tissue of animals treated 6 h after PH (figure 4C-D). For *Nf-κB* and *Tnf-α* expression were not observed differences between groups (supplementary figure 2).

Discussion

In a previous work, we have shown that encapsulated platelets improve survival of rats submitted to 90% PH[10]. We also showed that untreated animals (EC group) had swollen hepatocytes due to hydropic degeneration. Since the production of free radicals because of liver injury could be a cause of water accumulation in cells, here we investigate the role of encapsulated platelets in the oxidative stress response.

Oxidative stress is common in various types of liver injury and plays a critical role in the mechanism of ALF, contributing to hepatocyte injury and death by DNA, protein, and lipid damage[4]. The amount of carbonyls, a measure of oxidized proteins, was similar in animals treated with platelets or empty capsules. In addition, total sulfhydryl domains, that indicate the cellular redox/oxidative nature, were not different between groups (supplementary figure). Therefore, platelets do not influence protein damage or alter cell redox/oxidative ratio after partial liver resection.

On the other hand, encapsulated platelets reduce lipid peroxidation to normal values in all time points evaluated, indicating a protective effect against oxidative stress. The membrane lipids containing polyunsaturated fatty acids are predominantly susceptible to peroxidation which alter physicochemical properties of membrane lipid bilayers, resulting in severe cellular dysfunction and leads to spread of free radical reactions[20,21].

Encapsulated platelets also enhance the activity of antioxidant enzymes, such as catalase and SOD in the remnant liver. It is known that platelets are rich in antioxidant enzymes[22], but how encapsulated platelet stimulate the liver to augment these enzymes is still unclear.

ALF induces damages to cellular organelles and causes disruption of intracellular homeostasis. Also, the elicited immune response eventually activates liver cell death pathways[23]. Activation of caspase 3, a major mediator of both apoptotic and necrotic cell death[24], was 4-fold greater at 72 h in EC group compared to PLT group. These data suggest that platelets may inhibit apoptosis or they may protect liver cell from damage and therefore death pathway is not activated. Additional evidence suggesting a protective effect of encapsulated platelets was the increase in hepatic function, measured as the expression of *Alb* and *FV*.

In partial hepatectomy, the blood flows to a reduced remaining liver[25–27], leading to shear stress and eNOS activation by NF-κB[28]. In PLT group, *eNOS* expression was more than two-fold higher compared to EC group values. It is known that platelets express eNOS[29], and our data suggests that apparently they can also induce *eNOS* expression in the liver tissue. This increase could contribute to the observed reduction in lipid peroxidation. In addition, *Nf-κB* gene expression was more than twice in PLT group compared to EC group. It is important to point out that NF-κB present multiple roles in regulating genes involved both in promoting cell death and in survival[30]. In this case, it seems that NF-κB activation is associated with enhanced expression of NO synthase[28], which contributes to protection of hepatocytes from apoptosis[27].

KCs play a central role in the homeostatic response to liver injury, through the production and release of a wide array of mediators that provide physiologically diverse and key paracrine effects on all other liver cells[31]. The number of KC was increased in both groups at 6 h after PH but for PLT group this number decreased over time, contrary to what was observed for EC group. It has been demonstrated that platelets have a role in regulating KC activation, through 'touch-and-go' interactions under basal conditions[32,33]. It is also known that, in response to various pathologic conditions, including ischemia/reperfusion, cirrhosis and viral hepatitis, platelets accumulate in the liver, mainly in the place of Disse[8,34]. The key aspect in our study is that the platelets are encapsulated in sodium alginate and they are capable to regulate KC without direct cell-cell contact. Or, they could recruit endogenous platelets that play this role.

Co-culture experiments confirmed that platelets have an influence on KC activation by paracrine factors. *Il-6* levels decreased and *Il-10* levels increased in KC after 24 h of co-culture with encapsulated platelets. Interestingly, these same changes were observed in liver tissue from animals treated with encapsulated platelets at 6 h post-hepatectomy.

In general, our data suggests that the decreased number of KC diminishes ROS and in consequence, cell apoptosis is decreased, which results in higher survival. Noteworthy, all these events are mediated by paracrine factors released by encapsulated platelets and measured in the liver tissue (figure 5). The mechanism suggested here has to be confirmed by other studies and it may not apply to ALF of different etiologies.

In this work, we have suggested a possible mechanism for the increased survival observed with encapsulated platelets after 90% PH. The regulation of KC diminished oxidative stress and apoptosis resulting in healthier hepatocytes that are able to sustain life support during the first 72 h of liver regeneration.

Legends

Figure 1. Activity of antioxidant enzymes and lipid peroxidation in the remnant liver after 90% partial hepatectomy (PH) in platelet group (PLT) and empty capsules group (EC). (A) Superoxide dismutase (SOD) activity was higher in PLT group at 12 h compared to EC group (*P ≤ .01) or normal values at 12, 24 h (§P ≤ .02). At 48 and 72 h, both groups showed increased SOD activity compared to normal levels (§P < .001). (B) Catalase activity was increased in PLT group mainly at 24 and 48 h (*§P < .01) after PH compared to EC group or normal values. (C) PLT group presented less lipid peroxidation at 6 h (*P < .01), 24 h and at 72 h (*P < .001) compared to EC group. *difference vs. PLT group, § difference vs. normal value. One way ANOVA, Bonferroni post hoc. Horizontal shaded bar represents normal values.

Figure 2. Liver function and apoptosis in the remnant liver after 90% partial hepatectomy (PH) in platelet group (PLT) and empty capsules group (EC). (A) Caspase 3 activity was increased in EC group at 6 h compared to PLT group (P < .001), and increased in PLT group at 24 h (*P = .03) compared to EC group. EC group showed increased caspase activity at 48 h (*P = .02) until 72 hours (*P < .001). (B) Endothelial nitric oxide synthase (*eNOS*) expression was increased at all time points for PLT group (*P ≤ .04; *P < .01) compared to EC group. (C) Nuclear factor kappa B (*Nf-κB*) expression was increased in PLT group (*P ≤ .03) at all time points. (D-E) Albumin and Factor V expression were higher in PLT group (*P < .05) compared to EC group. Student's t-test or Mann-Whitney test. Horizontal shaded bar represents normal values.

Figure 3. Kupffer cells in the remnant liver after 90% partial hepatectomy (PH) in platelet group (PLT) and empty capsules group (EC). (A) Liver section immunostained with IgG-CD68 24 h after 90% PH, the arrow indicates $CD68^+$ cells in EC (above) and PLT (below) groups. (B) Quantification of $CD68^+$ cells over time after partial hepatectomy. PLT group presented diminished number of $CD68^+$ cells compared to EC group (*P < .01). Student's t test.

Figure 4. Cytokine expression of Kupffer cells (KC) in the co-culture system and in the liver of platelet group (PLT) and empty capsules group (EC). (A-B) Interleukin 6 (*Il-6*) was decreased and 10 (*Il-10*) was increased in KC co-cultured with encapsulated platelet (KC-PLT) compared to KC co-cultured with empty capsules (KC-EC) at 24 h of co-cultered (*P = .03 and *P = .01, respectively). (C-D) Similar changes were observed in liver tissue from animals 6 h after partial hepatectomy (HP) (*P < .01). Student's t-test or Mann-Whitney test.

Figure 5. Suggested mechanism for platelet action after 90% partial hepatectomy. (A) Time course of events in the 90% partial hepatectomy model. Platelets do not prevent such events but seem to reduce the intensity of damage. (B) Detailed scheme of events mentioned in A. Kupffer cells (KC) are activated and release ROS causing lipid peroxidation, which ultimately leads to cell death by caspase activation. (C) Same scheme as B, showing the proposed action of platelets. Encapsulated platelets are able to regulate KC and/or reduce ROS and consequently decrease cell apoptosis, which results in better hepatic function. Dotted lines represent suggested interactions.

Supplementary figure 1. Antioxidant enzymes activity and cell damage in the remnant liver after 90% partial hepatectomy in platelet group (PLT) and empty capsules group (EC). (A) Gluthatione reductase (GST) (B) and Gluthatione peroxidase 4 (GPX-4) were similar in both groups. (C) Carbonylated proteins did not show differences between groups. (D) Sulfhydryl domains increased over time equally in both groups, although PLT group showed lower levels at 24 h compared to EC group (*P = .001). *difference vs. PLT group, §difference vs. normal value. One way ANOVA, Bonferroni post hoc. Horizontal shaded bar represents normal values.

Supplementary figure 2. (A-B) Cytokine expression of Kupffer cells (KC) in the co-culture system and (C-D) in the liver of platelet group (PLT) and empty capsules group (EC). (A) Nuclear factor kappa B (*Nfκ-B*) expression and (B) Tumor necrosis factor alpha (*Tnf-α*) were similar for KC co-cultured with empty capsules (KC-EC) and co-cultured with encapsulated platelet (KC-PLT). (C) *Nfκ-B* was increased in PLT group compared to EC group (P < .01). (D) *Tnf-α* did not show differences between groups. Student's t-test.

Figures

Figure 1

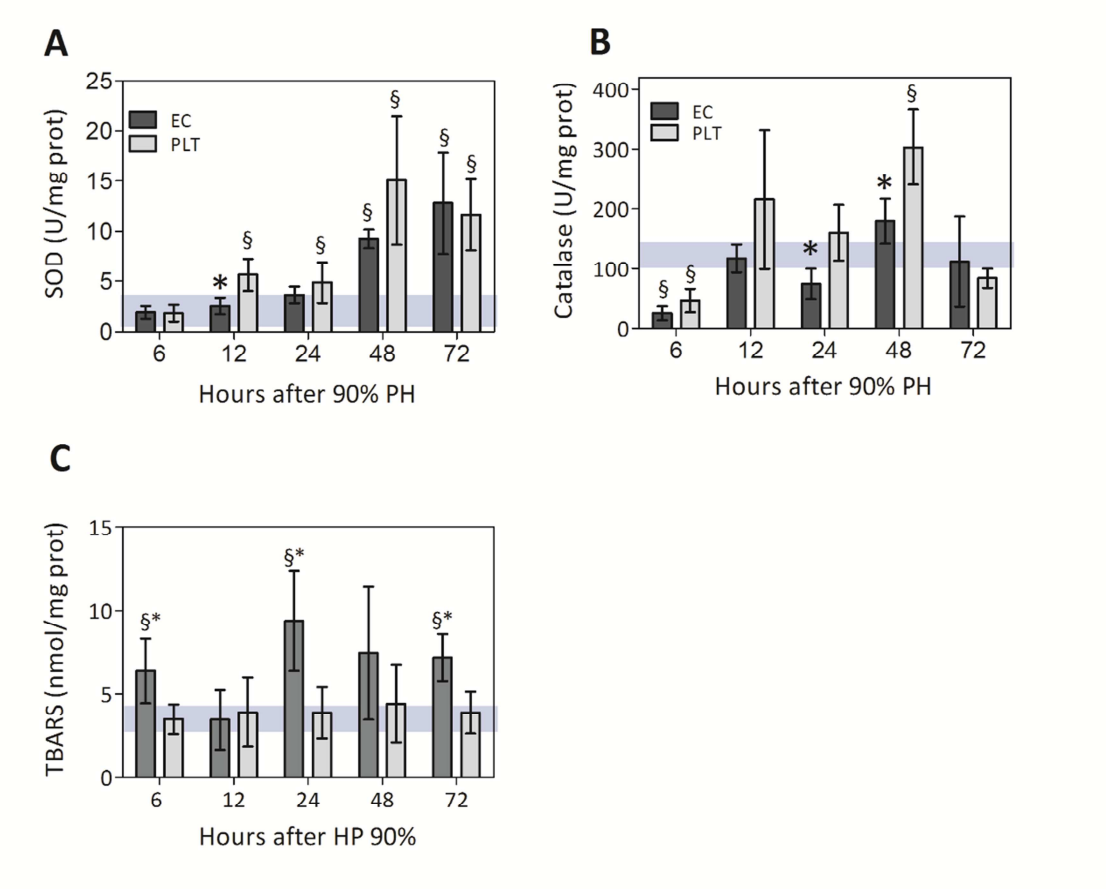


Figure 2

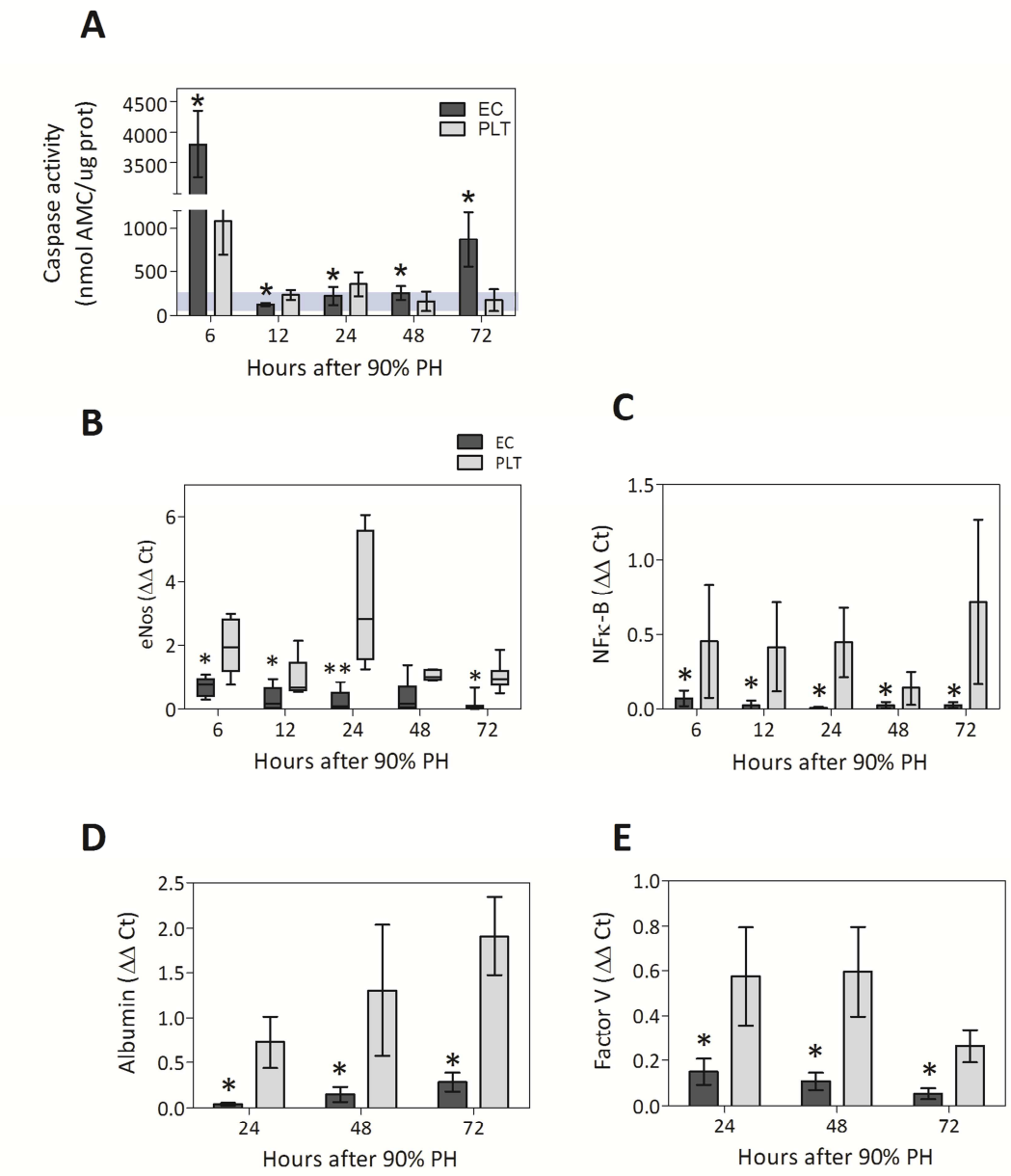

A
Caspase activity (nmol AMC/ug prot)
4500
4000
3500
1000
500
0
EC
PLT
6
12
24
48
72
Hours after 90% PH
B
eNos (ΔΔ Ct)
EC
PLT
Hours after 90% PH
C
NFκ-B (ΔΔ Ct)
1.5
1.0
0.5
0.0
Hours after 90% PH
D
Albumin (ΔΔ Ct)
2.5
2.0
1.5
1.0
0.5
0.0
Hours after 90% PH
E
Factor V (ΔΔ Ct)
1.0
0.8
0.6
0.4
0.2
0.0
Hours after 90% PH

Figure 3

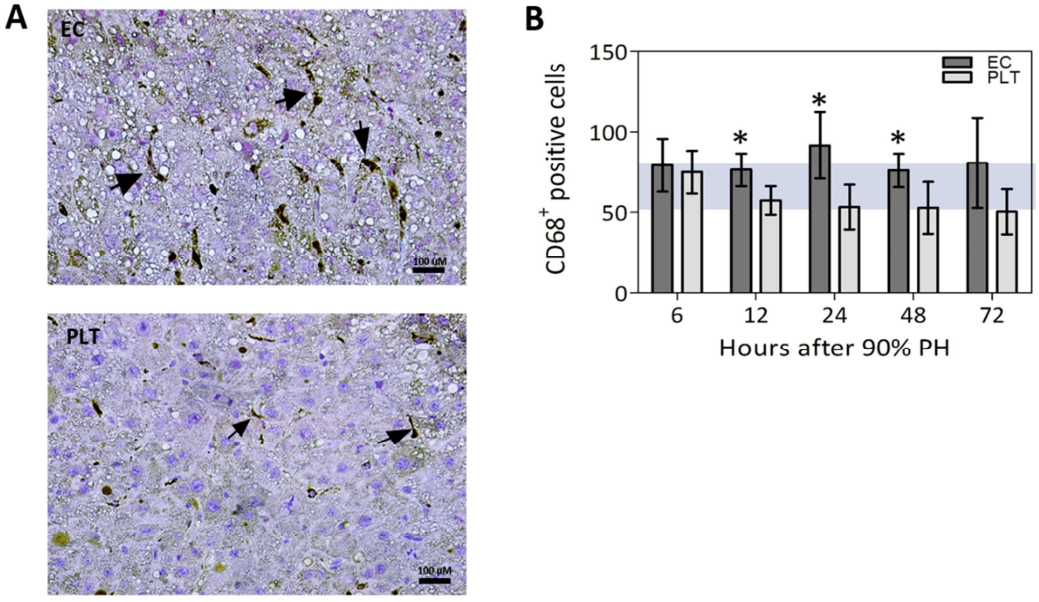

A
EC
100 uM
PLT
100 uM
B
CD68+ positive cells
150
100
50
0
EC
PLT
*
*
*
6
12
24
48
72
Hours after 90% PH

Figure 4

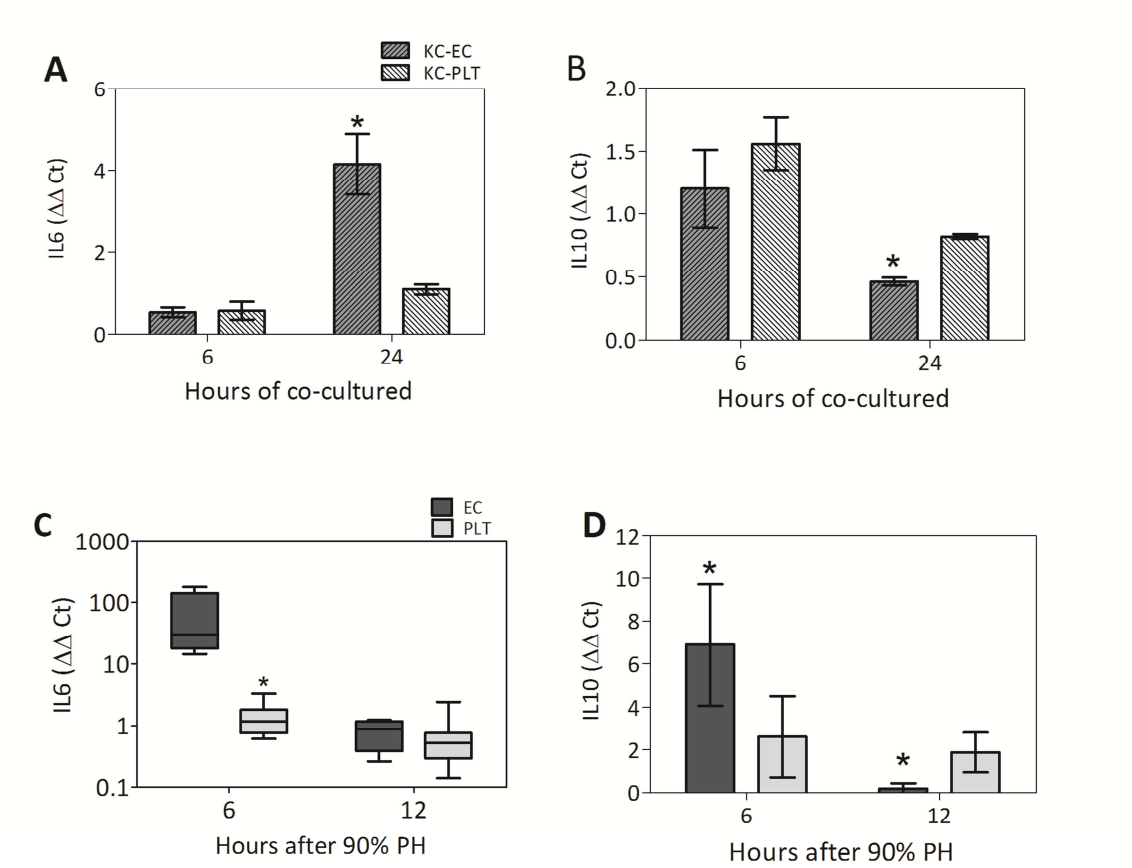
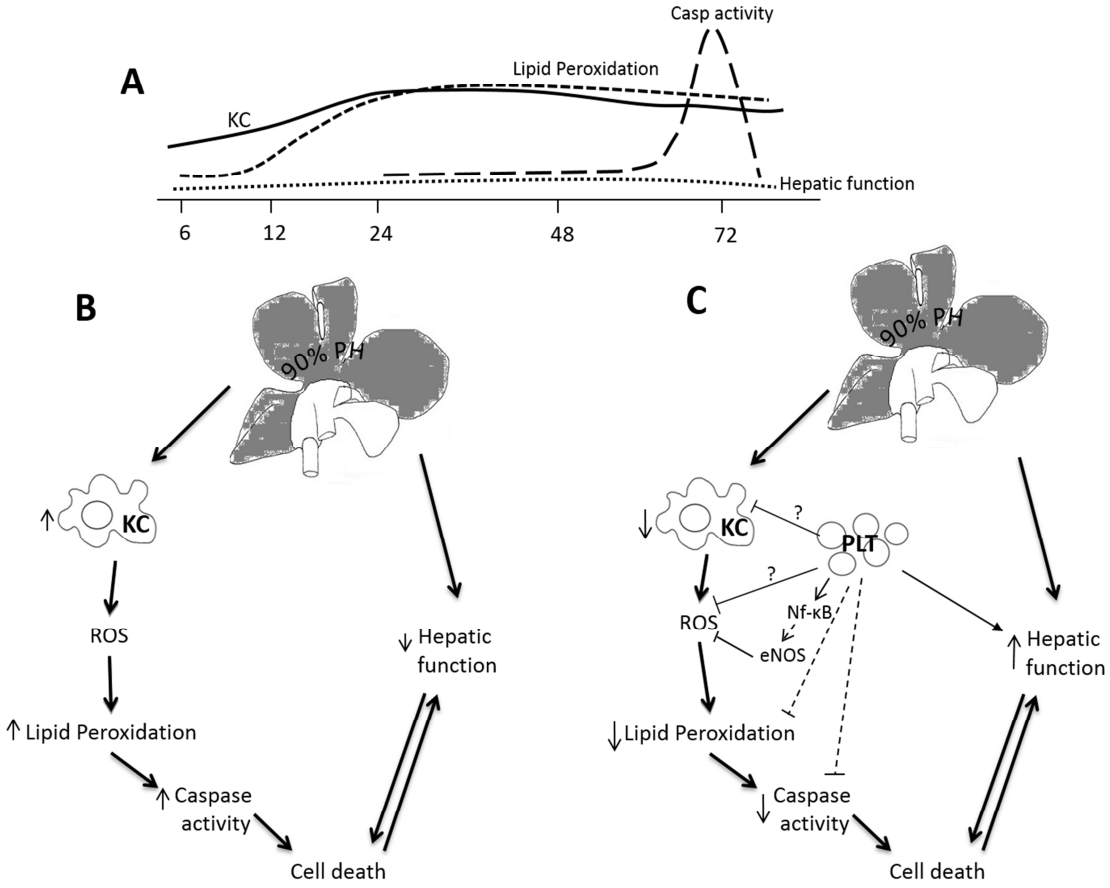
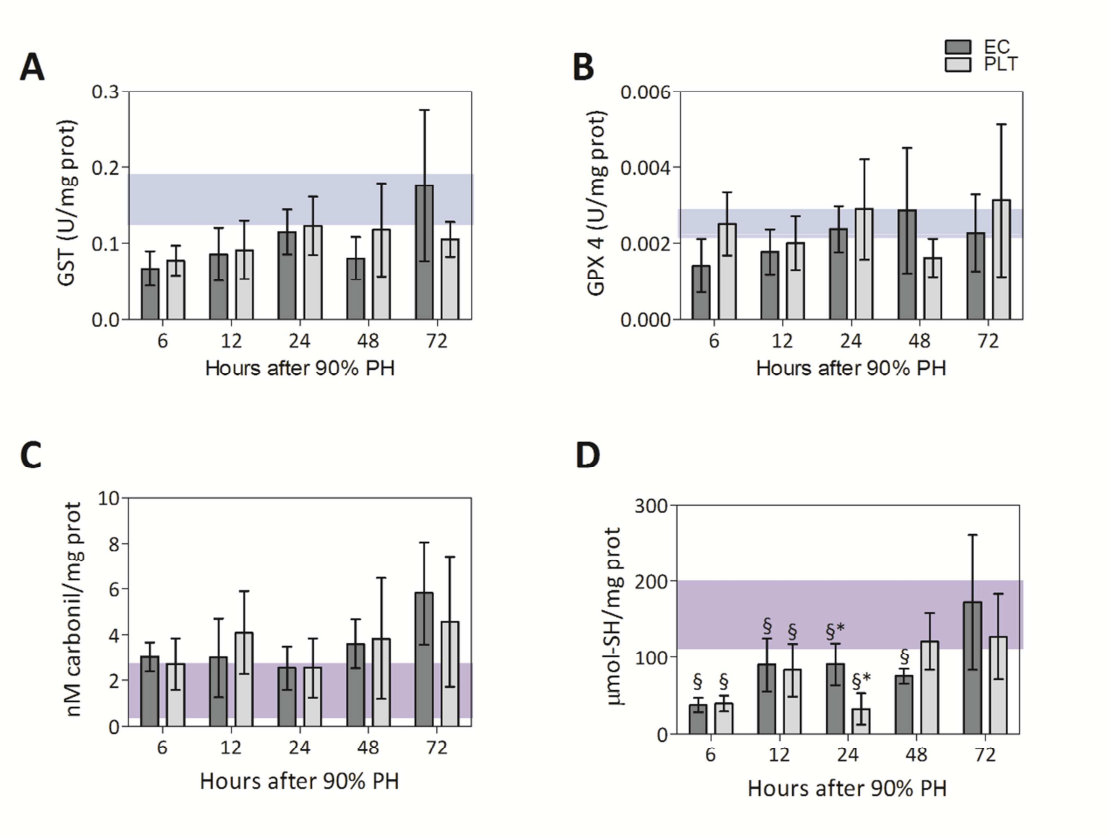

A
KC-EC
KC-PLT
IL6 (ΔΔ Ct)
Hours of co-cultured
B
IL10 (ΔΔ Ct)
Hours of co-cultured
C
EC
PLT
IL6 (ΔΔ Ct)
Hours after 90% PH
D
IL10 (ΔΔ Ct)
Hours after 90% PH

Figure 5


A
Casp activity
Lipid Peroxidation
KC
Hepatic function
6
12
24
48
72
B
90% PH
KC
ROS
↑ Lipid Peroxidation
↑ Caspase activity
Cell death
↓ Hepatic function
C
90% PH
KC
?
PLT
?
ROS
Nf-κB
eNOS
↓ Lipid Peroxidation
↓ Caspase activity
Cell death
↑ Hepatic function

Suplemmentary figure 1



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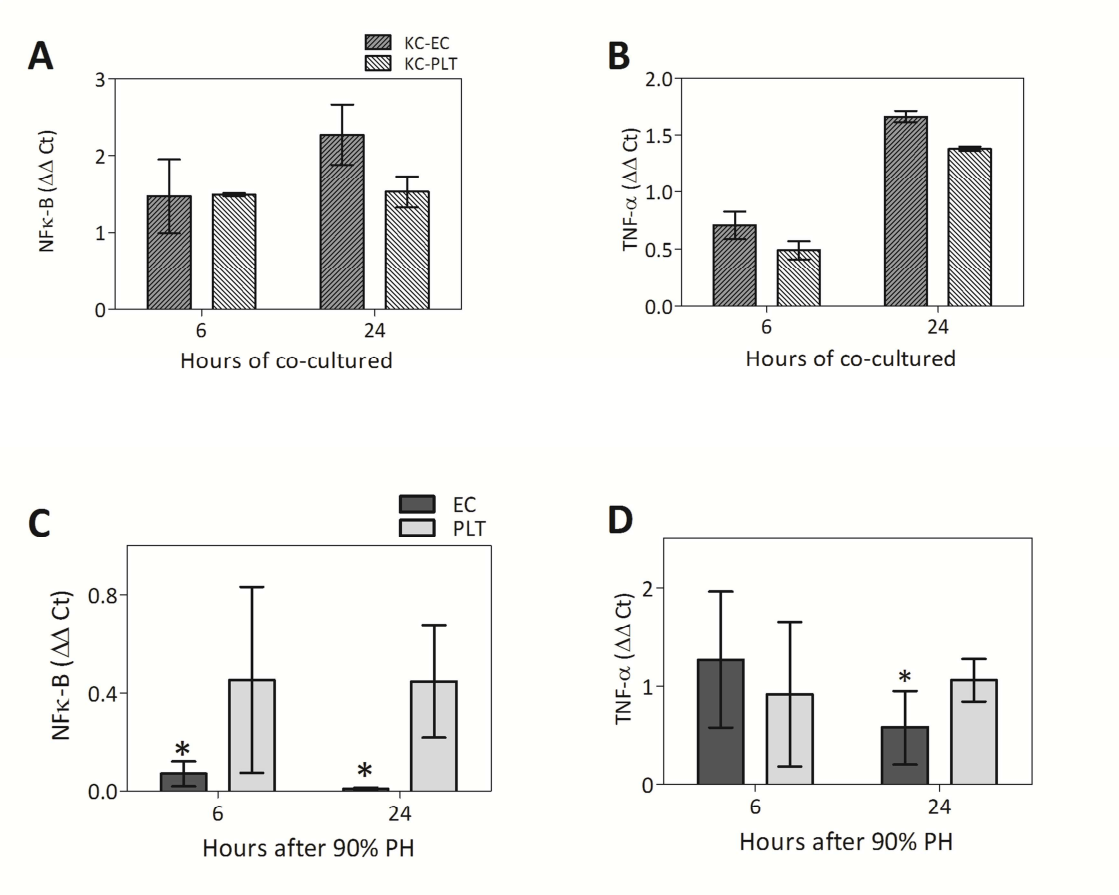

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4.2 Artigo 2

ENCAPSULATED PLATELETS ENHANCE AN IMMEDIATE RESPONSE TO ACUTE LIVER FAILURE

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Keywords: Platelet, hepatectomy, acute liver failure, capsules, Kupffer cells.

1. ABSTRACT

Backgorund and aims: Platelets increase survival and liver regeneration in animals with partial hepatectomy through paracrine effect. In this study, we investigate the early effects of microencapsulated platelets in a liver failure model. *Method*: Encapsulated platelets or empty capsules were implanted in rats submitted to 90% partial hepatectomy (PH). Animals were euthanized at 1 and 3 hours (n = 6/group/time) after hepatectomy to evaluate the number of Kupffer cells, gene expression of inflammatory cytokines and acute response in the liver, lipopolysaccharides (LPS) levels in serum and tissue and oxidative stress response. *Results:* Liver from treated group group presented increased number of Kupffer cells in 3 hours compared to empty capsules. LPS levels were similar in both groups, as well as *Tlr4* and *Myd88*, but *Lbp* expression was higher in platelet group. Moreover, *Il6* and *Nf-κB* were increased in platelet group compared to empty capsules. No differences were found in SOD and Catalase activities at 3 hours post PH. *Conclusion*: Encapsulated platelets regulate the number of Kupffer cells and cytokine expression by paracrine factors immediately after liver injury.

2. INTRODUCTION

Acute liver failure (ALF) is a severe liver disease resulting from rapid loss in hepatocyte function in patients with previously normal liver function. The clinical feature of ALF is encephalopathy and coagulopathy which are life-threatening conditions and require immediate hospitalization [1,2]. Orthotropic liver transplantation is still considered the most effective treatment, although the lack of a suitable donor in a short period can limit the success of this therapy[3]. Nevertheless, the liver has a unique ability to regenerate after injury or resection[4]. Cell therapy with bone marrow-derived cells is a promising alternative that can enhance the regenerative capacity of the liver[5-8].

There is accumulating evidence that platelets play an important role in wound healing and tissue regeneration[9]. In the last years, it has been reported that platelets prevent hepatic failure and stimulate liver regeneration after extensive hepatectomy by releasing growth factors[10-15]. In a previous study of our group[11], animals submitted to 90% partial hepatectomy (PH) and treated with platelets entrapped in alginate microcapsules showed a significant improvement in survival. Furthermore, encapsulated platelets regulate Kupffer cells number and enhance oxidative stress response 6 hours after hepatectomy[16].

The purpose of the present study was to investigate early effects of platelets on the liver. For that, immobilized platelets in a semipermeable membrane were implanted in the peritoneal cavity of animals submitted to 90% PH and 1 or 3 hours after livers were collected.

3. MATERIAL AND METHODS

3.1 Animals and Experimental Design

Ethics Committee in Animal Research of the Hospital de Clinicas de Porto Alegre approved the procedures and protocols here described. All animals received humane care according to the National Institute of Health guidelines.

Wistar rats were randomly divided in two groups. Control group (n=12) received empty alginate microcapsules (EC); treated group received microencapsulated platelets (PLT, n=13). The animals were euthanized at 1 and 3 hours after 90% PH (n=6/group/time point) to evaluate the early effects of treatments. For some assays, both experimental groups were

also compared to healthy animals (not submitted to any experimental procedure), considered as normal values. Another 8 animals were used as donors for platelet isolation.

3.2 Platelet Isolation

Donor Wistar rats (n=8) were euthanized with a lethal dose of anesthesia. Then, tibias and femurs were removed, and the bone marrow was flushed out of the bone cavities with acid citrate dextrose (pH 5) in a 1:4 volume and centrifuged at 150 g for 10 minutes. Platelets in the supernatant were then washed twice by centrifugation at 1000 g at 4°C for 15 minutes, and resuspended in citrate buffer (Mónica L López et al. 2014). They were counted in an automatic hematological counter (ABX Micros 60, Germany), diluted to $1.5x10^7$ platelets/mL and submitted to the encapsulation protocol.

3.3 Capsules Production

Platelets ($1.5x10^7$ platelets/mL) were mixed with 1.5% sodium alginate (Sigma-Aldrich, USA) in Dulbecco's Modified Eagle Medium (DMEM) (LGC, Brazil) and extruded through a Encapsulation Unit, type J1 (Nisco, Switzerland), attached to JMS Syringe Pump at a rate of infusion 40 mL/h. Droplets were sheared off with an air flow of 5 L/min delivered to the tip of a 27 G needle and they fell into a bath of 125 mM $CaCl_2$ where ionically cross-linked with $Ca^{2}+$ to form solid spherical hydrogel beads containing embedded platelets. In each well capsules were produced from a volume of 2 mL of alginate suspension. For control group, 2 mL of empty capsules were produced using the same approach without platelets. The resulting capsules were maintained under normal cell culture conditions, DMEM supplemented with 10% Fetal Calf Serum (Gibco®, USA) and Penicilin/Streptomycin 1% (Gibco®, USA) at 37ºC and 5% CO_2, for 24h prior to administration.

3.4 Surgical Procedure and Capsules Transplantation

Ninety percent hepatectomy was performed by the resection of the left lateral (30%), left median (40%), and the right superior lobes (20%), leaving only the caudate lobe. Hepatectomy was carried out under isoflurane (Forane®, Abbott SA, Buenos Aires, Argentina) anesthesia and by a single operator. Immediately after 90% PH, and before

complete laparotomy closure, microcapsules (either empty or containing platelets) were placed into the peritoneal cavity and glucose was supplemented i.p. (5% of body weight). Postoperatively, animals received 20% glucose in their drinking water and standard chow *ad libitum*.

3.5 Euthanasia

Euthanasia was performed by lethal anesthesia at 1 and 3 h after PH. Immediately, 2 mL of blood were collected by heart puncture and the liver was removed and weighted. Part of the liver was then flash frozen in liquid nitrogen and stored in -80ºC for RNA and protein extraction. The other part was fixed for histological analysis in 10% neutral-buffered formalin. Serum was separated by centrifugation and stored at -80ºC until analysis.

3.6 Histology and Immunohistochemistry

Paraffin-embedded liver specimens were cut in 4 µm sections and stained with hematoxylin and eosin (H-E). Microscopic liver aspect was observed by an expert pathologist blinded to the study groups. A semi-quantitative evaluation of necrosis was done in in 5 randomly selected high power fields (HPF, x400) per slide.

Kupffer cells were quantified by immunostaining for CD68. For that, the sections were treated with 0.3 % H_2O_2 in methanol for 20 min to block endogenous peroxide activity, and then incubated with a primary antibody rabbit IgG-CD68 (1:800, Abcam, USA) over night at 4 ºC. After washing with PBS-tween 20 biotinylated link universal and streptavidin-HRP were added (Dako, USA) and revealed with DAB kit (Dako, USA). The slides were counterstained with hematoxylin. The number of $CD68^+$ cells was counted in 5 randomly selected HPF (x400) per slide.

3.7 Lipopolysaccharides (LPS) levels

Serum and tissue levels of LPS were measured using Rat LPS ELISA kit (Cussabio, USA) according to manufacter's guidelines. Tissue levels of LPS were normalized by protein quantity.

3.8 Protein extraction and quantification

Total proteins were obtained by disrupting 100 mg of frozen liver in 800 µL of 1X PBS with protease inhibitor (Sigma, USA) solution (final concentration 10 µL/mL). Homogenates were centrifuged at 13000 g, for 30 minutes at 4ºC. The supernatant was collected and proteins were quantified using the Lowry assay.

3.9 Catalase activity

Catalase activity was determined as described by Li and Schellhorn[17]. Diluted samples were incubated with 10 µL of substrate solution (hydrogen peroxide 200 mM) and immediately scanned in a spectrophotometer (Spectramax M5, Molecular Devices) at λ 240 nm every 16 sec for 5 min at 30°C. Varying quantities of bovine liver catalase (Sigma, USA), ranging from 0.2 to 0.6 units were used for the standard curve. Catalase activity was calculated based on the rate of decomposition of hydrogen peroxide, which is proportional to the reduction of absorbance at λ 240 nm. Catalase activities of liver extracts were normalized to total protein in the lysate and expressed as units per mg of protein.

3.10 Superoxide dismutase activity

Superoxide dismutase (SOD) activity was measured as described by Misra and Fridovich[18]. In a 96-well microplate, glycine buffer 0.05 M and catalase 10 µM was pipetted in each well. Increasing quantities of SOD from bovine liver (Sigma, USA), ranging from 0.02 to 0.1 units, or liver homogenate (diluted 1:3) were added to glycine buffer 0.05 M and catalase 10 µM in a 96-well microplate. A volume of 5 µL of epinephrine (Sigma, USA) 0.02 M, pH 2.0 was rapidly pipetted and the plate was immediately scanned in a spectrophotometer (Spectramax M5, Molecular Devices) at λ 480 nm every 16 sec for 7 min at 32°C. SOD activity was calculated based on the oxidation of epinephrine, which is proportional to the increase of the absorbance. The SOD activity of liver homogenate were normalized to total protein and expressed as units per mg of protein

3.11 Quantitative Real-Time PCR

Total RNA was extracted from 50 mg of liver tissue using TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's guidelines. For each sample, 2 µg of total RNA were reverse-transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Gene expression was measured using TaqMan assays (Applied Biosystems, New Jersey, USA) for Nuclear Factor kappa B (*Nf-κB,* Rn01399583_m1*), Lipopolysaccharide binding protein (*Lbp,* Rn00567985_m1), Interleukin 6 (*Il-6,* Rn01410330_m1*), Toll-like receptor 4 (*Tlr4,* Rn00569848 m1*), Myeloid differentiation primary response gene 88 (*Myd88*, Rn01640049 m1*) and Tumor necrosis factor alpha (*Tnf-α,* Rn00562055_m1*). Differences in gene expression were calculated using the ΔΔCt method and β-actin (Rn00667869_m1) as internal control

3.12 Statistical Analysis

Results were expressed as means ± SD or medians when required. Student's t test or Mann-Whitney test were used in PASW Statistic 18 software. In some Fisher Exact test was used. $P < 0.05$ was considered to be significant.

4. RESULTS

4.1 Platelets increase the number of Kupffer cells

Liver sections were analyzed by a blinded pathologist. All groups presented hydropic degeneration and steatosis mainly in zone 3 (figure 1A). Necrosis of isolated cells was also observed, especially in EC group 1 hour after 90% PH, albeit not significant (Fisher Exact test, data not shown).

The number of CD68+ cells, a marker of KC, was assessed. PLT group showed more CD68+ cells per liver section compared to EC group 1 h after PH (P=0.03, figures 1B-C).

4.3 Platelets influence Kupffer cells response

It has been reported that after hepatectomy or liver injury LPS derived from intestine is upregulated in the blood flow(Taub 2004), therefore we assessed serum and liver LPS levels. Interestingly, there were no differences compared to normal values, nor between experimental groups. However, gene expression in the liver tissue of *Lbp,* the LPS-binding

protein, was upregulated in PLT group 3 hours after PH compared to EC group (P<0.01, figure 2).

LPS is also sensed by TLR4, which induces the MyD88-dependent downstream signaling pathways. Gene expression of *Tlr4* was similar in both groups at each time point analyzed (figure 3D-E). As well, *Myd88* increased over time equally in the groups.

Then, we assessed the downstream gene expression of LPS pathway in the liver. *Tnf-α* expression was similar in both group and did not change over time. Nevertheless, *Nf-κB* expression was increased 3 hours after PH in PLT group when compared to EC group (P=0.02, figure 3B). Moreover, *Il-6* expression was also increased in PLT group at 1 and 3 hours (P= 0.01 and P< 0.01, respectively). These results suggest that platelet activate inflammatory genes related to survival cell response.

4.4 Platelets have no effect in oxidative stress response immediately after PH

We have observed that encapsulated platelet enhance oxidative stress response [16]. Thus, we evaluated antioxidant enzymes activities such as SOD and catalase. It was not observed differences in the SOD activities between groups over time. Interestingly, catalase activity was increased in EC group 1 hour after PH (P<0.01, figure 4). This indicate that encapsulated platelet do not improve oxidative stress response immediately after a 90% PH.

5. Discussion

This study was designed to assess the early effect of microencapsulated platelets in an animal model of acute liver failure. In a previous study[11], we have shown that platelets enhance survival of rats submitted to PH and that this effect is mediated by paracrine factors. Moreover, the outcomes were observed at 6 hours after liver injury. Here, we investigated earlier effects of platelets in the liver after 90% PH, namely at 1 and 3 hours post PH.

ALF, like septic shock, is associated with an overwhelming activation of the immune response[19]. After partial hepatectomy or liver injury, gut derived factors such as lipopolysaccharide (LPS) are upregulated and reach the liver through the portal blood activating KC[20]. These cells are partly responsible for the initial pro-inflammatory

response to injury with cytokine production and the recruitment of multiple inflammatory effector cells that mediate tissue injury[21,22]. However, some evidence suggest that KC possess anti-inflammatory activity and may play a role in recovery from acute liver damage[22]. It has been demonstrated that platelets have a role in regulating KC activation[23,24]. We have shown that encapsulated platelets can downregulate KC *in vitro* and *in vivo* by paracrine factors, resulting in less oxidative stress and apoptosis in hepatocytes 6 hours after 90% PH[16]. In this work, KC number was increased in the first hour in PLT group after PH compared to EC group. This result suggests that platelet may be accelerating KC response to liver resection.

To rule out whether there were differences in LPS translocation between groups, we evaluated LPS levels in serum as in liver tissue. Surprisingly, there were no differences between groups and in both cases LPS was within normal range. It is worth noticing, however, that LPS values were very variable within groups, so we could be at presence on a statistical error type II. Nevertheless, we analyzed the cascade downstream LPS signaling.

Gene expression of *Lbp* was highly increased in PLT group three hours after PH. LBP has a concentration-dependent dual role in the pathogenesis of gram-negative sepsis: low concentrations of LBP enhance the LPS-induced activation of mononuclear cells, whereas the acute-phase rise in LBP concentrations inhibits LPS-induced cellular stimulation[25]. LBP upregulation suggests an increased host defense as an underlying mechanism[26].

LPS is recognized by LBP and facilitates the transfer to TLR4[27]. There were no differences in gene expression of *Tlr4* nor *Myd88* between groups at any time point. Yet, *Il6* and *Nf-κB* were increased in PLT group compared to EC group. It is known that the MyD88-independent pathway also activates NF-κB[27]. Besides that, after PH, the blood flows to a reduced remaining liver[28], leading to shear stress and activation of NF-κB[29]. Blockade of NF-κB activation followed by PH leads to hepatocyte apoptosis[30]. Thus, encapsulated platelets may enhance NF-κB role in cell survival. On the other hand, IL6 signaling has an important role in liver regeneration. However, IL6 is not a direct mitogen, instead it primes hepatocytes to respond to growth factors and enter in cell cycle[31,20]. Moreover, IL6-gp130-dependent signaling is not a direct influence on cell cycle progression after partial hepatectomy but is known to activate protective pathways important to enable hepatocyte proliferation[32].

Oxidative stress is common in various types of liver injury and plays a critical role in the mechanism of ALF, contributing to hepatocyte injury and death by DNA, protein, and lipid damage[33]. In a previous study, hepatocytes from a liver submitted to 90% PH were more swollen compared to the treated group with encapsulated platelets[11], so we evaluated oxidative stress in those animals, and we observed a better response to oxidative damage[16]. Here, surprisingly, EC group showed more catalase activities 1 hour after PH compared to PLT group, however, at 3 hours catalase activities returned to normal values. We were unable to measure reactive oxygen species (ROS) as they stimulate catalase activity, so we cannot discard the possibility of more ROS in EC group. This issue should be further investigated.

In conclusion, we have shown an early beneficial effect of encapsulated platelet after 90% PH by paracrine factors. Platelets improve the response to liver injury by regulating KC and cytokines expression immediately after liver injury and this effect is consistent throughout the liver regeneration.

LEGENDS

Figure 1. Liver aspect and Kupffer cells count after 90% partial hepatectomy (PH) in platelet group (PLT) and empty capsules group (EC). A) Liver section stained with HE 3 hours after PH. Head arrow indicates hydropic degeneration in zone 3 (Z3). B) Immunostaining for Kupffer cells marked with CD68 1 hour after PH. Arrow indicates CD68+ cells. C) PLT group showed more CD68+ cells compared to EC group 1 hour after PH (*P=0.03). Student-t test.

Figure 2. Quantitation of lipopolysaccharides (LPS) levels and its receptors after 90% partial hepatectomy (PH) in platelet group (PLT) and empty capsules group (EC). A) Serum LPS levels and B) liver LPS levels were similar in both group. C) LPS-binding protein (*Lbp*) gene expression in the liver was increased in PLT group 3 hours after PH (*P<0.01). D) Toll-like receptor 4 (*Tlr4*) did not show differences between group. E) Myeloid differentiation primary response gene 88 (*Myd88*) gene expression increased 3 hours equally in both group. (C and E) Student t test. (D) Man-Whitney test. Pointed lines represents normal range.

Figure 3. Cytokines expression in the liver after 90% partial hepatectomy (PH) in platelet group (PLT) and empty capsules group (EC). A) Tumor necrosis factor alpha (*Tnf-α*) did not show differences between groups. B) Nuclear factor kappa B (*Nf-κB*) was higher 3 hours after PH in PLT group (P=0.02). C) Interleukin 6 (*Il6*) expression was upregulated in PLT group 1 and 3 hours after PH compared to EC group (*P= 0.01 and *P< 0.01, respectively). Student t test.

Figure 4. Enzyme activities in the liver after 90% partial hepatectomy (PH) in platelet group (PLT) and empty capsules group (EC). A) Superoxide dismutase (SOD) activities were similar in both groups over time. B) Catalase activity was increased at 1 hour after PH in EC group compared to PLT group (*P<0.01). Student t test. Pointed lines represents normal range.

FIGURES

Figure 1

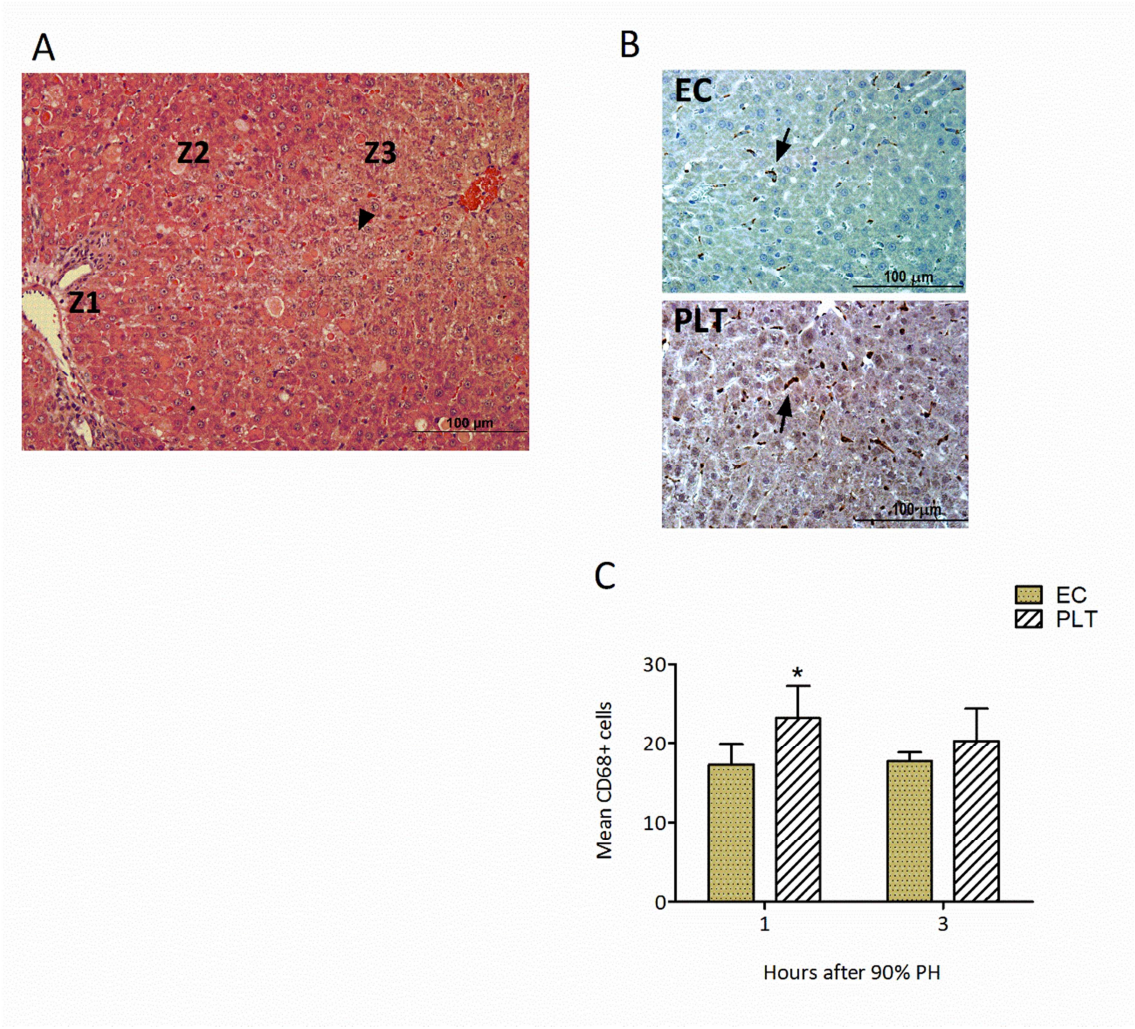


Figure 2

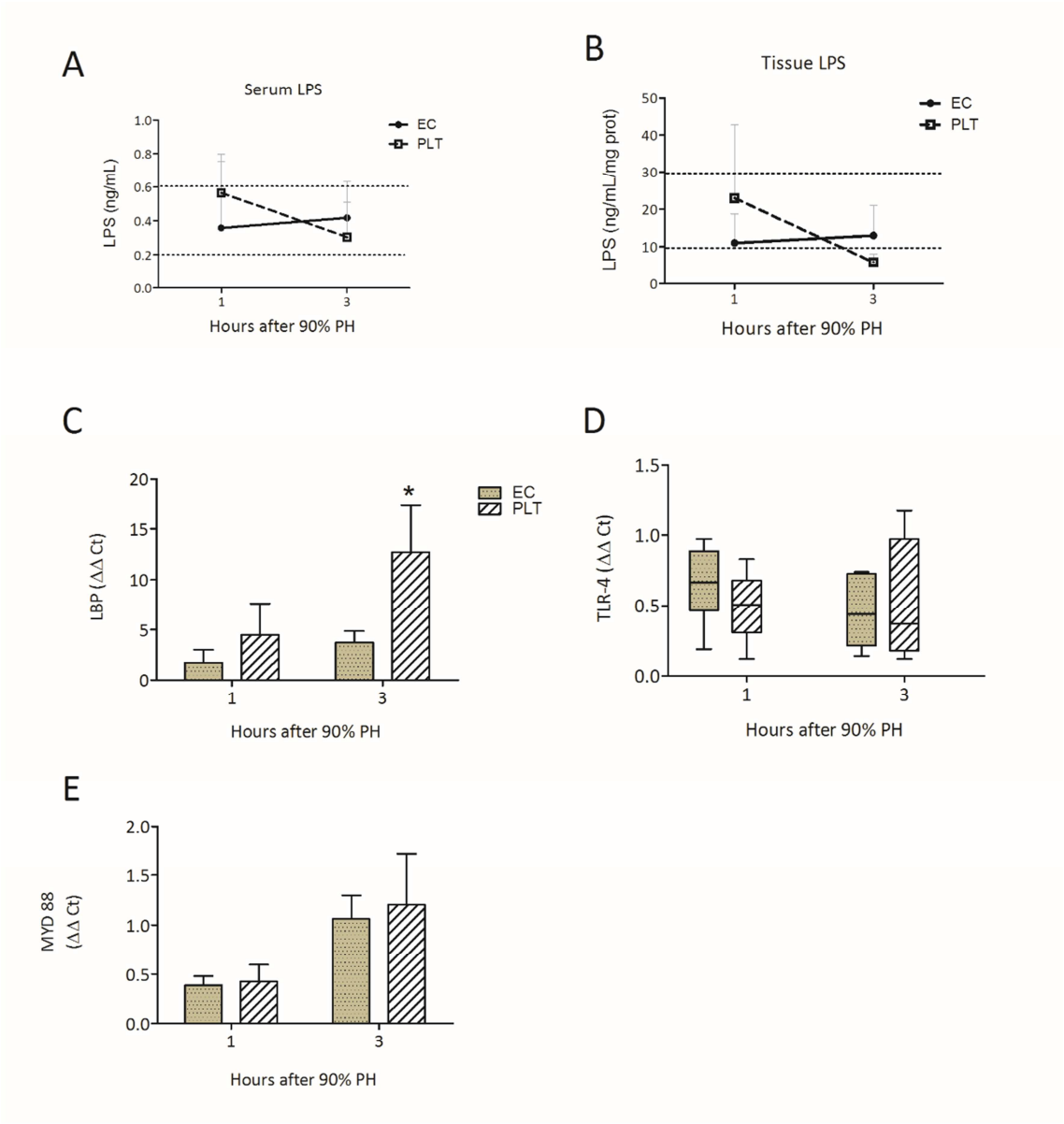

A
Serum LPS
LPS (ng/mL)
1.0
0.8
0.6
0.4
0.2
0.0
EC
PLT
1
3
Hours after 90% PH
B
Tissue LPS
LPS (ng/mL/mg prot)
50
40
30
20
10
0
EC
PLT
1
3
Hours after 90% PH
C
LBP (ΔΔ Ct)
20
15
10
5
0
*
EC
PLT
1
3
Hours after 90% PH
D
TLR-4 (ΔΔ Ct)
1.5
1.0
0.5
0.0
1
3
Hours after 90% PH
E
MYD 88
(ΔΔ Ct)
2.0
1.5
1.0
0.5
0.0
1
3
Hours after 90% PH

Figure 3

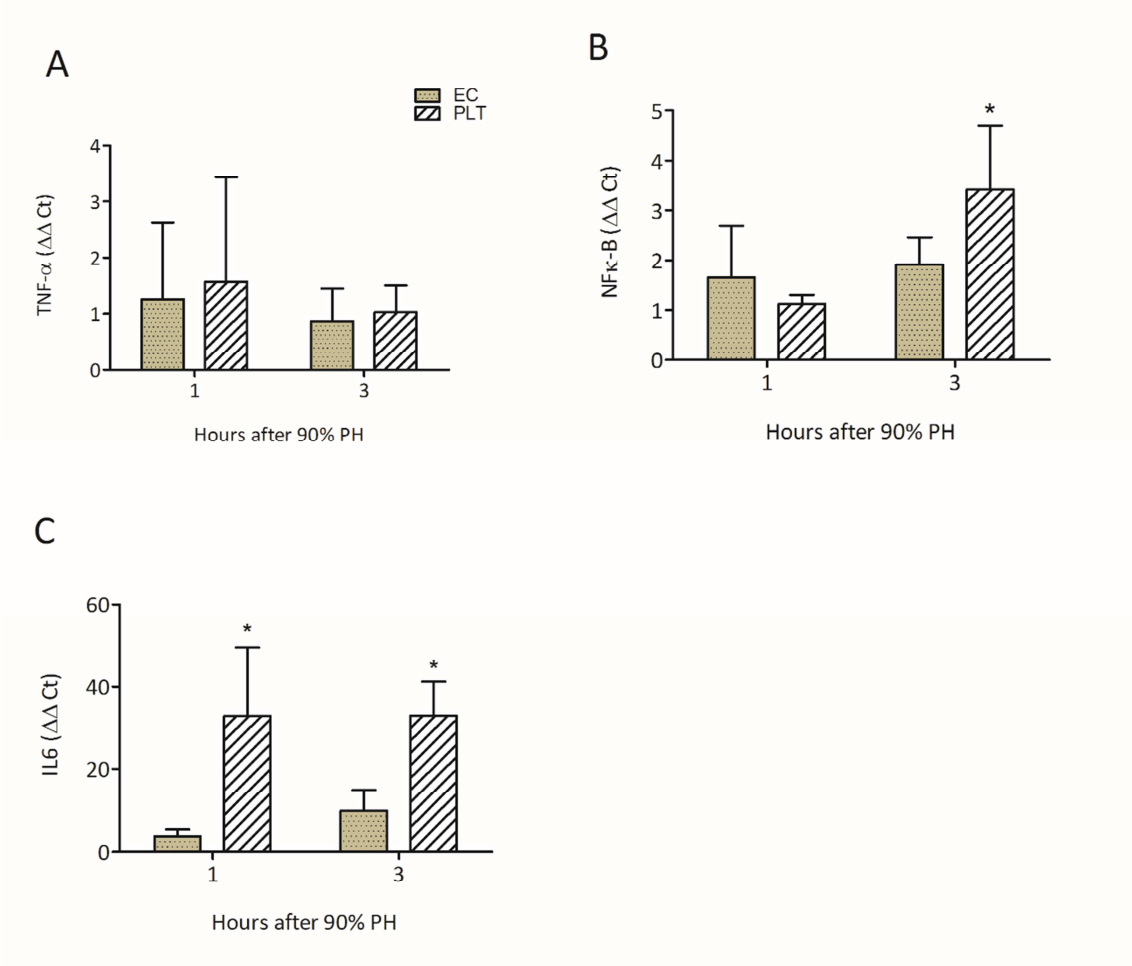
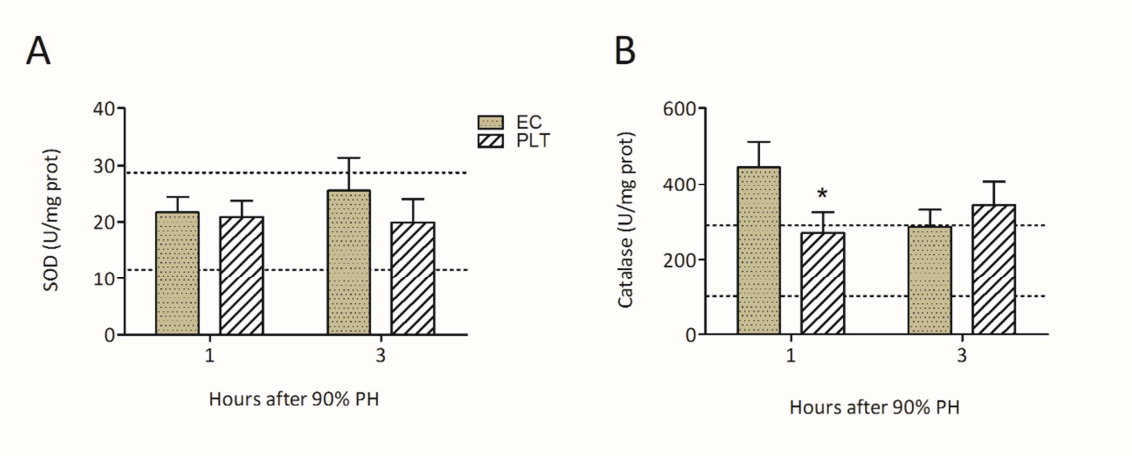

A
EC
PLT
TNF-α (ΔΔ Ct)
0
1
2
3
4
1
3
Hours after 90% PH
B
NFκ-B (ΔΔ Ct)
0
1
2
3
4
5
*
1
3
Hours after 90% PH
C
IL6 (ΔΔ Ct)
0
20
40
60
*
*
1
3
Hours after 90% PH

Figure 4


A
SOD (U/mg prot)
40
30
20
10
0
1
3
Hours after 90% PH
EC
PLT
B
Catalase (U/mg prot)
600
400
200
0
*
1
3
Hours after 90% PH

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5 DISCUSSÃO

A IHA pode ser consequência de várias etiologias desde hepatoxicidade até infeção por vírus das hepatites. Apesar dos avanços na área, o tratamento da IHA continua a ser um dos problemas mais desafiadores em medicina clínica (Maes et al. 2016). A terapia celular tem sido proposta como um método alternativo ao transplante hepático (Muraca 2011). As células transplantadas substituiriam ou melhorariam a função do tecido ou órgão danificado. O desenvolvimento e avaliação de uma nova terapia celular para doenças hepáticas requer o uso de um modelo animal apropriado (Goradel et al. 2015). Um bom modelo experimental de avaliação de terapias para IHA requer alta mortalidade antes da regeneração hepática e uma janela terapêutica adequada (Terblanche and Hickman 1991).

Nesse sentido, as hepatectomias extensas representam um bom modelo de dano hepático agudo (Goradel et al. 2015). Além disso, as ressecções hepáticas em roedores também são comumente usadas para estudar regeneração hepática, função hepática, resposta ao transplante hepático "*small-for-size*" e resposta metabólica ao dano hepático (Martins et al. 2008).

Os modelos de hepatectomia de 70% são os mais valiosos e mais estudados para a regeneração do fígado (Fausto and Riehle 2005; Martins et al. 2008). Neste modelo todos os animais sobrevivem, portanto, não é adequado como modelo para IHA (Makino et al. 2005). Panis et al. (Panis et al. 1997) mostraram que ressecções maiores levam a uma mortalidade proporcional ao aumento progressivo da hepatectomia. Após hepatectomia de 85 e 90%, as taxas de sobrevida são de 18 e 0%, respectivamente (Martins et al. 2008). Porém, a mortalidade elevada associada a grandes ressecções pode ser reduzida pela administração de glicose solúvel (Gaub and Iversen 1984; Kieling et al. 2012). Além disso, um controle na glicemia dos animais e a correção da hipoglicemia com a administração intraperitoneal de 5% glicose em ratos com 90% de ressecção, pode elevar a sobrevida até 80% (He et al. 2010). Em estudo prévio do nosso grupo (Kieling 2012b), observamos que no modelo de ressecção de 90% do fígado com reposição de glicose intraperitoneal e na água de beber a sobrevida foi de 5% em 10 dias; resultando em um bom modelo de IHA e com uma janela terapêutica adequada.

Este modelo foi usado para avaliar o efeito de plaquetas encapsuladas em alginato de sódio como terapia para IHA (López 2012). A sobrevida em 10 dias aumentou significativamente em comparação com animais controles que receberam capsulas vazias.

Os animais do grupo controle apresentaram uma mortalidade ao longo dos 10 dias, enquanto que no grupo tratado com plaquetas encapsuladas, a mortalidade concentrou-se nos primeiros três dias após a hepatectomia, o que nos levou a pensar em um efeito benéfico precoce das plaquetas (Anexo 1). Por este motivo, investigamos em detalhe o período imediatamente após a HP e durante 3 dias. Para isso, os animais foram eutanasiados em 1, 3, 6, 12, 24, 48 e 72 horas após cirurgia e implantação das cápsulas.

Após um dano hepático ou hepatectomia parcial, produtos derivados do intestino que chegam ao fígado pela veia porta, tais como os LPS estão aumentados em relação ao fígado funcional (Taub 2004). Pacientes com IHA tem aumentado os níveis séricos de LPS comparados a pessoas saudáveis (Wu et al. 2014). Portanto, quantificamos os níveis séricos no tecido hepático de LPS nas primeiras 3 horas após à HP. Não observamos diferenças entre os grupos, e estes se mantiveram dentro dos limites normais. Vale ressaltar que a variabilidade dentro de cada grupo foi alta levando, talvez, a um erro estatístico do tipo II. Também é notável que o comportamento nos níveis de LPS foi igual dentro de cada grupo tanto no soro como no tecido. No grupo EC, a concentração de LPS se mantém ao longo do tempo analisado; enquanto que no grupo PLT há um aumento na primeira hora com diminuição nas 3 horas após HP (P=0,3). Sabe-se que as bactérias são capazes de interagir com as plaquetas e induzir sua ativação. Esta interação pode ser direta, através de proteínas de superfície nas bactérias e receptores na membrana da plaqueta, ou pode ser uma interação indireta na qual proteínas plasmáticas se ligam à superfície bacteriana e subsequentemente a receptores nas plaquetas (Engstad et al. 1995; Fitzgerald et al. 2006; Kerrigan and Cox 2010). Nossa hipótese portanto era de que as plaquetas pudessem estar atuando sobre a translocação bacteriana ou sobre a produção de LPS pelas bactérias. Porém, nosso dados não nos permitem comprovar experimentalmente esta hipótese. O fato dos animais não serem isogênicos, o que aumenta a variabilidade entre os grupos, pode ter contribuído para essa ausência de diferença.

De qualquer forma, quantificamos o número de CK no tecido hepático, já que são as primeiras células que ficam expostas ao material proveniente do trato gastrointestinal, e portanto são as primeiras em se ativar após um dano hepático (Kolios et al. 2006; Taub 2004). Estas células são responsáveis pela resposta inicial pró inflamatória ao dano através da produção de citocinas e o recrutamento de múltiplas células efetoras que promovem a lesão do fígado (Kolios et al. 2006; Possamai et al. 2010). Porém, há evidências que sugerem que as CK possuem também atividade anti-inflamatória e desempenham um papel

na recuperação de lesão hepática aguda (Possamai et al. 2010). Observamos que há um aumento no número de CK na primeira hora após HP no grupo PLT em comparação ao grupo EC, mas que essa diferença diminui a partir das 12 horas. Tem sido demonstrado que as plaquetas são capazes de regular a ativação das CK em condições normais através da interação '*touch-and-go*' (Jenne et al. 2011; Wong et al. 2013). Além disso, vários estudos mostram que as plaquetas se acumulam no espaço de Disse em resposta à isquemia e reperfusão, cirrose, colestase e hepatite viral (Takahashi, Kozuma, et al. 2013; Tamura et al. 2012). O aspecto chave do presente trabalho é que as plaquetas estão encapsuladas e são capazes de regular as CK sem contato direto; ou elas atuam recrutando plaquetas endógenas que exercem esse papel. Apesar de não podermos comprovar este recrutamento de plaquetas para o fígado, medimos a quantidade destas no sangue periférico antes e após hepatectomia. Não foram achadas diferenças entre os grupos (dados não mostrados), pelo que inferimos que as plaquetas encapsuladas não promovem o aumento de plaquetas endógenas circulantes. No entanto, uma análise mas precisa envolveria avaliar a presença de plaquetas autólogas no fígado remanescente.

Para demonstrar que as plaquetas têm um efeito parácrino realizamos um o experimento *in vitro* de co-cultura com CK. Medimos a expressão de citocinas nas CK após 6 e 24 horas de co-cultivo. A expressão gênica de *Il-6* nas CK estava diminuída sendo que a expressão de *Il-10* estava aumentada 24 horas após o co-cultivo com as plaquetas encapsuladas. Esses dados se correlacionaram com os observados no tecido hepático de animais tratados com plaquetas 6 horas após hepatectomia. É sabido que as plaquetas secretam vários fatores importantes para hemostasia, regeneração do tecido e ativação do sistema imune (De Pascale et al. 2015; Golebiewska and Poole 2015), mas quais são esses fatores que influenciam na ativação das CK ainda não está bem esclarecido. Em nosso caso, tivemos dificuldades em avaliar esses fatores parácrinos pois o material biológico de dentro das cápsulas recuperadas após o transplante não é de boa qualidade, impedindo uma adequada padronização dos métodos e interpretação dos dados.

A partir dos dados sobre as CK, avaliamos a cascata de sinalização do LPS. Observamos um aumento na expressão gênica da proteína de ligação ao lipopolissacarideo (*Lbp*) três horas após HP. A LBP é uma proteína de fase aguda produzida pelos hepatócitos que se liga ao LPS sérico (Su et al. 2000). A LBP facilita a associação entre o LPS e CD14, que irá se acoplar ao TLR4. A LBP tem um duplo papel dependente da concentração do LPS, concentrações baixas de LBP aumentam a ativação das células mononucleares

induzida por LPS, enquanto que o aumento agudo de concentrações de LBP inibe a estimulação celular induzida por LPS (Gutsmann et al. 2001): As plaquetas encapsuladas parecem estimular a expressão de *Lbp* o que pode estar relacionado com o menor número de CK a partir das 3 horas após HP.

Quando avaliamos a expressão de *Tlr4* e *Myd88* não encontramos diferenças entre os grupos nas primeiras 3 horas. No entanto, a expressão de *Nf-κB* estava aumentada no grupo PLT em todos os tempos avaliados. As vias independentes de MyD88 também ativam NF-κB (Lu et al. 2008). Por exemplo, o aumento do fluxo sanguíneo em relação ao tamanho do fígado remanescente após hepatectomia (Michalopoulos 2010), leva a um estresse por fricção e consequentemente ativação de NF-κB (Grumbach et al. 2005). Este fator de transcrição tem múltiplas funções tanto fisiológicas como patológicas, promovendo a sobrevida ou morte celular. Tem sido demonstrado que o bloqueio da ativação de NF-κB seguida de HP promove a morte celular de hepatócitos (Iimuro et al. 1998). Assim, podemos inferir que as plaquetas encapsuladas promovem a expressão de *Nf-κB* e seu papel na sobrevida celular. Além disso, a ativação de NF-κB leva à ativação de eNOS (Grumbach et al. 2005), o qual tem um papel importante na regeneração hepática após HP e na IHA (Leifeld et al. 2002; Mei and Thevananther 2011). Observamos que as plaquetas encapsuladas promoveram a expressão de *eNos* no fígado a partir das 6 h após HP, e esta ativação pode estar relacionada com a expressão elevada de *Nf-κB*, corroborando com a literatura.

As CK ativadas expressam IL6, a qual tem um papel importante na regeneração hepática (Taub 2004). Embora esta citocina não tenha um papel mitogênico direto, estimula os hepatócitos para responder aos fatores de crescimento e assim entrar no ciclo celular (Michalopoulos 2007; Taub 2004). A IL6 é uma citocina pleiotrópica com efeitos benéficos para o fígado promovendo a regeneração hepática e protegendo contra o dano hepático de diferentes etiologias (Klein et al. 2005). A expressão gênica de *Il6* no fígado remanescente estava aumentada nas primeiras 3 horas após HP no grupo PLT e depois diminui consideravelmente nas horas subsequentes em relação ao grupo EC. Há evidencias que IL6 também previne a apoptose e tem efeitos positivos nos modelos de isquemia e reperfusão, de cirrose e de transplante hepático (Klein et al. 2005; Taub 2003; Tiberio et al. 2008). O tratamento com IL6 em um modelo de HP de 85% foi associado com menor bilirrubina e marcadores de dano hepático, reduziu o dano por estresse oxidativo, melhorou a função mitocondrial, diminuiu o edema celular e acelerou a regeneração da massa

hepática (Jin et al. 2007). Baseado nisto, nossos dados sugerem que as plaquetas encapsuladas estariam promovendo uma resposta imediata ao dano hepático e consequentemente inibindo a morte celular por redução do estresse oxidativo.

No estudo anterior, foi observado que a taxa de regeneração hepática foi similar nos animais tratados com plaquetas encapsuladas e nos animais que receberam cápsulas vazias. No entanto, os fígados remanescentes dos animais que receberam capsulas vazias eram mais pesados que o grupo que recebeu plaquetas encapsuladas nas 72 horas após HP. A diferença de peso era causada principalmente por acumulo de agua. Além disso, no grupo de cápsulas vazias os hepatócitos estavam edemaciados devido à degeneração hidrópica (López et al. 2014). O edema hepatocitário pode ser causado por mudanças nas concentrações de íons intracelulares (Takahasi and Yamaguchi 1996). Foi demonstrado que a hepatectomia de 87% leva a lesão oxidativa e consequentemente a edema de hepatócitos e estreitamento sinusoidal (Jin et al. 2007). Tendo em vista que a produção de radicais livres, como consequência de lesão hepática poderia ser uma causa do acúmulo de água nos hepatócitos, neste trabalho investigamos o papel das plaquetas encapsuladas na resposta ao estresse oxidativo.

Em situações fisiológicas normais os hepatócitos estão continuamente expostos a níveis variáveis de ROS que são gerados por múltiplas fontes, como por exemplo resultado da respiração mitocondrial. Porém, os hepatócitos tem um sistema de defesa antioxidante de enzimas e não enzimas para neutralizar estes ROS (Czaja 2002). As CK quando se ativam por citocinas ou LPS liberam ROS ao meio extracelular como defesa de invasão bacteriana (Murata et al. 2008). O estresse oxidativo ocorre quando os níveis celulares de ROS excedem a capacidade neutralizante, sendo este prejudicial, e pode resultar da produção excessiva de ROS, de uma diminuição nos níveis de antioxidantes, ou de uma combinação destes dois efeitos (Czaja 2007). Na IHA, o estresse oxidativo tem um papel critico, já que causa danos no DNA, proteína e lipídeos e consequentemente morte celular (Czaja 2002).

Uma medida de dano por estresse oxidativo é a medida de grupos carbonilas nas proteínas. Estes grupos carbonilas (aldeídos e cetonas) são produzidos por oxidação das cadeias laterais dos aminoacidos (Dalle-Donne et al. 2003). Neste trabalho, foi avaliada a quantidade de grupos carbonilas presentes no tecido hepático após hepatectomia. Não

achamos diferença entre os grupos experimentais; e surpreendentemente, nem com os valores normais em todos os tempos analisados.

Por outro lado, os domínios sulfidrilos que indicam a natureza redox/oxidativo da célula tem um papel critico na determinação da estrutura e função de proteínas, regulação da atividade enzimática, controle da atividade de fatores de transcrição e proteção antioxidante (Włodek 2002). Observamos que os grupos sulfidrilos totais em ambos os grupos experimentais foram mais baixos que os valores normais, embora tenham aumentado ao longo do tempo. Estes resultados mostram que as plaquetas não influenciam no dano à proteína nem alteram a relação redox/oxidativa da célula após uma hepatectomia.

Além disso, medimos a quantidade de peroxidação lipídica no tecido hepático. A peroxidação lipídica é o resultado do ataque dos radicais hidroxila às cadeias de ácidos graxos de fosfolipídeos e triglicerídeos, e podem alterar a fluidez da membrana e sua permeabilidade, o que leva a efeitos na função celular (Hauck and Bernlohr 2016). Não observamos diferenças nas primeiras 3 horas (dados não mostrados), talvez porque o dano ainda não tenha ocorrido neste intervalo de tempo. No entanto, as plaquetas encapsuladas preveniram a peroxidação lipídica a partir de 6 horas pós HP quando comparado com o grupo EC, indicando que as plaquetas têm um efeito protetivo contra a estresse oxidativo.

Do mesmo modo avaliamos a atividade de enzimas antioxidantes e observamos que as enzimas que previnem a formação de ROS (catalase e SOD) estavam aumentadas 6 horas após HP no grupo PLT. Porém, foi observado um aumento na atividade de catalase no grupo EC uma hora após HP em comparação ao grupo PLT e aos valores normais ($P<0,05$). Porém, após 3 horas esses valores estavam normalizados. Não podemos descartar a possibilidade de maior produção de ROS no grupo EC imediatamente após HP, o que levaria um aumento na atividade de catalase. No entanto, esta suposição é um pouco controversa, já que o grupo EC apresentou menor quantidade de CK na primeira hora após o dano, sendo estas as principais células de produção de ROS. Para descartar esta hipótese teríamos que medir a quantidade de ROS que está sendo produzida no tecido hepático, o que não foi possível realizar neste trabalho por falta de reagentes e tempo.

As enzimas relacionadas com a remoção de subprodutos da peroxidação (Rana et al. 2002) mostraram atividades similares nos dois grupos. As plaquetas são ricas em enzimas antioxidantes e sua função também está regulada pela interação com ROS

(Freedman 2008; Strange et al. 2012). No entanto, como as plaquetas encapsuladas estimulam enzimas antioxidantes no fígado ainda não está bem claro, porém tudo sugere que esse estímulo está mediado por fatores parácrinos, já que as plaquetas estão encapsuladas no alginato.

O estresse oxidativo pode evoluir para morte celular (Czaja 2002; Hauck and Bernlohr 2016). Tanto ROS como caspases são potenciais mediadores da morte celular (Higuchi et al. 1998), sendo que a caspase 3 é o principal ativador da morte celular por apoptose e necrose (McIlwain et al. 2013). A atividade de caspase 3 estava altamente elevada nas 6 horas após HP em ambos os grupos, mostrando que a ressecção do fígado causa uma lesão grave. Já nas 72 horas, a atividade de caspase 3 no grupo EC foi quatro vezes mais elevada no grupo PLT. As análises histológicas mostraram que no grupo EC houve mais necrose de células isoladas, embora não tenha havido diferença estatística devido ao baixo tamanho amostral dos grupos. Estes dados sugerem que as plaquetas inibem a apoptose ou podem estar protegendo as células do fígado dos danos e, portanto, a via de morte celular não se ativa. Não obstante, há evidências que as plaquetas inibem a morte celular por apoptose através da ativação da via Akt no modelo de hepatite (Hisakura et al. 2011) ou pela liberação de um agonista do receptor de EGF que ativa vias de reparação do DNA (Au et al. 2014).

Evidencia adicional ao efeito protetor das plaquetas encapsuladas foi observado no aumento da expressão de genes relacionados à função hepática tais como albumina e fator V. Infusão de plaquetas em pacientes com doença hepática crônica e cirrose aumentam os níveis séricos de albumina e colinoesterase, melhorando assim a função hepática (Maruyama et al. 2013). A esplenectomia em pacientes com cirrose melhorou a síntese de proteínas assim como o aumento do tamanho do fígado (Murata et al. 2008). Esses dados corroboram que as plaquetas além de ter função na hemostase, ajudam na recuperação do tecido após um dano.

Além disso, observamos que as plaquetas ajudam na regulação do lactato sanguíneo, uma vez que os níveis de lactato se mantiveram normais nas primeiras três horas no grupo PLT, enquanto no grupo EC o lactato aumentado ($P=0,03$) em relação a valores normais. A hiperlactemia é frequente em pacientes críticos e é comumente usada como marcador de adversidade (Khosravani et al. 2009). O fígado, após esgotar suas

reservas de glicogênio, perde a via gluconeogênica para gerar glicose e, portanto, sua capacidade de remover o lactato, causando hiperlactemia (Oldenbeuving et al. 2014).

Outra medida que reflete o estado do fígado é através do nível de glicemia nos animais, já que a IHA está associada a uma hipoglicemia pronunciada (Karim et al. 2012). O fígado é o principal órgão de armazenamento e regulação da disponibilidade do glicogênio (Ishibashi et al. 2009). Neste trabalho, não observamos diminuição na concentração de glicose sanguínea nos animais após HP. Porém, avaliamos a expressão do transportador de glicose tipo 2 (*Glut2*) no fígado remanescente nas primeiras três horas e foi observado que estava aumentada no grupo PLT (P=0,03). O Glut2 promove o rápido fluxo da glicose seguida de gluconeogênese, mas também de outros açúcares como a galactose, manose e frutose com alta afinidade pela glucosamina (Karim et al. 2012). É importante ressaltar que todos os animais recebem glicose i.p. imediatamente após HP e na água de beber, o que pode estar mascarando uma possível hipoglicemia. De qualquer forma estes resultados sugerem que as plaquetas melhoram uma resposta metabólica ao dano hepático.

As plaquetas são necessárias para uma resposta total do fígado ao dano, tanto em animais como em pacientes (Kurokawa et al. 2015; Alkozai et al. 2010; Murata et al. 2007). Uma contagem baixa de plaquetas em pacientes com carcinoma hepático que sofreram uma ressecção parcial está associada com um atraso na recuperação das funções hepáticas e aumento da mortalidade (Amano et al. 2011). Além disso, tem se demonstrado as propriedades benéficas do plasma rico em plaquetas na regeneração de vários tipos de tecido, inclusive na regeneração hepática após hepatectomia parcial (De Pascale et al. 2015; Matsuo et al. 2011; Hisakura et al. 2010). Em nosso trabalho, demonstramos que as plaquetas exercem seu papel benéfico mediado pela liberação de fatores e não é preciso o contato direto com o tecido lesionado.

Concluindo, neste trabalho foi possível elucidar um dos mecanismos pelos quais as plaquetas aumentam a sobrevida dos animais submetidos a HP 90% como modelo de IHA. Nossos dados sugerem que as plaquetas encapsuladas têm um efeito benéfico imediato após HP de 90% e este efeito é mediado por fatores parácrinos. As plaquetas regulam a ativação das CK e a expressão de citocinas relacionadas com a sobrevida celular. Essa regulação também está relacionada à diminuição do estresse oxidativo e da apoptose

resultando em hepatócitos mais saudáveis que são capazes de manter a vida durante as primeiras 72 horas da regeneração hepática.

6 CONCLUSÕES

Neste trabalho foi avaliado o mecanismo de ação das plaquetas em animais submetidos à lesão hepática aguda induzida por hepatectomia parcial de 90% (HP 90%).

As plaquetas encapsuladas reduzem o estresse oxidativo no tecido hepático após HP 90%.

As plaquetas encapsuladas regulam a ativação das células de Kupffer tanto *in vitro* como *in vivo*, promovendo a expressão de citocinas relacionadas com a sobrevida celular e regeneração hepática

As plaquetas encapsuladas possuem um efeito benéfico imediatamente após HP 90%.

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ANEXOS

Anexo 1: PLATELET INCREASES SURVIVAL IN A MODEL OF 90% HEPATECTOMY IN RATS.

Liver INTERNATIONAL
OFFICIAL JOURNAL OF THE INTERNATIONAL ASSOCIATION FOR THE STUDY OF THE LIVER

Liver International ISSN 1478-3223

ORIGINAL ARTICLE

Platelet increases survival in a model of 90% hepatectomy in rats

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Keywords
acute liver failure – animal model of acute liver failure – capsules – hepatectomy – platelets

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Received 12 February 2013
Accepted 29 August 2013

DOI:10.1111/liv.12326

Abstract

Background & Aims: Ninety per cent hepatectomy in rodents is a model for acute liver failure. It has been reported that platelets have a strong effect enhancing liver regeneration, because of the production of several growth factors such as serotonin. The aim of this study was to investigate the role of microencapsulated platelets on 90% hepatectomy in rats. *Methods:* Platelets (PLT) were microencapsulated in sodium alginate and implanted in the peritoneum of rats after 90% partial hepatectomy (PH). Control group received empty capsules (EC). Animals were euthanized at 6, 12, 24, 48 and 72 h post PH (n = 9–12/group/time) to evaluate liver regeneration rate, mitotic index, liver content, serum and tissue levels of Interleukin 6 (IL-6) and serotonin and its receptor 5-hydroxytryptamine type 2B (*5Ht2b*). Survival rate in 10 days was evaluated in a different set of animals (n = 20/group). *Results:* Platelets group showed the highest survival rate despite the lowest liver regeneration rate at any time point. Mitotic and BrdU index showed no difference between groups. However, the number of hepatocytes was higher and the internuclear distance was shorter for PLT group. Liver dry weight was similar in both groups indicating that water was the main responsible factor for the weight difference. Gene expression of IL-6 in the liver was significantly higher in EC group 6 h after PH, whereas *5Ht2b* was up-regulated at 72 h in PLT group. *Conclusions:* Platelets enhance survival of animals with 90% PH, probably by an early protective effect on hepatocytes and the increase in growth factor receptors.

Acute liver failure (ALF) is a severe and sudden hepatocellular loss and dysfunction associated with jaundice, coagulopathy and hepatic encephalopathy (1, 2). Liver transplantation remains the most promising treatment for ALF. However, organ availability is limited and a significant percentage of patients die while waiting for a transplant (2, 3). Nevertheless, adult hepatocytes maintain the ability to proliferate in response to toxic injury and infection (4). After a partial hepatectomy (PH), hepatocytes are the first cells to enter the S phase for DNA synthesis, peaking at 24 h, whereas for non-parenchymal cells this happens around 36–48 h (5). Because of this unique ability to regenerate after injury or resection, bridge strategies to keep the patient alive or to abbreviate the liver recovery time have to be employed.

Cell therapy with bone marrow-derived cells is a promising alternative that can enhance the regenerative capacity of the liver (6–9). Recently, it has been shown that encapsulated cells transplanted in animal models of ALF improve survival rate and liver regeneration (10–13). The advantages of this strategy are the isolation of the transplanted cells from the host and the possibility to study paracrine effects of cell therapy (14, 15).

It is well known that platelets play an important role in wound healing and tissue regeneration (16). Platelets contain proteins needed for haemostasis and growth factors including platelet-derived growth factor (PDGF),

hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), tumour growth factor (TGF) and serotonin (5HT), required for tissue regeneration (17–22). Recently, it has been reported that platelets prevent hepatic failure and stimulate liver regeneration after extensive hepatectomy by releasing growth factors (23–28). For instance, platelet-derived serotonin improves liver regeneration through its liver receptor 5HT2B (23). *In vitro* studies have also shown that PDGF and insulin growth factor type 1 (IGF-1) enhance hepatocyte proliferation (29). In addition, the administration of exogenous platelet improves liver regeneration after 70% hepatectomy in rats via Akt and ERK1/2 pathways (30). Most of these studies have shown that platelets accumulate in the space of Disse where they are in contact with liver cells. However, it is likely that platelets exert their effects through paracrine mechanisms. In order to test that, we have immobilized platelets in a semipermeable membrane to prevent direct cell contact and isolate the effect of their location within the liver. Therefore, microencapsulated platelets were implanted in the peritoneal cavity in an animal model of 90% PH.

Material and methods

Animals

Two-month-old male Wistar rats, weighing 314 ± 29 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 into two groups. Control group ($n = 23$) received empty alginate microcapsules (EC); treated group received microencapsulated platelets (PLT, $n = 20$). Survival was observed for 10 days after 90% PH. An additional set of animals was sacrificed at 6, 12, 24, 48 and 72 h after 90% PH ($n = 9$–12/group/time point) to evaluate the early effects of treatments. Another 33 animals were used as bone marrow-derived platelet donors.

Platelet isolation

Donor Wistar rats were sacrificed in CO_2 chamber and both femurs were isolated. Whole bone marrow was flushed out with acid citrate dextrose (pH 5) in a 1:4 volume and centrifuged at 150*g* for 10 min. Platelets in the supernatant were then washed twice by centrifugation at 1000*g* at 4°C for 15 min, and resuspended in citrate buffer (modified from Matsuo *et al.*) (30). They were counted in an automatic haematological counter (ABX Micros 60; Axonlab, Baden, Germany), diluted to 1.5×10^7 platelets/ml and submitted to the encapsulation protocol without prior activation.

Capsules production

Platelet encapsulation was performed according to our laboratory protocol, as previously described by Lagranha *et al.* (31). Briefly, platelets were mixed with 1.5% sodium alginate (Sigma-Aldrich, St Louis, MO, USA) in Dulbecco's Modified Eagle Medium (DMEM) (LGC, Cotia, São Paulo, Brazil) 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 platelets. In each well capsules were produced from a volume of 2 ml of alginate suspension, containing 3×10^7 platelet/animal. Platelet encapsulation was carried out under sterile conditions. For control group, 2 ml of empty capsules were produced using the same approach without platelets. The resulting capsules were maintained under normal cell culture conditions, DMEM supplemented with 10% Foetal Bovine Serum (Gibco®, Grand Island, NY, USA) and Penicilin/Streptavidin 1% (Gibco®, USA) at 37°C and 5% CO_2, for 24 h prior to administration.

Surgical procedure and capsules transplantation

Ninety per cent hepatectomy was performed by a single operator (32). In brief, the left lateral (30%), left median (40%) and the right superior lobes (20%) were removed, leaving only the caudate lobe. Hepatectomy was carried out under isoflurane (Forane®, Abbott SA, Buenos Aires, Argentina) anaesthesia (33). Immediately after 90% PH, and before complete laparotomy closure, microcapsules (either empty or containing platelets) were placed into the peritoneal cavity with a 60 ml syringe, and glucose was supplemented i.p. (5% of body weight). Post-operatively, 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, 2 ml of blood was collected by heart puncture and the liver was removed and weighted. Part of the liver was then flash frozen in liquid nitrogen for RNA, protein and lipid extraction and then stored in −80°C. The other part was fixed for histological analysis in 10%

neutral-buffered formalin. Serum was separated by centrifugation and stored at −20°C until analysis.

Liver regeneration rate

The liver regeneration rate was calculated as follows;

Liver Regeneration Rate (%) = 100 × [C- (A–B)]/A where A is the estimated liver weight before 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 euthanasia (34).

Liver dry weight

A liver fragment was weighed immediately after removal and then placed in a chamber at 70°C for 3 days to determine the dry weight.

Protein quantification

Total proteins were obtained by disrupting 100 mg of frozen liver in 800 µl of 1X PBS with protease inhibitor (Sigma, USA) solution (final concentration 10 µl/ml). Homogenates were centrifuged at 17 000*g*, for 30 min at 4°C. The supernatant was collected and submitted to protein quantification using the Lowry protein assay (35).

Nile red assay

Lipid content in the liver was determined by the Nile Red method. To prepare liver homogenates, small pieces of tissue were disrupted in PBS 1X. Homogenates were diluted 1:10 in PBS and an aliquot of 25 µl was incubated with Nile Red (Sigma-Aldrich) solution (final concentration of 1 mg/ml in PBS) at 37°C for 15 min in 96-well plates. After incubation, fluorescence was directly measured at 488 nm excitation and 550 nm emission on U-2001 Spectrophotometer (Hitachi, Japan) and normalized by a normal liver lipid content measured at the same time (36).

Histology and immunohistochemistry

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

To assess the rate of hepatocyte proliferation, the number of hepatocytes undergoing mitosis was counted in 10 high-power fields (HPF). In addition, 5-bromo-2′-deoxyuridine (BrdU) immunostaining was done using BrdU staining kit (Invitrogen, USA). Two hours before sacrifice, rats (n = 3/group) were injected with BrdU (1 ml/g). Thereafter, liver sections were incubated with BrdU antibody and the number of positive hepatocytes was counted in 5 HPF.

To determine the size and the number of parenchymal cells per slide, the internuclear distance was measured and hepatocytes nuclei were counted in 5 HPF using Cell Imaging Software for Life Science Microscopy (Olympus).

Enzyme-linked immunosorbent assay

Serum levels of Serotonin were determined by ELISA using Rat-Serotonin kit (GenWay, USA), according to the manufacturer's instructions. Serum and tissue Interleukin-6 were also detected using Rat-IL-6 Kit (BD Bioscience, USA) according to the manufacturer's instructions.

Quantitative real-time PCR

Total RNA was extracted from 50 mg of liver tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Two micrograms of RNA were reverse-transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Hammonton, NJ, USA). Gene expression was measured using TaqMan assays (Applied Biosystems) for serotonin receptor (*5-Ht2b*, Rn00568450-m1) and Interleukin-6 (*Il6*, Rn01410330_m1). Messenger RNA expression levels were normalized to *Actb* RNA (Rn00667869_m1) using the $2^{-\Delta\Delta Ct}$ method (37) and a normal liver as calibrator group.

Statistical analysis

Results were expressed as means ± SD or medians when required. Statistical analyses were carried out using IBM SPSS, Armonk, NY, USA. Parametric data were analysed with Student's t-test and for non-parametric variables Mann–Whitney was used. Kaplan–Meier estimator was used for survival rate evaluation. $P < 0.05$ was considered to be significant.

Results

Survival rate and liver regeneration rate

Firstly, overall survival rate was observed during 10 days after hepatectomy. The survival rate was higher for the PLT group (85%) than for EC group (30%) (P = 0.001). Animals in PLT group died predominantly during the first 3 days, whereas in the other group deaths occurred over time after 90% PH (Fig. 1A). Therefore, the remaining analyses were performed until 72 h post 90% PH.

Liver weight is usually used to determine the liver regeneration rate in the remnant lobe. Surprisingly, the liver regeneration rate did not show a correlation with survival rate. Liver weight increased gradually after surgery, without differences between groups at 6, 12, 24 or 48 h. However, at 72 h EC group showed a higher

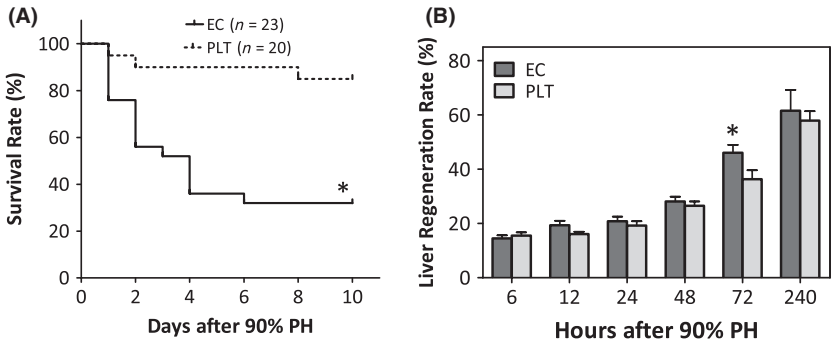

Fig. 1. (A) Kaplan–Meier 10-day survival curve after 90% partial hepatectomy. EC=Empty capsule group, PLT=Platelet group. *P = 0.001. (B) Liver regeneration after 90% partial hepatectomy. Values are expressed as means ± SD. *P = 0.049, Student's t-test.

regeneration rate compared to PLT (P = 0.049). At day 10, the liver regeneration rate for EC group was 61% and for PLT group was 58% (P = 0.626) (Fig. 1B).

Histological findings

Hydropic and necrotic changes were observed in both groups. Congestion was predominant at 6 h after PH. Presence of steatosis was observed until 10 days after PH (Fig. 2A–B). Interestingly, BrdU labelling index did not show differences among the groups (Fig. 2C–D), as well as mitotic index at any time point (data not shown). However, at 72 h after PH the number of hepatocytes in PLT group was increased (P = 0.005) and the internuclear distance was shorter (P < 0.001) compared to EC group (Fig. 2E-F).

Liver content

In order to determine the cause of the higher weight and the larger hepatocyte size in EC group, liver content of protein, lipid and water were evaluated. There was no difference in lipid content of livers at 72 h (Fig. 3A). On the other hand, PLT group had more total protein than EC group (P = 0.009) (Fig. 3B).

Although liver weight at sacrifice was higher for EC group, liver dry weight assessed at 72 h did not show difference between groups (Fig. 3C). Altogether, these results suggest that water is a main factor affecting liver weight and therefore regeneration rate after PH.

Liver regeneration factors

We then searched for factors that could be related to a protective effect of platelets, either promoting liver regeneration or reducing early damage to hepatocytes. No difference between groups was detected on serum IL-6 levels that was elevated 6 h after PH and decreased at 12 h, as expected. On the contrary, tissue IL-6 was reduced at 6 hours and slightly increased at 12 h, again without difference between groups. Surprisingly, liver gene expression of *Il6* was significantly increased at 6 h for EC group (P = 0.004), while at 12 h no difference was observed (Fig. 4).

Serum serotonin did not show differences between groups at 48 and 72 h after liver injury (Fig. 5A). On the other hand, the gene expression of serotonin receptor *5-Ht2b* in PLT group was 20 times higher at 72 h compared to EC group (P = 0.028, Fig. 5B).

Discussion

This study was designed to assess the role of microencapsulated platelets in animals submitted to 90% PH as an acute liver failure model. Cell therapy protocols using bone marrow-derived cells (6–8), and hepatocytes alone or combined with stem cells (12–15) have been tested in animal models of acute liver failure. The role of platelets has been investigated by the induction of thrombocytosis through the administration of thrombopoietin (27, 28), or pegylated recombinant human megakaryocyte growth and development factor-induced thrombocytosis (24–26).

In this study, the role of exogenous platelets and their paracrine effect were investigated using the advantage of microencapsulation technology. Our results suggest that platelet effect is independent of direct cell to cell contact and platelet location within the liver. Although the viability of platelets within the capsules was not assessed during the treatment, the implant of encapsulated platelets significantly increased survival rate in 10 days. The mortality rate for PLT group occurred in the first 72 h after hepatectomy, which indicates an early beneficial effect of platelets after massive liver injury. One may assume that this effect was mediated by secreted factors by viable platelets but we do not have direct evidence of that.

Liver regeneration rate was assessed at different time points and it was observed that both groups recovered over than 50% of their total liver weight on day 10. Nevertheless, PLT group had the lowest regeneration rate and it presented a gradual increase over time. This is in accordance to the findings of Ninomiya *et al.* (38) that have demonstrated that deceleration of regenerative

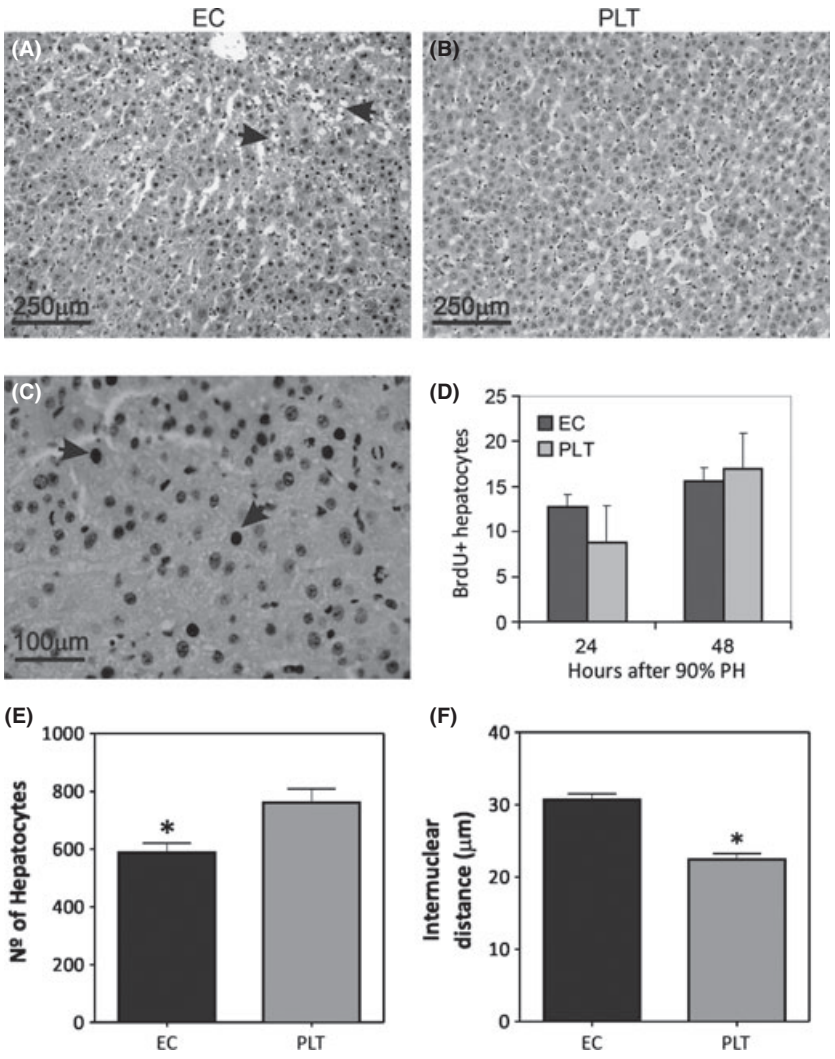

Fig. 2. Histological analysis. (A–B) H&E stained liver section 10 days after 90% partial hepatectomy showing diffuse steatosis in EC group (arrows). (C) Liver section showing BrdU-positive nuclei in hepatocytes (arrows). (D) Number of BrdU-positive hepatocytes 48 h after hepatectomy. (E) Number of hepatocytes counted in 5 High-Power Fields (HPF), $*P = 0.005$, Student's *t*-test. (F) Internuclear distance between hepatocytes in 5HPF, $*P < 0.001$, Student's *t*-test. Values are expressed as means ± SD, $n = 3$-8/group.

response after a massive hepatectomy improves survival. Accordingly, liver aspect on different time points was better in the PLT group than EC group, suggesting that transplanted platelets protect the animals from other types of stress imposed by liver injury (39).

Despite these observations in liver regeneration rate measured by weight, no differences were found in BrdU labelling or in the mitotic index. Interestingly, the numbers of hepatocytes in PLT group were higher and the distances among nuclei were smaller than in EC group at 72 h. Liver content analysis suggested that water accumulation in hepatocytes of EC group could be responsible for their larger size and heavier liver weight, which impacts the liver regeneration rate.

It has been shown that hepatocytes can swell as a protective cellular adaptation after liver injury (40). The events responsible for swelling may be related to changes in the intracellular concentrations of ions like Potassium and Calcium (41, 42). Furthermore, high concentrations of Calcium induce oxidative stress in the mitochondria which in turn appears to induce and amplify mitochondrial permeability transition (MPT) (43, 44). The MPT and the elevated production of oxidants activate apoptotic and necrotic process (45, 46). Platelets are rich in the antioxidant catalase and enzymes of the glutathione redox cycle and may prevent cell injury by neutralizing oxygen radicals (47). Hence, encapsulated platelets may prevent hepatocyte swelling by promoting a response to oxidative stress and improving hepatocyte functioning. This would lead to better outcome of animals after 90% PH.

Hepatocytes present mitogenic response to various growth factors and cytokines such as HGF, IL-6, PDGF

and serotonin. Activated Kuppfer cells secrete IL-6 as a response to liver injury, and therefore activate neighbouring hepatocytes, which activate the mitogen-activated protein kinase (MAPK) pathway and the signal transducer and activator of transcription (STAT)3 pathway (4). As expected, serum levels of IL-6 increased very early after 90% PH in both groups. Meanwhile, this was not observed for tissue levels, in which there was an increase 12 h after PH. On the other hand, the expression of IL-6 at 6 h was significantly increased in EC group. It is possible that encapsulated platelets led to an

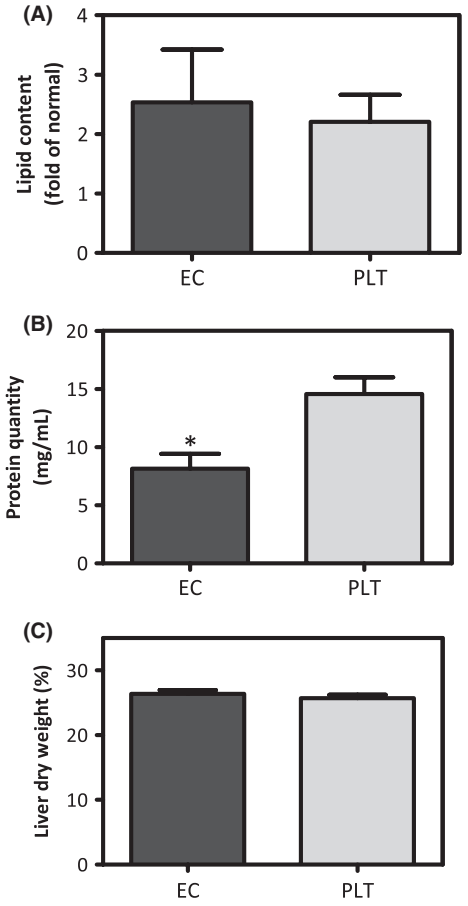

Fig. 3. Liver content assessed at 72 h after 90% partial hepatectomy (n = 5–7 per group). (A) Lipid content as fold of normal. (B) Protein quantification, *P = 0.009, Student's t-test. (C) Liver dry weight. Values are expressed as means ± SD.

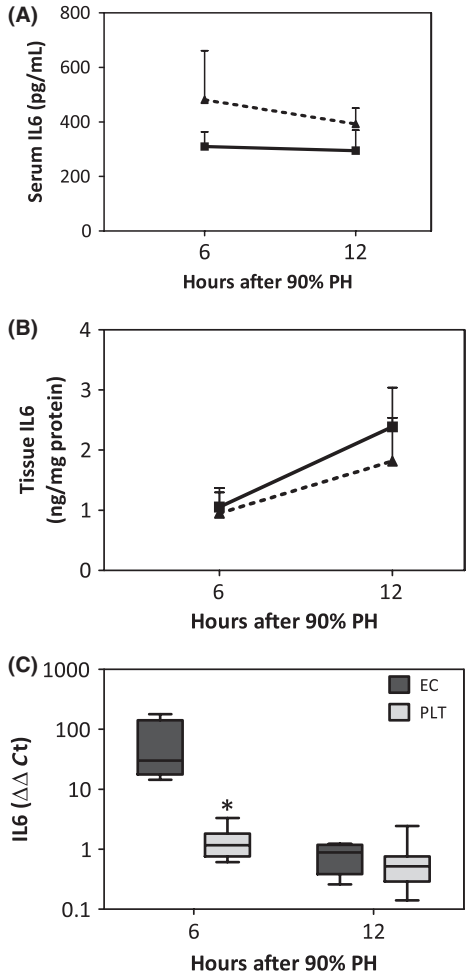

Fig. 4. Interleukin 6 at 6 and 12 h after 90% partial hepatectomy (n = 5–8/group). (A) Serum levels and (B) liver tissue levels (EC group = solid line and PLT group = dotted line). (C) Liver gene expression, *P = 0.004, Mann–Whitney test.

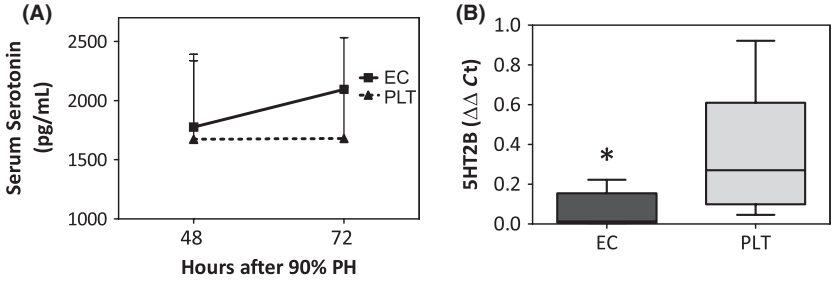

Fig. 5. Serotonin and its receptor *5-Ht2b* after 90% partial hepatectomy (n = 5–8/group). (A) Serum levels of serotonin at 48 and 72 h (EC group = solid line and PLT group = dotted line). (B) Liver gene expression of *5-Ht2b*, *P = 0.028, Mann–Whitney test.

earlier increase of IL-6 in PLT group that was no longer detectable at 6 h after PH (48). It is known that IL-6 increase very rapidly after PH and the down-regulation of components of the pathway appeared sequentially during the first 12 h after PH (49). It cannot be ruled out that platelets caused a diminished increase in IL-6 even in earlier times after PH as the first time point to be analysed was 6 h.

Serotonin is not only a neurotransmitter but also has mitogenic properties (23) and is secreted by platelets. The 5HT2B receptor is mainly expressed in the liver and it is related to liver regeneration in 70% liver resection (23, 50). We observed a significant up-regulation of *5Ht2b* in livers from PLT group at 72 h, suggesting a possible positive feedback mechanism mediated by platelet-derived serotonin. Although, there was no difference in serum levels of serotonin, animals treated with encapsulated platelets appear to be more capable to respond to serotonin.

In conclusion, platelets are required for full hepatic response to injury both in patients and animals (24, 51). Moreover, administration of exogenous platelet has recently been shown to improve liver regeneration (30). Our findings showed that encapsulated platelets enhance the survival rate of rats submitted to 90% PH by paracrine factors, independent of direct cell contact or location within the liver. This outcome may be because of a platelet-protective effect on the liver cells that takes place early after liver injury. In addition, a positive feedback related to 5HT2B gene expression was also observed.

Acknowledgements

Financial support: This work received financial support from FIPE/HCPA, PRONEX/FAPERGS 10/0039-3. MLL and UM are recipients of CNPq scholarships.

Conflict of interest: The authors do not have any disclosures to report.

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Anexo 2: BONE MARROW-DERIVED MONONUCLEAR CELLS DIFFERENTIATE INTO HEPATOCYTE-LIKE CELLS WITHIN FEW HRS WITHOUT FUSION

Cell Science & Therapy

Matte et al., J Cell Sci Ther 2014, 5:3
http://dx.doi.org/10.4172/2157-7013.1000163

Research Article Open Access

Bone Marrow-Derived Mononuclear Cells Differentiate into Hepatocyte-Like Cells within Few Hrs without Fusion

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Rec date: Jan 30, 2014; **Acc date:** Apr 05, 2014; **Pub date:** Apr 17, 2014

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, 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 hrs after CCl_4 administration and capsules were collected within 6, 24 and 48 hrs ($tCCl_4$ 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. 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 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 hrs after treatment. On the other hand, BMMC from cCCl4 group showed Albumin expression 6 hrs 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

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 hrs. 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 hrs (Figure 1).

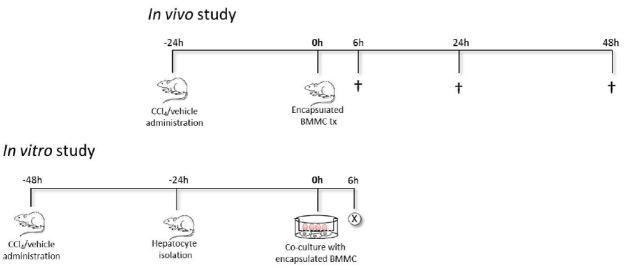

Figure 1: Experimental design of in vivo and in vitro studies. † Sacrifice of animals and retrieve of capsules; Ⓧ Interruption of co-culture and recovery of encapsulated BMMC

Animals

Adult male Wistar rats weighing 200 to 250 gr 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 hrs 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 hrs 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 $Ca2^+$ 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 37°C and 5% CO_2 for 24 hrs 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 hrs 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 hrs 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 hrs 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 hrs 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 µm 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 hrs.

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 Quanti ChromTM 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.

Primer name	Sequence	PCR condition	Fragment length
Alb	For: 5'GGTATGAATATGCAAGAAG3' Rev: 5'CACTCTTCCCAGGTTTCTTG3'	48°C	350 bp
Ck-18	For: 5'GGACCTCAGCAAGATCATGGC3' Rev: 5'CCAGGATCTTACGGGTAGTTG3'	50°C	518 bp
Afp	For: 5'CCCACCCTTCCACTTTCCAGA3' Rev: 5'GCTGGAACTGCCTTGTCATA3'	54°C	164 bp
Gapdh	For: 5'GAGTTGCTGTTGAAGTCACAGG3' Rev: 5'CAGCAATGCATCCTGCAC3'	42°C	429 bp
Alb- Albumin; Ck-18, Cytokeratin 18; Afp, Alpha-fetoprotein; Gapdh, Glyceraldehyde 3-phosphate dehydrogenase			

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

Statistical analysis

Statistical comparison of urea levels of the cControl and $cCCl_4$ 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 hrs after CCl_4 or olive oil administration. In animals from $tCCl_4$ 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 hrs 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 $tCCl_4$ retrieved after 48 hrs, 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 hrs after injections 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.

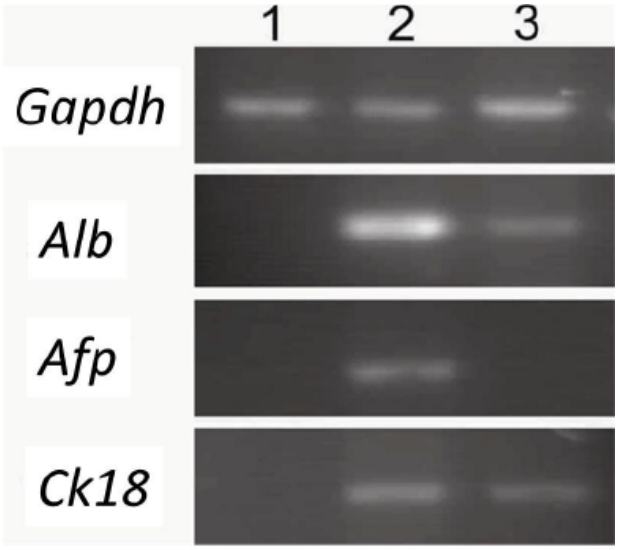

Figure 2: BMMC expression of hepatocyte markers 48 hrs after implantation in animals with CCl_4-induced acute liver injury. RT-PCR analysis of gene expression in encapsulated BMMC from tControl (lane 1) or $tCCl_4$ (lane 3) retrieved 48 hrs after implantation. Positive control (hepatocytes, lane 2)

In vitro experiments

Since encapsulated BMMC showed the expression of hepatocyte markers 48 hrs 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 hrs showed Albumin expression, whereas those in contact with control hepatocytes were negative (Figure 3). No expression of Ck18 or Afp was detected.

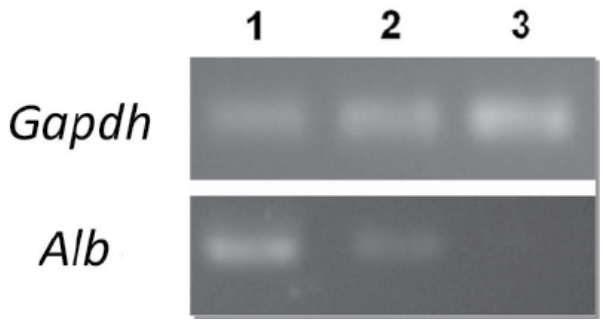

Figure 3: RT-PCR analysis of Alb gene expression in BMMC after 6 hour co-culture. Hepatocyte (positive control, lane 1). Encapsulated BMMC from $cCCl_4$ group (lane 2). Encapsulated BMMC from cControl group (lane 3). Gapdh: internal control

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 hrs 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).

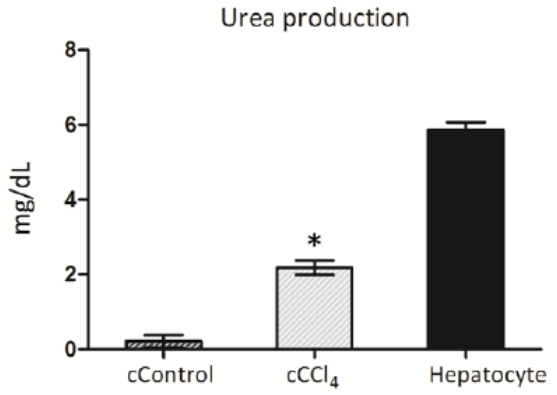

Figure 4: Quantification of urea production in the culture medium from encapsulated BMMC. Urea production was increased in BMMC from $cCCl_4$ group but not in cControl. Medium from hepatocyte culture was used as reference value. $^*p$=0.033 compared to cControl group (Student's T-test)

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 hrs of transplantation in CCl_4 treated animals. In vitro, these cells express Albumin and produce urea after only 6 hrs 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 capsule's 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 and changes in gene expression at early times like this are not reported by studies with MSC, which usually differentiate after 3 to 40 days in culture [22,36]. On the other hand, BMMC are readily available, and does not need a culture step prior to administration.

Our results showed that BMMC co-cultured with injured hepatocytes for six hrs showed expression of Albumin and urea production, both characteristics of hepatocyte activity. It has been reported that kidney tubular epithelium also produces urea, while extraembryonic cells express albumin, however, only hepatocytes can do both [40,41]. In contrast, when BMMC were co-cultured with hepatocytes isolated from healthy animals, no Albumin expression or urea production was detected after 6 hrs. 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. On the one hand, BMMC are composed of phenotypically and functionally different cell populations and we have preliminary data indicating that is the non-adherent fraction of bone marrow mononuclear cells that differentiates. However, further characterization of these cells is still pending. On the other hand, it is possible that injured hepatocytes secrete paracrine factors that lead to BMMC reprogramming sooner than healthy hepatocytes [42,43].

Interestingly, in vivo differentiation occurred only after 48 hrs. 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.

Acknowledgements

This work was supported by: Research Incentive Fund of Hospital de Clínicas de Porto Alegre (FIPE-HCPA No.08-695); The National Council for Scientific and Technological Development of Brazil (CNPq), Fund for Research Support of the State of Rio Grande do Sul (FAPERGS) and Program for Support of Centers of Excellence (PRONEX – FAPERGS/CNPq n. 008/2009).

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Anexo 3: ENCAPSULATED WHOLE BONE MARROW CELLS IMPROVE SURVIVAL IN WISTAR RATS AFTER 90 % PARTIAL HEPATECTOMY

Hindawi Publishing Corporation
Stem Cells International
Volume 2016, Article ID 4831524, 9 pages
http://dx.doi.org/10.1155/2016/4831524

Research Article

Encapsulated Whole Bone Marrow Cells Improve Survival in Wistar Rats after 90% Partial Hepatectomy

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Received 10 December 2014; Revised 18 January 2015; Accepted 28 January 2015

Academic Editor: Kenichi Tamama

Background and Aims. The use of bone marrow cells has been suggested as an alternative treatment for acute liver failure. In this study, we investigate the effect of encapsulated whole bone marrow cells in a liver failure model. *Methods*. Encapsulated cells or empty capsules were implanted in rats submitted to 90% partial hepatectomy. The survival rate was assessed. Another group was euthanized at 6, 12, 24, 48, and 72 hours after hepatectomy to study expression of cytokines and growth factors. *Results*. Whole bone marrow group showed a higher than 10 days survival rate compared to empty capsules group. Gene expression related to early phase of liver regeneration at 6 hours after hepatectomy was decreased in encapsulated cells group, whereas genes related to regeneration were increased at 12, 24, and 48 hours. Whole bone marrow group showed lower regeneration rate at 72 hours and higher expression and activity of caspase 3. In contrast, lysosomal-β-glucuronidase activity was elevated in empty capsules group. *Conclusions*. The results show that encapsulated whole bone marrow cells reduce the expression of genes involved in liver regeneration and increase those responsible for ending hepatocyte division. In addition, these cells favor apoptotic cell death and decrease necrosis, thus increasing survival.

1. 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 [1]. 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 [2]. In addition to that, the lifelong use of immunosuppressant after the transplant possesses side effects in the short and long term [3, 4]. 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 [5, 6] and acute liver disease [7–9]. They present several advantages when compared to hepatocytes as they are readily available and can be expanded *in vivo* or *in vitro* [10]. In addition, the use of autologous cells would eliminate the need for immunosuppressants [11]. In animal models, heterologous transplantation of mesenchymal stem cells [12] or encapsulated bone

marrow cells [13] is also performed without immunosuppressants. However the mechanisms by which these cells exert their beneficial effect on liver regeneration are not completely well understood. They may involve an increase in the number of hepatocytes by either transdifferentiation, fusion, and/or the secretion of paracrine factors that stimulate cell division, inhibit apoptosis, or modulate local and systemic inflammatory state [10, 14].

Several proinflammatory 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 Nfκ-B (nuclear factor kappa B) and the release of Tnf-α (tumor necrosis factor-α) and Il-6 (interleukin-6) [15]. Il-6 plays a key role in liver regeneration, activating acute phase genes and priming hepatocytes to growth factors [16, 17]. Hgf (hepatocyte growth factor) then stimulates hepatocytes to pass from G0 to G1, thus initiating the cell cycle [18, 19]. The increase in molecules such as Socs3 (suppressor of cytokine signaling 3) and Tgf-β1 (transforming growth factor-beta) contributes to the decrease in stimulating factors and the halt of liver regeneration [18, 20].

After partial hepatectomy, there is a complex remodeling of the liver tissue with a transient disruption of the lobular architecture [21]. Agglomerates of poorly vascularized hepatocytes are formed in the periportal area before invasion of sinusoidal cells [20, 22]. Some authors have suggested that at the early stages of liver regeneration a very fine tuning in the rate of proliferation of parenchymal and nonparenchymal cells is needed. Ninomiya et al. [22] showed that a slowed hepatocyte regeneration rate increased the survival in a model of 90% partial hepatectomy.

Our goal was to investigate the paracrine effects of bone marrow cells and the mechanisms by which they increase survival in a rat model of 90% partial hepatectomy.

2. Methods

2.1. Animals. Two-month-old male outbred 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 the Experimental Animal Unit at Hospital de Clínicas de Porto Alegre (HCPA). Handling, care, and processing of animals were carried out according to regulations approved by our local ethics committee (protocol number 10-0062) and complied with the National Guidelines on Animal Care.

2.2. Experimental Design. Animals were submitted to 90% partial hepatectomy (90% PH) and randomly divided in two groups. Treated group received encapsulated whole bone marrow cells (WBM, $n = 11$) and control group ($n = 15$) received empty capsules (EC). Survival was observed for up to 10 days after 90% PH. An additional set of animals from both groups was sacrificed at 6, 12, 24, 48, and 72 hours after 90% PH ($n = 6$/group/time point) to evaluate the early effects of treatments.

2.3. Isolation of Whole Bone Marrow Cells and Encapsulation. Thirty-three animals without liver injury were used as donors of WBM 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 was performed according to our laboratory protocol, previously described [23]. Briefly, WBM cells were mixed with 1.5% sodium alginate (Sigma-Aldrich, USA) in complete medium and extruded through an 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. For control group, 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 37°C and 5% CO_2 for 24 h prior to transplantation.

2.4. Surgical Procedure and Capsules Transplantation. Ninety percent hepatectomy was performed by a single operator as described by Gaub and Iversen [24]. 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 [25]. Immediately after 90% PH and before complete suture, microcapsules (containing 3×10^7 WBM cells [26] or empty) 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*.

2.5. Euthanasia. Euthanasia was performed in CO_2 chambers. To evaluate survival, the animals were euthanized 10 days after 90% HP. To evaluate the early effects of treatments the animals were euthanized 6, 12, 24, 48, and 72 h after 90% HP and immediately blood was collected, the liver was removed and weighed, and part was flash frozen in liquid nitrogen or set at paraffin.

2.6. 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 was 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 following

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

Gene symbol	Assay ID
Act-β	Rn00667869_m1
Hgf	Rn00566673_m1
Il-6	Rn01410330_m1
Myd88	Rn01640049_m1
Nfκ-B	Rn01399583_m1
Socs3	Rn00585674_m1
Tgf-β	Rn01475963_m1
Tlr-4	Rn00569848_m1
Tnf-α	Rn00562055_m1
Casp3	Rn00563902_m1

formula: percent β-actin = (100) $\times$ $2^{\Delta CT}$ [27]. 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. Livers of animals without injury were used as calibrator group ($n = 5$).

2.7. Liver Regeneration Rate. The liver regeneration rate was calculated as follows: liver regeneration rate (%) = $100 \times [C - (A - B)]/A$, where A is the estimated liver weight before 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 [28].

2.8. Histology. Paraffin-embedded liver specimens were cut in 4 μm sections and stained with hematoxylin and eosin (H-E). To assess the rate of hepatocyte proliferation, the number of hepatocytes undergoing mitosis was counted in 10 high-power fields (HPF) in 72 hs after HP (mitotic index) [29].

To determine the number and the size of parenchymal cells per slide, the hepatocytes nuclei were counted and internuclear distance was measured in 5 HPF using Cell Imaging Software for Life Science Microscopy (Olympus) at 72 h after HP.

2.9. Enzyme Assays. Fluorimetric caspase activity (Sigma-Aldrich, USA) assays were performed according to manufacturer's instruction. Briefly, approximately 100 μg of liver was placed in an opaque 96-well plate and 200 μL of mixture reaction solution (containing acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin) was added in each well. The plate was incubated in dark at 25°C and every 10 minutes the fluorescence was read at 360 nm of excitation and 460 of emission. Caspase activity was normalized by protein.

For lysosomal-β-glucuronidase (Gusb) measurement, livers were homogenized in PBS buffer with proteases inhibitor cocktail 1%. Assays were performed using the chromogenic substrates 4-methylumbelliferyl-β-L-glucuronide (Sigma-Aldrich) at pH 4.5. One unit of enzyme activity converts 1 nmol of substrate to product per hour at 37°C.

2.10. Statistical Analysis. Results were expressed as means ± standard deviation (SD) or medians when required. Statistical differences were assessed by Student's t-test and for nonparametric variables Mann-Whitney test 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 .05 were considered statistically significant.

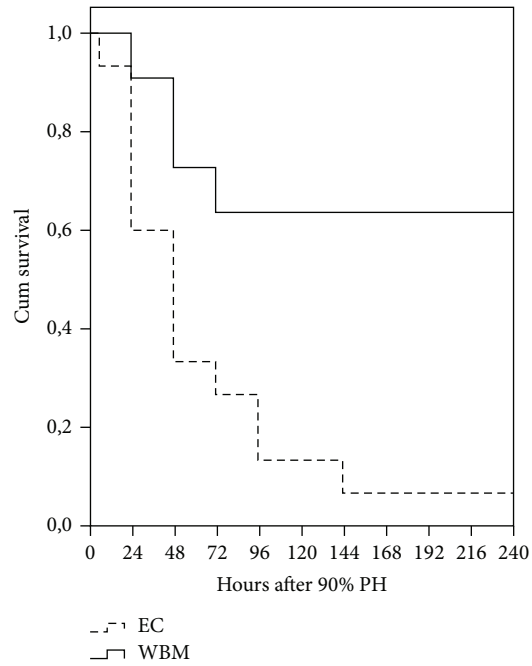

Figure 1: Spontaneous survival according to therapeutic regimen in rats after 90% partial hepatectomy (PH). EC: empty capsules, WBM: whole bone marrow, and Cum: cumulative. Log rank = .002.

3. Results

3.1. 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%) ($P = .002$). 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, to evaluate the effect of encapsulated WBM on the regeneration pathway the remaining analyses were performed in the first 72 hours after 90% PH.

3.2. Expression of Genes Involved in Liver Regeneration. First we assessed the expression levels of genes related to the early phase of liver regeneration. The expression of *Tnf-α* ($P = .01$) and *Nfκ-B* ($P = .01$) was markedly decreased in WBM group at 6 hours after 90% PH (Figures 2(a) and 2(b)). As a result, the expression of *Il-6* was also decreased ($P = .04$) in the WBM group compared to EC group (Figure 2(c)). Interestingly, LPS receptor (*Tlr-4*) and its mediator (*Myd88*) showed no differences in gene expression between groups 6 hours after 90% HP (Figures 2(d) and 2(e)).

We then analyzed genes related to the progress of liver regeneration. At 12, 24, and 48 hours after 90% PH other

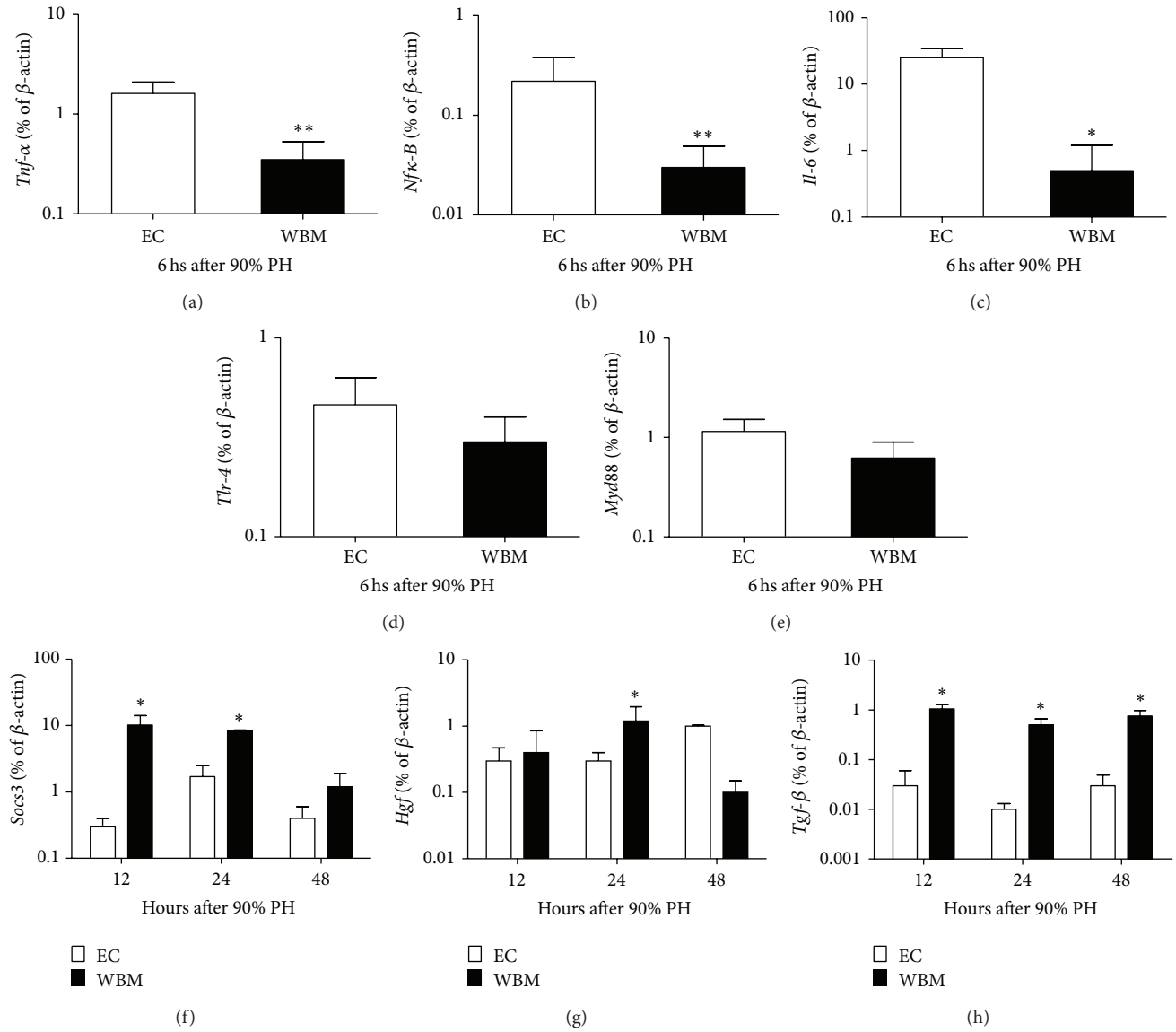

Figure 2: Liver gene expression after 90% partial hepatectomy (PH). Liver gene expression of *Tnf-α* (a), *Nfκ-B* (b), *Il-6* (c), *Tlr-4* (d), and *Myd88* (e) 6 hours after 90% partial hepatectomy and *Socs3* (f), *Hgf* (g), and *Tgf-β* (h) 12, 24, and 48 hours after 90% partial hepatectomy. WBM: whole bone marrow; EC: empty capsules. Values are expressed as means ± SD in log scale. Student's *t*-test, $^{*}P < .05$, $^{**}P < .01$.

genes were also differently expressed between WBM and EC groups. *Socs3*, which inhibits signaling via *Il-6*, was increased in the WBM group at 12 and 24 hours after 90% PH ($P \leq .05$, Figure 2(f)). *Hgf* was slightly increased in WBM only 24 hours after 90% PH ($P = .04$, Figure 2(g)), whereas the expression of *Tgf-β* was increased in WBM group in 12–48 hrs ($P \leq .03$, Figure 2(h)).

3.3. Liver Regeneration Rate and Histology Analysis. Interestingly, genes that promote liver regeneration were decreased in WBM group, whereas genes that halt hepatocyte division were increased. On the other hand, liver regeneration rate increased gradually after surgery, but without differences between groups at 6, 12, 24, or 48 hours. However, as shown in Figure 3(a), at 72 hours WBM group showed a lower regeneration rate compared to EC group (44% versus 59%, $P = .003$). Nevertheless, no differences were found in the number of mitotic cells in both groups (Figure 3(b)) and the number of hepatocytes at 72 hours after PH was also similar (Figure 3(c)). Surprisingly, the internuclear distance among hepatocytes was higher in EC group compared to WBM group at 72 hours ($P = .003$; Figure 3(d)), indicating that hepatocytes in WBM group were smaller than in EC group, resembling that of normal liver (data not shown). This could explain the lower regeneration rate, measured by changes in the remnant liver weight.

3.4. Mechanisms of Cell Death. Since no differences were found regarding cell proliferation, we then investigated if encapsulated WBM cells could lead to differential cell death.

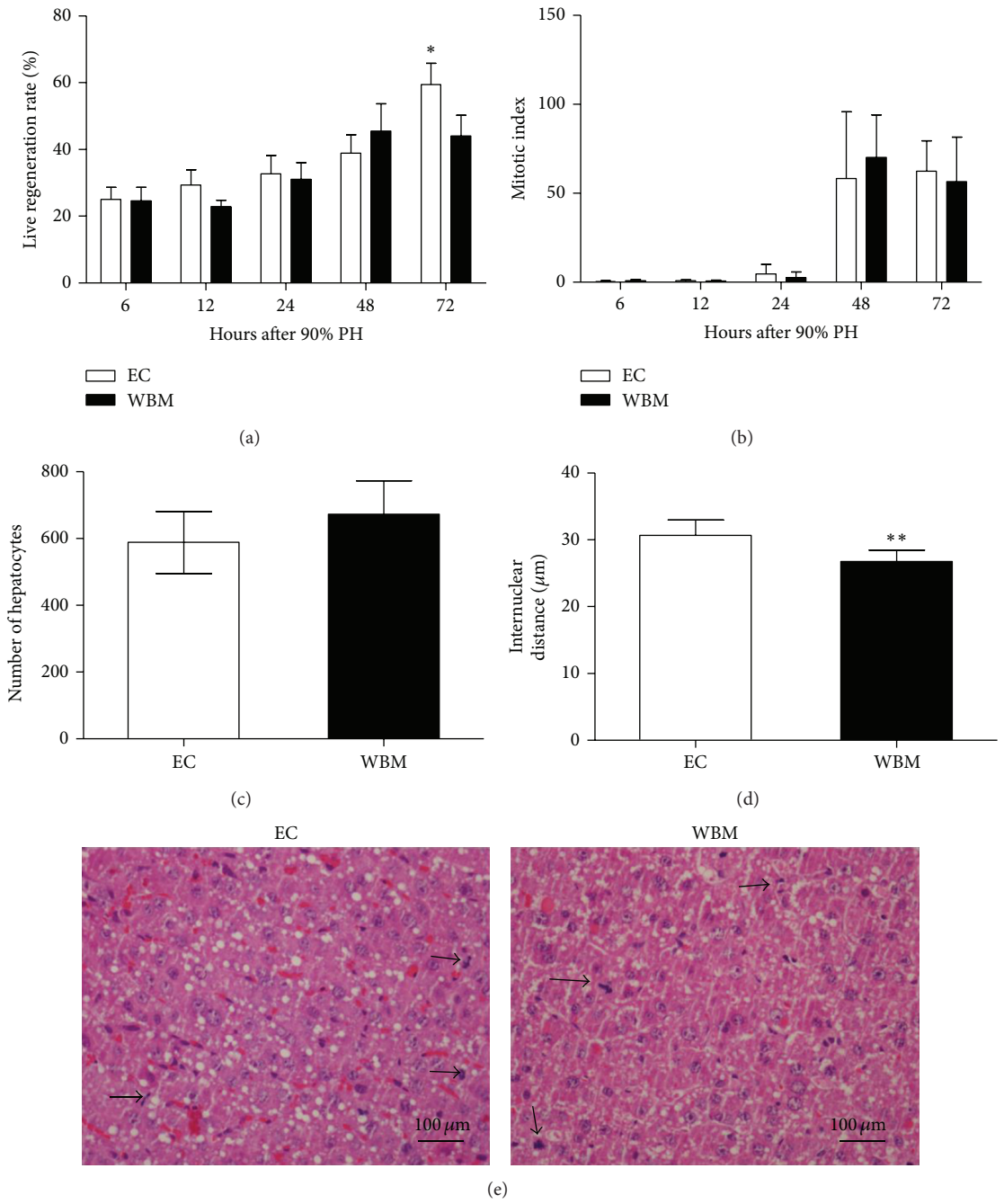

FIGURE 3: Liver regeneration rate after 90% partial hepatectomy (a). Mitotic index of hepatocytes after 90% partial hepatectomy (b). Number of hepatocytes (c) and internuclear distance (d) at 72 hours after partial hepatectomy. (e) Histology of mitotic hepatocytes (arrows) 72 hours after 90% partial hepatectomy; liver slides were stained with H-E. WBM: whole bone marrow; EC: empty capsules. Values are expressed as means ± SD. Student's t-test, $^{*}P < .05$, $^{**}P < .01$.

In order to assess possible mechanisms of cell death associated with our results, we quantified Caspase 3 as a measure of apoptosis and Gusb activity as an indicator of necrosis. We observed that WBM group had higher levels of *Casp3* at all times points ($P < .05$, Figure 4(a)), except at 72 hours where there was no difference between groups. Caspase 3 activity was also assessed in liver homogenates at 24, 48, and 72 hours. It was increased in WBM compared to EC group only at 48 hours ($P = .013$; Figure 4(b)), suggesting that cells from WBM group are dying by apoptosis. Interestingly when we evaluated Gusb activity at the same time point, WBM group presented less activity at 72 hours than EC group ($P = .009$; Figure 4(c)) suggesting that hepatocytes from EC group are dying by necrosis.

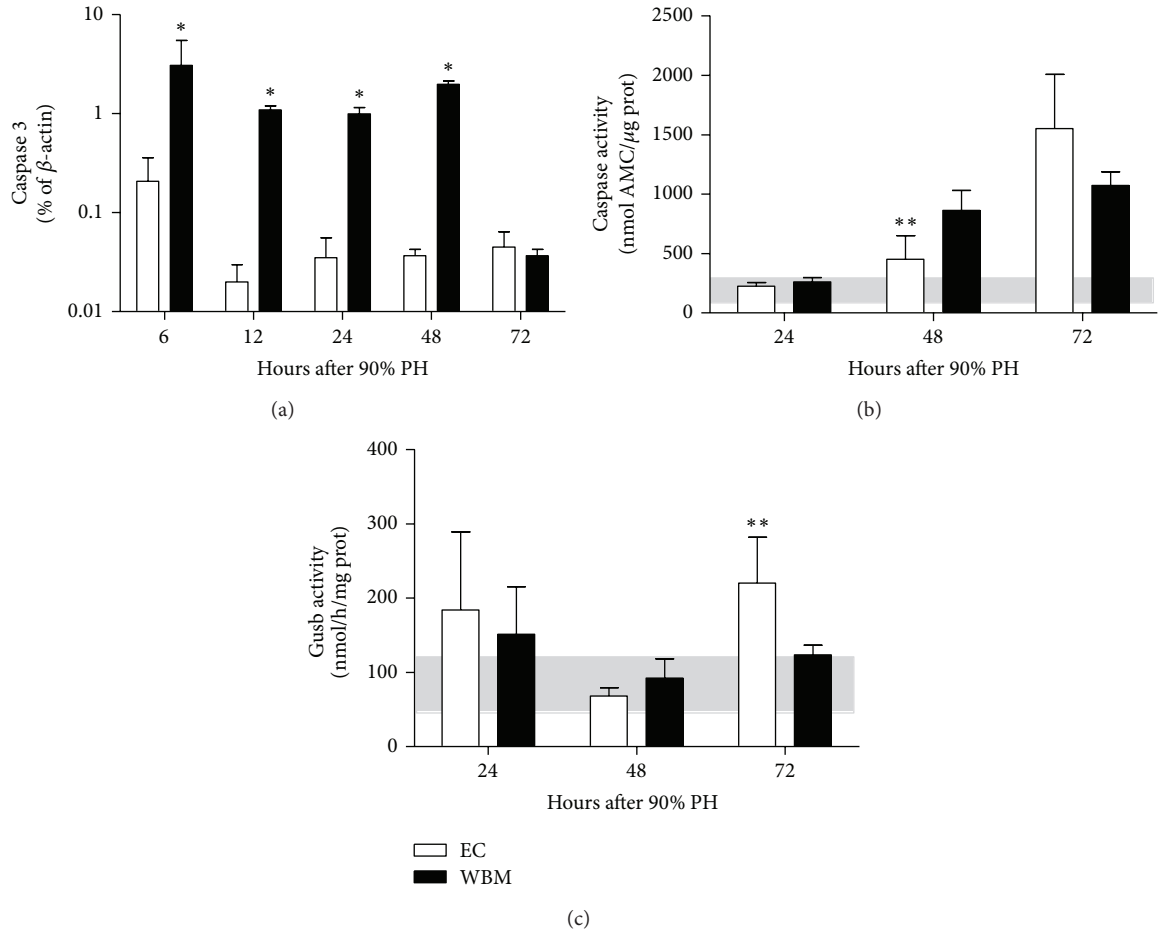

Figure 4: Mechanisms of cell death after 90% partial hepatectomy (PH). (a) Liver gene expression of *Caspase 3* at 6, 12, 24, 48, and 72 hours after 90% PH. (b) Caspase 3 activity and (c) lysosomal-β-glucuronidase (Gusb) activity at 24, 48, and 72 hours after 90% PH. Gray bar indicates normal values. WBM: whole bone marrow; EC: empty capsules. Values are expressed as means ± SD. Student's t-test, $^{*}P < .05$; $^{**}P < .01$.

4. Discussion

In the present study we showed that encapsulated WBM cells increase ten-day survival in a model of 90% PH by acting very early in the regenerative process. At 6 hours after 90% PH the synthesis of inflammatory cytokines in the liver was reduced. Moreover, the expression of factors abrogating liver regeneration (such as *Tgf β* and *Socs3*) was increased from 12 hours on, thus suggesting a decrease in the pace of liver regeneration through the secretion of paracrine factors. Our results corroborate the findings of Liu and Chang [13, 28, 29, 32], who showed that encapsulated WBM cells increased survival in rats after 90% PH.

The increase in 10-day survival rate from 6.7% in EC group to 63.6% in the treated group may not be directly comparable to other data in the literature. Indeed the survival rate after 90% PH is quite variable. It depends on many factors, including the surgeon's experience, the use of glucose, and the type of anesthesia [25]. In fact, some authors report 100% survival in one week [30] whereas others have 0% survival after 2 days [31], both using glucose supplementation as in the present study. Therefore, it is important to compare the differences between treated and untreated animals within the same research group, as all animals are submitted to the same surgeon, anesthetic protocol, and glucose administration. Also, there is no group without empty capsules (EC); therefore an influence of alginate itself in the survival curve cannot be ruled out. Yet, the results reported here can be compared to those of Liu and Chang [32] who reported 35% survival in empty capsules group in 10 days (and 100% in those treated with whole bone marrow cells). However, they showed an increase in the secretion of Hgf suggesting that it stimulated liver regeneration [32].

We evaluated the expression of inflammatory cytokines *Il-6*, *Tnf-α*, and *Nfκ-B* that are pivotal for the beginning of liver regeneration [33]. We observed that these cytokines were all decreased in WBM group at 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 Tlr-4 triggers the regenerative process [34]. 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 would not be 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 after 90% PH. On the other hand, the expression of *Tgf-β*, an inhibitor of Hgf [35], 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 [36, 37], was also increased in WBM group. Taken together, these data suggest that encapsulated WBM cells are increasing survival by decreasing liver regeneration rate.

Nevertheless, the liver regeneration rate was similar in both groups until 48 hours. Only at 72 hours did WBM group show a decreased regeneration rate compared to EC group. Ninomiya et al. [22] suggested that the abrupt regenerative response after PH causes a derangement of the lobular architecture that is damaging to hepatocytes. In their work, the deceleration of liver regeneration increases survival rate after 90% PH. Accordingly, in the present study WBM group survival rate was 63% compared to 6.7% in EC group at 10 days after surgery.

It is important to stress that the rate of regeneration mentioned above is evaluated by the weight of the remaining liver. Thus, a more precise measure of regeneration rate would be mitotic index or hepatocyte number. However, when we evaluate these parameters we found no differences between the WBM group and EC group. Nevertheless, the internuclear distance was smaller in WBM group, suggesting that hepatocytes were smaller when compared with EC group. Therefore, these results point to the fact that hepatocytes of EC group are swelled and this may contribute to an increase in the reminiscent liver weight.

Cell swelling is an indication of hydropic degeneration, as observed by López et al. [37] in the 90% PH model. This led us to hypothesize that WBM group's hepatocytes are healthier than EC group's hepatocytes, maybe due to protective cell death. Both *Caspase 3* gene expression and activity were increased in the WBM group. Furthermore, Gusb activity, a marker of necrosis [38], was lower in WBM group. These results indicate that in the WBM group the predominant mechanism of cell death is apoptosis whereas in the EC group it is necrosis.

Apoptosis may be considered a controlled process to eliminate malfunctioning cells and results in apoptotic bodies that will be phagocyte by other cells [39]. Necrosis, on the other hand, is a traumatic cell death in which cells swell until the lysis and spread of intracellular components, which will trigger the immune response, leading to inflammation [39]. We observed that in both groups liver cells died as a consequence of injury; nonetheless in WBM group the death is cleaned and controlled.

It is worth noticing that donor and recipient animals were not related, as our experiments were performed in Wistar rats, which are outbred animals. However, as the cells are encapsulated in alginate beads no immune reaction against the cells is expected; that is the function of the capsules. The allograft model seems to be a better option as in a clinical setting one may not expect a patient in acute liver failure to be able to provide cells for transplantation or to wait for a match donor to be found.

In summary the results presented here show that encapsulated WBM cells increase survival in a model of 90% PH, reduce the expression of genes involved in liver regeneration, such as *Tnf-α*, *Nfκ-B*, *Il-6*, and *Hgf*, and increase those responsible for ending hepatocyte division, such as *Tgf-β* and *Socs3*. In addition to that, these cells favor apoptotic cell death and decrease necrosis, thus increasing long term survival. Although there is no definitive answer on how these cells exert their beneficial effects, a few hypotheses may be ruled out. There is no immunomodulatory effect of stem cells, as data on systemic cytokine levels did not differ between groups (Supplementary Figure 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/4831524). Differences related to genes involved in liver regeneration were found but point to the opposite direction (as one would expect survival to be related to a faster regeneration). Also, no difference was found on cell proliferation. Unfortunately we were unable to retrieve enough RNA from recovered capsules in order to investigate what kind of changes happened in WBM cells, although preliminary data from an ongoing study from our group suggest that they may be compensating for some liver function, as well as which specific cell types are involved in this response.

Abbreviations

ALF: Acute liver failure
WBM: Whole bone marrow
EC: Empty capsules
PH: Partial hepatectomy
LPS: Lipopolysaccharides
Tlr-4: Toll like receptor 4
MYD88: Myeloid differentiation factor
Tnf: Tumor necrosis factor
Il-6: Interleukin-6
Hgf: Hepatocyte growth factor
Tgf-β: Transforming growth factor-beta
Nfκ-B: Nuclear factor kappa B
Socs3: Suppressor of cytokine signaling 3
Gusb: Lysosomal-β-glucuronidase
SD: Standard deviation.

Conflict of Interests

The authors have no conflict of interests to declare.

Authors' Contribution

Carolina Uribe-Cruz and Carlos Oscar Kieling contributed equally to this work.

Acknowledgment

This work was supported by FIPE/HCPA, PRONEX/FAPERGS 10/0039-3.

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Anexo 4: INJURED HEPATOCYTE-RELEASED MICROVESICLES INDUCE BONE MARROW-DERIVED MONONUCLEAR CELLS DIFFERENTIATION

Differentiation 90 (2015) 40–47

Contents lists available at ScienceDirect

Differentiation

journal homepage: www.elsevier.com/locate/diff

Injured hepatocyte-released microvesicles induce bone marrow-derived mononuclear cells differentiation

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ARTICLE INFO

Article history:
Received 2 April 2015
Received in revised form
4 August 2015
Accepted 14 September 2015
Available online 26 September 2015

Keywords:
Cell plasticity
Hepatocyte-like cells
Acute liver failure
Extracellular vesicles
Co-culture system

ABSTRACT

The ability of bone marrow-derived mononuclear cells (BMMCs) to differentiate into hepatocyte-like cells under different conditions has been demonstrated previously. In the present study, we investigated the effect of CCl_4-injured hepatocytes on the differentiation of the non-adherent (NAD) fraction of BMMCs. Differentiation (cell fate) was analyzed after 2, 6 and 24 h of co-culture by gene and protein expression and by urea production. We also evaluated the presence of microvesicles (MVs) in the supernatant of differentiated cells, their content and the ability of these cells to absorb them. Hepatocyte-like characteristics were observed in the NAD cells after 24 h of co-culture with injured hepatocytes. Cells that were co-cultured with healthy hepatocytes did not present signs of differentiation at any analyzed time point. Analysis of the supernatant from differentiated cells revealed the presence of MVs carrying hepatocyte-specific mRNAs, including *Albumin*, *Coagulation factor V*, *Alpha-fetoprotein*, and *Cytokeratin 18*. The incorporation of injured hepatocyte-derived MVs by NAD cells was shown at 24 h, suggesting a possible role for MVs in the induction of cell plasticity.

1. Introduction

Bone marrow-derived mononuclear cells (BMMCs) represent a potential source of material for cell-based therapies (Muraca, 2011). BMMC transplantation has shown great benefits in the treatment of patients with and in experimental models of acute liver failure (Park et al., 2013; Baldo et al., 2010; Belardinelli et al., 2008). However, the mechanisms by which these cells contribute to the recovery of the damaged liver remain controversial. Thus far, bone marrow-derived cells are believed to act by fusion with host cells, differentiation into hepatocyte-like cells and/or secretion of paracrine factors (Quintana-Bustamante et al., 2012; Lagasse et al., 2000; Liu and Chang, 2006).

The ability to differentiate into hepatocyte-like cells and into other hepatic cells has been attributed to various cell types, particularly bone marrow-derived mesenchymal stem cells and peripheral monocytes (Lee et al., 2004; Ruhnke et al., 2005). In a previous study from our group, we observed that microencapsulated BMMCs are inducible into hepatic lineages both *in vitro* (6 h) and *in vivo* (48 h) when exposed to injured liver cells for a short period (Matte et al., 2014).The events of rapid differentiation after injury exposure have usually been interpreted as the humoral induction of differentiation; however, several studies have suggested that this differentiation could be due to the microvesicular transfer of information (Aliotta et al., 2007; Quesenberry et al., 2010).Cells release diverse types of membrane vesicles into the extracellular environment; these vesicles represent an important mode of intercellular communication by serving as vehicles for the transfer of membrane and cytosolic proteins, lipids, and RNA between cells (Raposo and Stoorvogel, 2013).Examples of extracellular vesicles include exosomes (50–100 nm in diameter) that originate from endosomal membranes,

Abbreviations: BMMCs, Bone marrow-derived mononuclear cells; CCl_4, Carbon tetrachloride; NAD, Non-adherent; MVs, Microvesicles; MSCs, Mesenchymal stem cells; $ED1^+$, Monocyte/macrophage marker; MACS, Magnetic-activated cell sorting; DMEM, Dulbecco's modified eagle medium; FBS, Fetal bovine serum; P/S, Penicillin/streptomycin; CO_2, Carbon dioxide; FITC, Fluorescein isothiocyanate; PE, Phycoerythrin; CD29, Integrin β1; CD90, Thy-1; CD45, lymphocyte common antigen; CD31, Platelet endothelial cell adhesion molecule; qPCR, Real-time polymerase chain reaction; Alb, Albumin; CK18, Cytokeratin 18; FV, Coagulation factor V; Afp, Alpha-fetoprotein; β-act, β-actin; PBS, Phosphate buffered saline; DAPI, 4′,6-diamidino-2-phenylindole; HSCs, Hematopoietic stem cells

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http://dx.doi.org/10.1016/j.diff.2015.09.001
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microvesicles (MVs, 100–1000 nm in diameter) that originate from plasma membranes, and apoptotic bodies (50– 5000 nm in diameter) that form only during programmed cell death (Yang et al., 2014).

The tissue-specific expression of lung, brain, heart, and liver mRNAs has been demonstrated in bone marrow cells after co-culture with radiation-injured tissue. This effect on phenotype change is promoted both by the delivery of mRNAs to bone marrow cells and by bone marrow cell transcription of tissue-specific mRNAs *in vitro* (Aliotta et al., 2007, 2010). The release of extracellular vesicles from the damaged tissue has also been detected in *in vivo* studies. Circulating liver-specific mRNAs that are packaged in MVs serve as molecular markers of hepatic injury in rat liver fibrosis models (Kudo et al., 2008) and of severe drug-induced acute liver injury (Wetmore et al., 2010).

To our knowledge, although sufficient evidence of BMMC differentiation exists, the plasticity of the non-adherent BMMC components (other than MSCs and monocytes) has not yet been investigated. In the present study, we analyzed the effect of injured hepatocytes on the differentiation of the non-adherent (NAD) subset of BMMCs after the depletion of the monocytic lineage ($ED1^+$) by magnetic-activated cell sorting (MACS). Additionally, we employed an *in vitro* co-culture model to exclude the possibility of cellular fusion. We also evaluated the presence of RNA shuttled by MVs in the supernatant of differentiated cells and the ability of these cells to absorb the MVs. This study attempted to clarify the capacity of the hepatic differentiation of NAD BMMCs and to define the role of the injury microenvironment in cell plasticity.

2. Materials and methods

2.1. Cells isolations and CO-culture

2.1.1. Animal experimentation

The procedures and protocols here described were approved by the Ethics Committee in Animal Research of the Hospital de Clinicas de Porto Alegre. All animals received humane care according to the National Institute of Health guidelines.

2.1.2. Bone marrow mononuclear cells (BMMCs) isolation

BMMCs were isolated from male Wistar rats ($n=12$) at the age of 2–3 months. The animals were sacrificed with a lethal dose of anesthesia. Then, tibias and femurs were removed, and the bone marrow was flushed out of the bone cavities. Mononuclear cells were obtained by density gradient centrifugation with Ficoll Histopaque®-1077 (Sigma-Aldrich, USA) at 2000 rpm for 20 min at room temperature.

2.1.3. Monocytic lineage depletion and non-adherent (NAD) fraction separation

Mononuclear cells were collected, and the monocytic lineage was depleted by negative magnetic cell sorting using a MiniMACS system (Miltenyi Biotec, Germany). For negative selection, the cells were labeled with biotin mouse anti-rat mononuclear phagocyte (ED1) primary antibody (1:100 v/v, BD Biosciences Pharmingen, USA), incubated with anti-biotin microbeads and then passed through a column attached to a strong magnetic field according to the manufacturer's instructions. The labeled cells were retained in the column, while $ED1^-$ cells passed freely through the magnetic field. The exclusion of $ED1^+$ cells was confirmed by flow cytometry based on forward/side scatter (FSC/SSC) properties using an Attune® Acoustic Focusing Cytometer (Applied Biosystems, USA). $ED1^-$ BMMCs were seeded (1×10^6 cells/insert) in 6-well cell culture inserts with basic medium (low-glucose DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin/streptomycin (P/S, Gibco, USA). The cells were kept in a chamber at 37 °C with 5% CO_2 for 72 h, and then the supernatant containing NAD cells was collected and centrifuged. Next, the pelleted cells were transferred to a fresh insert with basic medium. The cells were collected for flow cytometry analysis immediately after separating the NAD fraction. The cells were fixed in 2% formaldehyde for 20 min and then incubated with primary antibody (1:100 v/v) for 30 min at 4 °C according to the manufacturer's instructions. Fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated primary antibodies against rat cell-surface markers CD29, CD90, CD45, and CD31 (all from BD Biosciences, USA) were used to label the cells. The detection of PE and FITC labeling was performed using an Attune® Acoustic Focusing Cytometer (Applied Biosystems, USA). At least 10,000 events were collected.

2.1.4. Hepatocyte isolation from acute liver failure model or healthy animals

Acute liver failure was induced in male Wistar rats at the age of 2–3 months by the administration of CCl_4 (Merck, Germany) at 1.25 mg/kg weight in olive oil through gavage (Baldo et al., 2010) at 24 h before hepatocyte isolation. Control animals received only vehicle. Liver injury was assessed macroscopically based on the appearance of nutmeg liver and microscopically based on the deposition of lipid droplets stained with Oil Red-O (Merck, Germany).

Hepatocytes were isolated from male Wistar rats with acute liver failure ($n=6$) or from healthy animals ($n=6$) by the two-step collagenase perfusion method as described by Seglen (Seglen, 1976), with modifications (Rodrigues et al., 2012), using collagenase type II (Gibco, USA). Hepatocytes were seeded in 6-well plates coated with type I collagen (3×10^5 cells/well) and cultivated in low-glucose DMEM (Gibco) supplemented with 20% FBS (Gibco) and 1% P/S (Gibco) for 12 h to allow the cells to adhere before co-culture experiments. For some experiments, the hepatocytes were labeled with PKH26 dye (Sigma-Aldrich), with the lipid bilayers intercalating red fluorescent chromophores, before cell seeding. Membrane labeling was confirmed by flow cytometry and by fluorescence microscopy.

2.1.5. CO-culture system

To analyze the effect of injured hepatocytes on NAD cell differentiation, we used a two-chamber co-culture system in which hepatocytes were placed in the lower chamber and NAD cells were placed in the upper chamber and isolated using a 6-well cell culture insert with 0.4 μm pore sizes (BD Biosciences). After the hepatocytes adhered, the medium was changed to basic medium, and the inserts were coupled in the wells containing the hepatocytes. NAD cells were transferred for co-culture immediately after separation (72 h). Co-culture was maintained for 2, 6 and 24 h at 37 °C with 5% CO_2 in basic medium (Fig. 1A).

2.1.6. RNA purification and gene expression analysis

After the co-culture periods cited above, NAD cells were collected, washed once with PBS 1X, and total RNA was extracted using an RNeasy Mini Kit (Qiagen, Germany). cDNA was synthesized using a SuperScript® II Reverse Transcriptase kit (Invitrogen, USA) according to the manufacturer's instructions and then used as a template (100 ng) for real-time polymerase chain reaction (qPCR). qPCR was performed on a Stratagene Mx3000P analyzer using TaqMan® Assays (Applied Biosystems) according to the manufacturer's instructions to analyze the expression of hepatocyte-specific genes, including *Albumin (Alb*; Rn00592480_m1), *Cytokeratin-18 (Ck-18*; Rn01533363_g1), *Coagulation Factor V (FV*; Rn01483178_m1), and *Alpha-fetoprotein (Afp*; Rn00560661_m1),

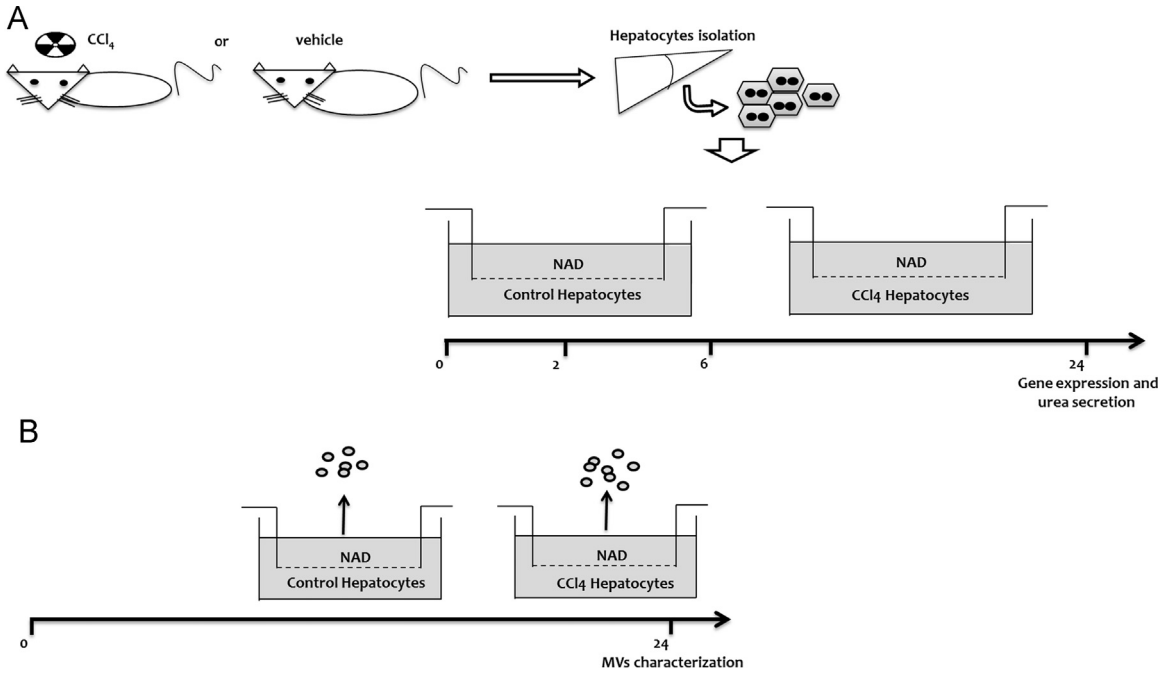

Fig. 1. Summary of experimental design. (A) Co-culture system and differentiation analysis of NAD cells. (B) MV isolation and characterization after differentiation.

with *β-actin* (*β-act*; Rn00667869_m1) as the internal control. All samples were run in duplicates using identical amounts of cDNA. Gene expression was calculated based on the normalized mean differences between each target gene and the internal control (*β-act*). The $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression of each target gene. The calibrator group, which was composed of NAD cells cultured alone, was used for all comparisons.

2.1.7. Urea quantification

The measurement of urea production in culture medium was used to evaluate specific hepatic function by NAD cells. After co-culture with hepatocytes, NAD cells were removed, washed with 1X PBS and placed in fresh basic medium. After 24 h, 1 mL of medium was collected, and urea was quantified using a QuantiChrom™ Urea Assay Kit (DIUR-500, BioAssay Systems, USA) according to the manufacturer's instructions.

2.1.8. Immunofluorescence

NAD cells were collected after co-culture, transferred to histological slides using a cytospin centrifuge and fixed with cold methanol. The cells were permeabilized with 0.25% Triton X-100 and blocked with normal rabbit serum. Sheep polyclonal antibody directed against serum albumin (1:400 dilution, Abcam, UK) was incubated with each sample overnight at 4 °C. Secondary antibody rabbit anti-sheep IgG-FITC (1:400 dilution, Sigma-Aldrich, USA) was added and incubated for 1 h at room temperature. Fluoroshield™ with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) was used to counterstain the nuclei. Freshly isolated hepatocytes were used as a positive control for staining.

3. Microvesicles

3.1. Isolation of microvesicles (MVs)

We analyzed cell culture supernatants of NAD cells that showed signs of differentiation into hepatocyte-like cells after co-culture based on the results of liver-specific gene expression, urea production and albumin synthesis (differentiated NAD cells) to investigate the influence of MVs on cell fate. We also used the supernatants of undifferentiated NAD cells (NAD cells cultured alone or co-cultured with healthy hepatocytes) and of healthy and injured hepatocytes (CCl_4) to isolate MVs after 24 h in culture (Fig. 1B). The presence of MVs was investigated as previously described (Aliotta et al., 2010). Briefly, the medium was collected from the upper chamber and centrifuged at 2000 × *g* for 20 min to remove debris. Cell-free supernatants were ultracentrifuged (100,000 × *g*, 1 h, 4 °C) in a HIMAC CP80WX ultracentrifuge (Hitachi, Japan). The pelleted materials were fixed in 2% formol for flow cytometry and for Zetasizer analysis or stored at −80 °C for RNA analysis.

3.2. Characterization of MVs

The estimated sizes of the MVs were determined by electrophoretic light scattering technology using a Zetasizer NZ50 (Malvern Instruments, Great Britain). The MVs were diluted in filtered water for size measurement, whereas the MVs were diluted in 1 mM NaCl for zeta potential determination. The MVs were also analyzed by flow cytometry using an Attune Acoustic Focusing Cytometer (Applied Biosystems). Attune Performance Tracking Beads (2.4 and 3.2 μm, Applied Biosystems) and NAD cells were used as size markers, and the analysis was performed using a log scale for forward scatter and side scatter parameters as described previously (Bruno et al., 2009). The suspended MVs were stained with propidium iodide to examine the presence of apoptotic bodies.

3.3. RNAse A treatment and RNA content analysis

Pelleted MVs were treated with RNAse A (40 μg/mL) for 30 min at 37 °C to evaluate whether RNA was enclosed by a membrane and therefore protected against enzymatic degradation. RNA extraction was performed using an RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Then, the RNA content was measured by spectrophotometry (NanoDrop ND-1000, USA). Total RNA was used as a template for cDNA synthesis using Super Script

III reverse transcriptase (Invitrogen, USA), followed by qPCR for *β-act* as the internal control and for the target genes *Alb*, *Afp*, *Ck-18*, and *FV* using TaqMan® Assays (Applied Biosystems) as described above. The calibrator group, which was composed of MVs that were isolated from NAD cells cultured alone, was used for all comparisons.

4. Statistical analysis

The results are expressed as the means ± SD. Statistical comparisons were performed using Student's *t*-test with SPAW v.18 software (IBM, USA). The results were considered statistically significant when $p < 0.05$.

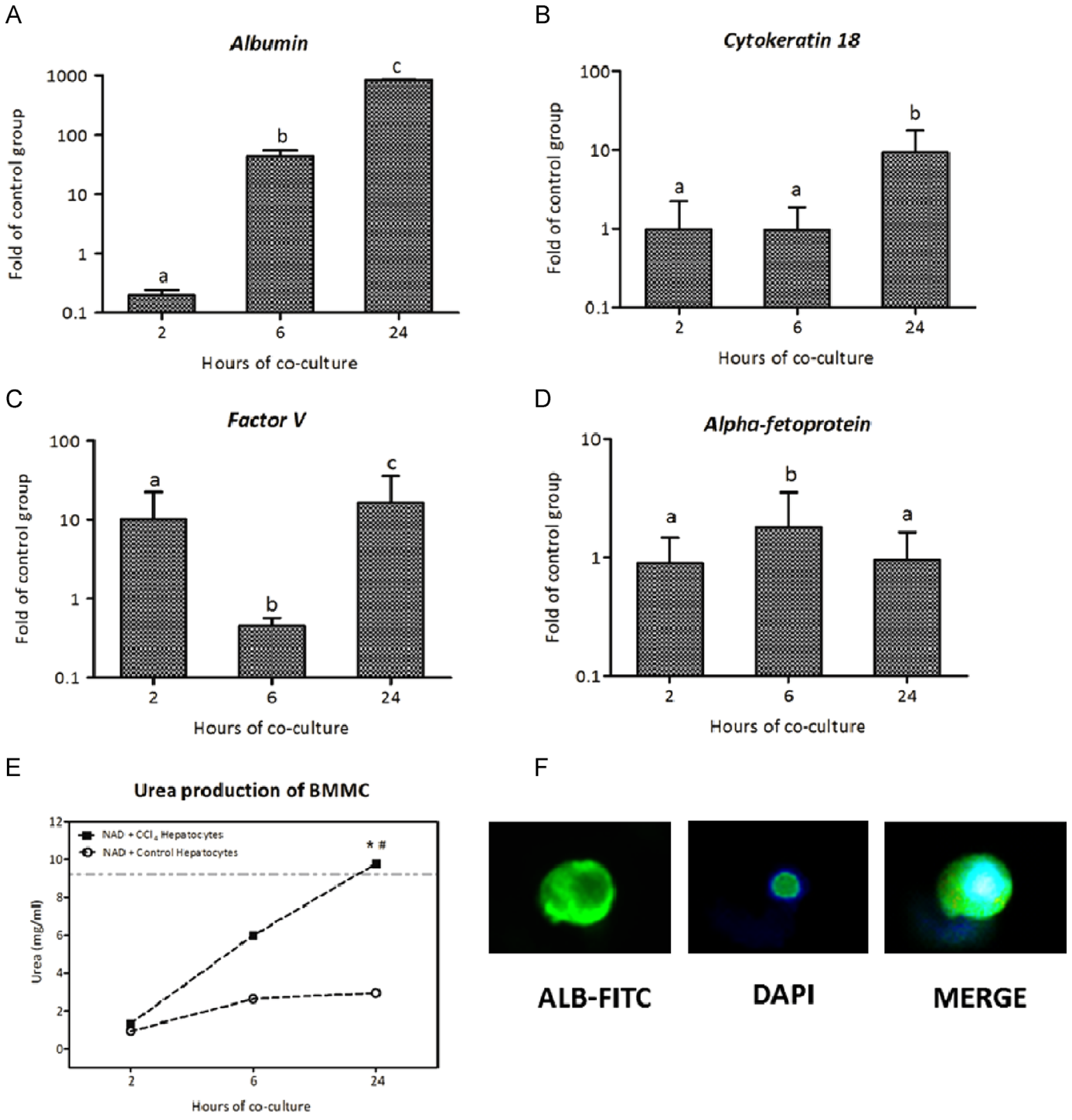

Fig. 2. Differentiation of NAD cells into hepatocyte-like cells. (A–D) Expression of hepatic markers in NAD cells after co-culture with CCl_4-injured hepatocytes. We observed increased expression of *Albumin* ($p < 0.05$), *Cytokeratin 18* ($p < 0.05$), and *Factor V* ($p < 0.05$) at 24 h of co-culture compared to shorter periods of co-culture. The values are expressed as the fold change compared to the control group (NAD cells co-cultured with healthy hepatocytes). Different letters (a, b, c) represent differences in the statistical analysis. **(E)** Urea synthesis was higher at 24 h of co-culture compared to the control group ($^*p < 0.05$) and to earlier time points analyzed ($\#p < 0.05$). **(F)** Immunofluorescence revealed Albumin-positive cells in the NAD fraction from the CCl_4 group at 24 h. Magnification, 400 ×.

5. Results

NAD cells differentiate into hepatocyte-like cells after 24 h of co-culture with damaged hepatocytes

NAD cells were obtained from ED1$^-$ BMMCs after 72 h in culture. Flow cytometry analysis showed that these cells were positive for CD29 (24.2%), CD90 (23.5%), CD45 (45.2%), and CD31 (20.25%) expression, presenting heterogeneous marker expression.

The differentiation cell fate was assessed by specific gene expression and by functional assays. RNA was obtained from NAD cells exposed to 2, 6 and 24 h of co-culture with hepatocytes that were isolated from control or CCl_4-treated rats. cDNA was synthesized and used as a template to verify the expression of liver-specific genes. *Albumin*, *Ck-18* and *FV* expression was elevated at 24 h compared to the earlier time points that were analyzed ($p < 0.05$) Fig. 2A–D). In contrast, the cells that were exposed to healthy hepatocytes did not express hepatic markers.

Urea production was assessed as a specific measure of hepatocyte function. NAD cells showed increases in urea production relative to the period of exposure to injured hepatocytes. Remarkably, the urea levels were higher in the CCl_4 group after 24 h of co-culture compared with those in the primary hepatocytes that were used as a positive control and significantly differed from those in the control group (NAD cells that were co-cultured with healthy hepatocytes; $p=0.004$). In addition, urea production was much lower in the control group compared with that in the CCl_4 group (Fig. 2E). Moreover, immunofluorescence revealed the presence of Albumin-positive cells in the NAD fraction after 24 h of co-culture only when the cells were exposed to injured hepatocytes (Fig. 2F).

5.1. Evidence of MVs released by NAD cells and hepatocytes in the co-culture medium

Because we observed signs of differentiation in the NAD fraction of the CCl_4 group after 24 h of co-culture, we used the supernatant of this experimental group in further experiments to investigate the presence of MVs. Supernatants were also collected from the media from NAD cells and hepatocytes cultured alone and from NAD cells that were co-cultured with healthy hepatocytes. The culture media were collected from the upper chambers of the co-culture plates to analyze the presence of MVs. The supernatant-isolated MVs were composed of a heterogeneous population with sizes ranging from 100 nm to >1 μm. Zeta potential analysis showed similar values between groups (-20 mV $\pm$ 5), which is representative of the cell membrane potential in physiological solution. MVs were detected by flow cytometry below the forward scatter signal corresponding to 2.4 μm beads (Fig. 3A). Propidium iodide staining showed the absence of apoptotic bodies in the supernatant-isolated MVs.

5.2. Liver-specific mRNAs are contained in MVs released by CCl_4-injured hepatocytes

Total RNA was extracted from MVs after treatment with RNase A, indicating that the RNA was protected against RNase A degradation because the MV membrane encompassed the RNA (Fig. 3B). Similar amounts of RNA were detected in all MV groups that were tested (NAD cells alone, healthy hepatocytes alone, CCl_4-injured hepatocytes alone, and NAD cells that were co-cultured with healthy or CCl_4-injured hepatocytes).

However, the expression of the hepatocyte-specific genes *Alb*, *Ck18*, *Afp*, and *FV* was detected only in supernatant MVs that were isolated from the co-culture of differentiated cells. A 7.7 $\pm$ 4.7-fold (SEM) increase in *Alb* mRNA expression was observed in the CCl_4 group compared to that of the control group ($p < 0.05$). Interestingly, no evidence of MVs carrying hepatocyte-specific genes was observed in the supernatant of undifferentiated cells, including NAD cells that were co-cultured with healthy hepatocytes (Fig. 3C). In this group, gene expressions were 1.41 $\pm$ 0.65-fold (SEM), 0.39 $\pm$ 0.31-fold, 1.45 $\pm$ 0.65-fold, 1.45 $\pm$ 0.65-fold of *Alb*, *Ck18*, *Afp* and *FV*, respectively. Internal control (*β-actin*) mRNA was detected in all groups.

5.3. NAD cells that differentiated into hepatocyte-like cells incorporate MVs

We stained hepatocytes with the membrane fluorescent dye PKH26 before co-culture to track the vesicular transfer of information between hepatocytes and BMMCs. Flow cytometry and

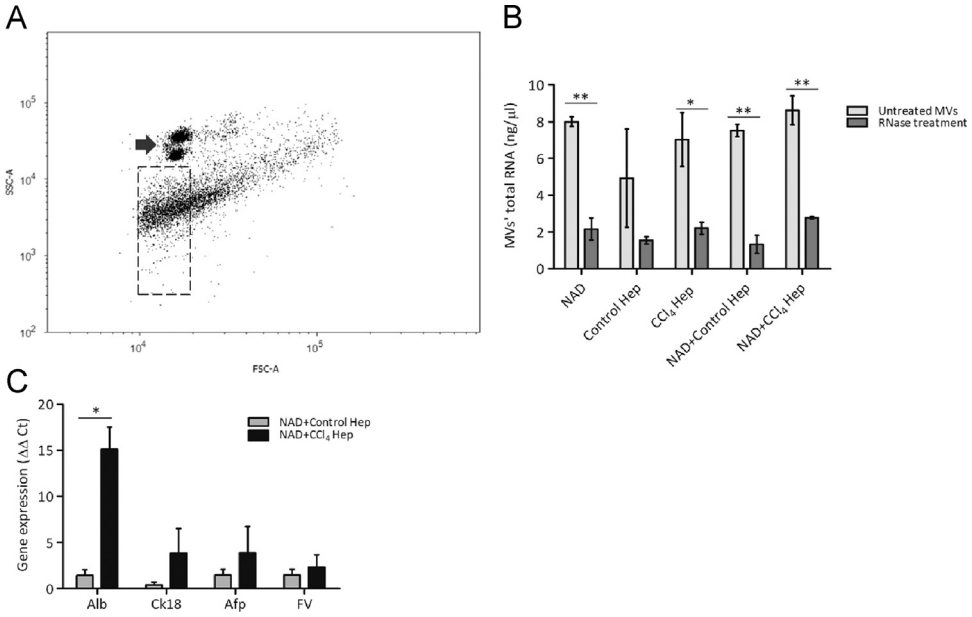

Fig. 3. Characterization of supernatant-isolated MVs. (A) MVs (gated population) were detected by flow cytometry and determined as smaller events than internal control beads (arrow). (B) RNA quantification before (light gray bars) and after (dark gray bars) RNase A treatment indicating that RNA is protected from degradation. (C) Liver-specific genes were found in the co-culture medium of the CCl_4 group (black bars) and were absent in the control group (gray bars) ($^*p \leq 0.05$; $^{**}p \leq 0.01$).

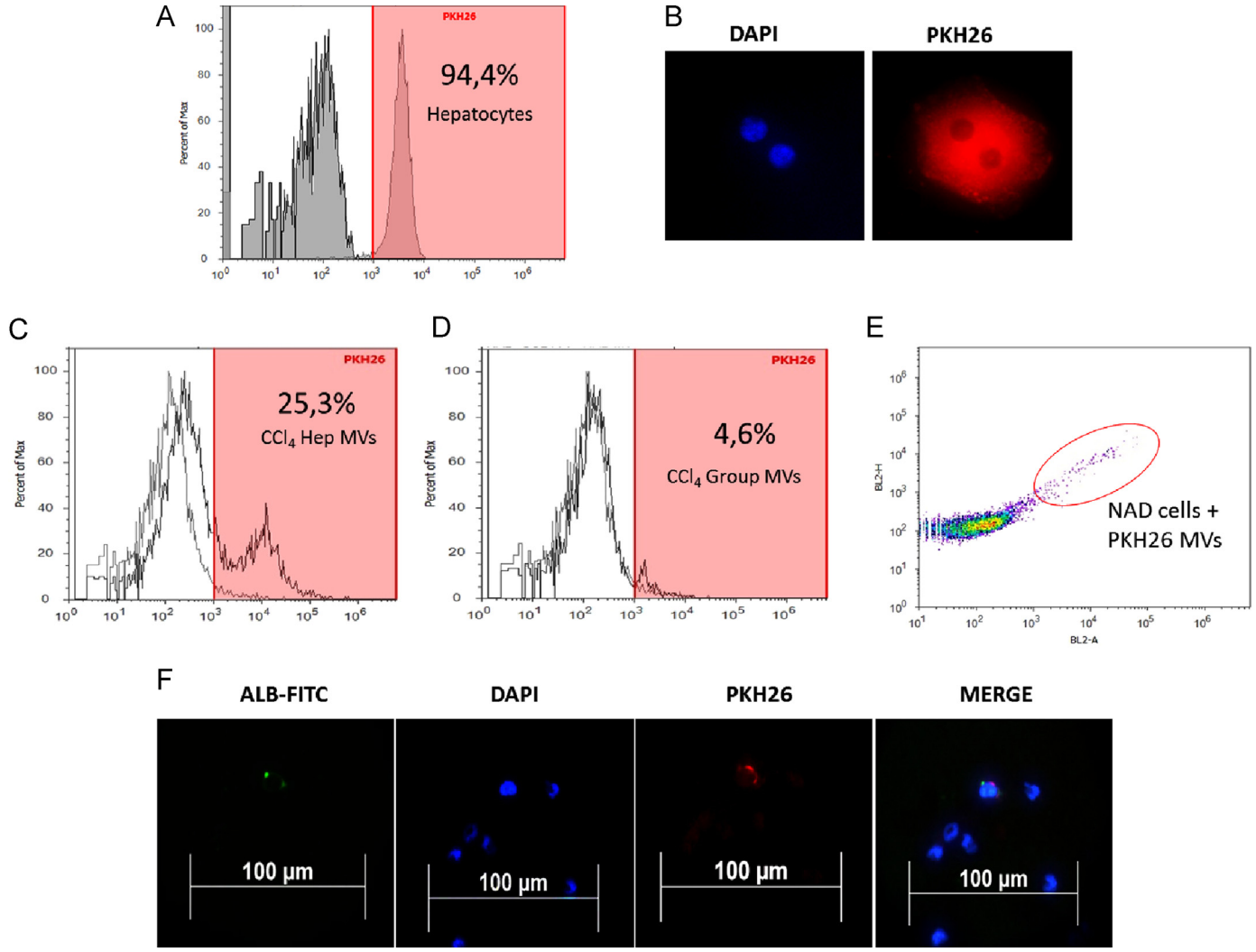

Fig. 4. Evidence of MV uptake by NAD cells. (A) PKH26 fluorescence was observed in hepatocytes before co-culture experiments by flow cytometry and (**B**) by microscopy. **(C)** From total MVs that were isolated from hepatocytes in culture, approximately 25% were stained with PKH26. **(D)** After 24 h of co-culture with NAD cells, less than 5% of MVs that were isolated from supernatants presented PKH26 staining. **(E)** PKH26-positive NAD cells were detected by flow cytometry (circled area). **(F)** Immunofluorescence also revealed the colocalization of PKH26 and FITC fluorescence (Albumin-positive cells). Magnification, 400 × . (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

immunofluorescence analyses confirmed that the hepatocytes were properly stained (Fig. 4(A and B)). Approximately 25% of the hepatocyte-derived MVs that were isolated from the supernatant of hepatocytes cultured alone were positive for PKH26 staining (Fig. 4C). However, less than 5% of the supernatant-isolated MVs in co-culture plates were positive for PKH26 (Fig. 4D). Therefore, we investigated whether NAD cells were positive for PKH26, and we confirmed that approximately 1% of these cells featured the red–orange fluorescence (Fig. 4E). Taken together, these findings suggest that hepatocyte-derived MVs may be incorporated by NAD cells.

In addition, Albumin-positive NAD cells were observed by immunofluorescence after 24 h of co-culture with injured hepatocytes. The presence of red-orange fluorescence was also evident in Albumin-positive NAD cells after co-culture with injured hepatocytes and staining with PKH26 (Fig. 4F). These findings suggest that Albumin-positive cells from bone marrow incorporated membrane from the hepatocytes, most likely due to the uptake of MVs.

6. Discussion

Early events of differentiation have been reported under several experimental conditions. Jang et al. (2004) demonstrated that HSCs expressed hepatocyte-specific genes and proteins, such as Albumin, after 8 h of co-culture with injured liver cells (Jang et al., 2004). In a previous study by our group, we observed that microencapsulated BMMCs expressed *Albumin* after 6 h of co-culture with CCl_4-injured hepatocytes and were able to produce and secrete urea (Matte et al., 2014). Both studies have shown that BMMCs that are exposed to damaged liver tissues are capable of taking on many characteristics of liver cell types without cell fusion.

In the present study, we observed hepatocyte-like characteristics in NAD cells from BMMCs after co-culture with damaged hepatocytes, which was not observed when NAD BMMCs were co-cultured with healthy hepatocytes. The expression of hepatocyte-specific genes was noted after a period of 24 h of co-culture with injured cells, which is a longer period than that observed in the differentiation of HSCs (8 h) (Jang et al., 2004) and whole BMMCs (6 h) in our previous study (Matte et al., 2014).

Extracellular vesicles may represent an important vehicle by which one cell communicates with another, delivering macromolecules (mRNAs, microRNAs, and proteins) and modulating recipient-cell phenotypes. In addition to a constitutive release of

MVs by cells, MV secretion is enhanced upon activation or apoptosis (Pap et al., 2009). Previous evidence has demonstrated the increased vesicular transfer of information under specific conditions, including irradiation, hypoxia, oxidative injury, diabetes, sepsis, thromboembolic disease, rheumatoid arthritis and cancer (Aliotta et al., 2007; Distler et al., 2005; Logozzi et al., 2009). The evidence of this mechanism of communication in cell differentiation was demonstrated by apoptotic bodies from mature endothelial cells influencing the differentiation of adult human endothelial progenitor cells (Hristov et al., 2004). Later studies showed the transfer of mRNA and microRNA among different cells through MVs (Baj-Krzyworzeka et al., 2006) and exosomes (Valadi et al., 2007), which represented a major breakthrough in the discovery of their functions and interactions with target cells.

In our model, we analyzed MVs that were present in the cell-free supernatants of co-cultured NAD cells and CCl_4-injured hepatocytes after the differentiation of NAD cells into hepatocyte-like cells. The ultracentrifugation method employed in the present study (100,000 × *g* for 60 min) is widely accepted for the isolation of MVs larger than 100 nm (Pap et al., 2009). Zetasizer analysis showed that the MVs formed a rather heterogeneous population with sizes ranging from 100 nm to more than 1 μm. In addition, the zeta potential for MVs presented slightly negative values compared to double-layer membrane under physiological conditions, which might be due to the presence of a higher content of phosphatidylserine in the outer lipid monolayer of the membrane (Bondar and Saifullina, 2012). Interestingly, phosphatidylserine has been described as an MV marker in situations of cell surface and cytoskeleton reorganization, leading to outward blebbing of the plasma membrane, which is usually associated with early stages of apoptosis (Lemoinne et al., 2014).

Because MVs originate from many cell types, including BMMCs (Collino et al., 2010) and hepatocytes (Aliotta et al., 2010), MVs from both types of cells were most likely present in the co-culture supernatant. We marked the hepatocyte membrane with the fluorescent dye PKH26 before co-culture to trace the exchange of MVs. Supernatants were collected from the upper chamber of the co-culture experiments where NAD cells were plated and isolated from hepatocytes by a cell-impermeable barrier, which allowed the exchange of medium and molecules smaller than 0.4 μm. This experiment allowed us to estimate that approximately 5% of the isolated MVs from the upper chamber of co-cultures of differentiated cells were positive for this marker. This result supports the fact that MVs that were released by hepatocytes were able to cross the insert and reach the compartment where NAD cells were plated. Furthermore, the uptake of MVs from NAD cells was confirmed by the presence of PKH26-positive cells in the upper chamber.

Phenotype alteration by cell-derived MVs may occur by direct interactions (delivery of mRNAs and proteins), cell surface receptor transfer, epigenetic reprogramming via transcriptional regulators, and/or post-transcription regulation by microRNAs (Quesenberry and Aliotta, 2010). MVs in the supernatant of differentiated hepatocyte-like cells carried liver-specific mRNAs that included *Albumin*, *Ck18*, *Afp*, and *Fv*. These tissue-specific mRNAs were not found in the control group (co-culture with healthy hepatocytes). The direct incorporation of MVs carrying liver-specific mRNAs by NAD cells may be responsible for the transdifferentiation event observed in this study. However, the possibility that transcription regulators are influencing cell reprogramming cannot be excluded. Albumin-positive NAD cells were also positive for PKH26, thus reinforcing the evidence for cell phenotype alteration driven by the incorporation of MVs that are secreted by hepatocytes. Albumin could also be transferred as protein, in addition to the *Albumin* mRNA, which could have been translated by the NAD cell machinery. Notably, the presence of Albumin mRNA and protein has been reported within MVs that were released by hepatocytes (Aliotta et al., 2010; Conde-vancells et al., 2008).

Finally, our data regarding urea production suggest that the hepatocyte-like phenotype may last for some time despite this phenotype acquisition being mediated by MV uptake. NAD cells co-cultured with injured hepatocytes were assessed for urea production at 24 h after contact with injured hepatocytes to indicate the persistence of the acquired phenotype. Notably, the MV content from injured and healthy hepatocytes must be distinct because NAD cells that were co-cultured with healthy hepatocytes failed to present hepatocyte-like characteristics.

In conclusion, this study demonstrates that the NAD fraction of BMMCs express hepatocyte-like characteristics within a short period after co-culture with CCl_4-injured hepatocytes. Our findings suggest that MVs that are released from damaged tissue may be one of the mechanisms that affect cell plasticity.

Conflict of interest

The authors declare no conflict of interest.

Financial support

This work was made possible by support from FIPE-HCPA (#12-0208), PRONEX (FAPERGS/CNPq #008/2009), and CNPq (#457394/2013-7).

Acknowledgments

The authors thank Juliana Bidone for helping with zetasizer analysis and Guilherme Baldo for critical reading of the manuscript.

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Anexo 5: ERK 1/2 ACTIVATION MEDIATED BY ENCAPSULATED BONE MARROW CELLS AFTER IN 90% PARTIAL HEPATECTOMY

(submetido para *Annals of Hepatology*)

ERK 1/2 activation mediated by encapsulated bone marrow cells after in 90% partial hepatectomy

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Abstract:

In a previous study using encapsulated bone marrow cells we have shown an increase in survival in an animal model of acute liver failure by 90% partial hepatectomy. The mechanisms underlying such benefits are still unknown, but a possible candidate involves ERK1/2 pathway, that is involved in proliferation, survival and stress response. We investigated the influence of paracrine factors released by bone marrow cells upon activation of ERK 1/2 in animals with acute liver failure. Whole bone marrow cells were microencapsulated in sodium alginate beads and implanted in the peritoneum of Wistar rats submitted to 90% partial hepatectomy. A control group was submitted to hepatectomy but received capsules without cells. Animals were euthanized at 6, 12, 24 and 72h after the implant (n=5/group). Phosphorylated ERK 1/2 was evaluated by western blot. No differences were found on the ERK 1/2 phosphorylation between the groups. In Conclusion the hypotheses that the increase in survival could be related to activation of ERK 1/2 pathway was not corroborated by this study. Therefore, even though whole bone marrow treated animals show increased survival, this does not seem to be mediated by ERK 1/2 activation.

Keywords:

MAP Kinase Signaling System, stem cells, acute liver failure.

Introduction:

Acute liver failure (ALF) is defined as a sudden loss of hepatic function leading to jaundice, coagulopathy and encephalopathy in previously health individuals. ALF can originate from different causes, such as toxic or viral agents and resection of liver mass(1,2),. In most severe cases the loss of hepatocytes overcomes the liver regenerative capacity and the online therapeutic alternative is orthotopic liver transplantation. Due to the many limitations associated to this procedure, there is an active search for therapeutic approaches that can stabilize patients, thus postponing orthotopic liver transplantation or eliminating its need (3,4)..

Cell therapy protocols with bone marrow derived cells have shown promising results increasing the speed of hepatic regeneration (5,7). The mechanism of action of these cells is not completely understood but different mechanisms have been suggested, including paracrine effects(8,9). In the 90% partial hepatectomy (90%HP) ALF model, Liu *et al.* (2006) showed an increase in serum levels of hepatocyte growth factor in animals treated with encapsulated whole-bone marrow cells (WBM) that correlated to increased survival (10). In a previous study from our group we have shown that encapsulated WBM increase 10-day survival after 90%HP in 50% when compared with de control group (5%) by decreasing the expression of liver regenerating factors (11). A previous observation that decreased liver regeneration rate is beneficial after massive hepatocyte loss has been reported by Ninomyia *et al*. (12).These authors suggest that inhibition of ERK1/2 leads to a balanced cell division between hepatocytes and sinusoids epithelial cells. In order to investigate if this mechanism could be operating in our model we analyzed ERK 1/2 and AKT phosphorylation status and mitotic index in 90%HP animals receiving encapsulated WBM.

Methods:

Animals used in this study are the same that have been reported by Uribe *et al*. (2015). Briefly, male *Wistar* rats were submitted to 90%PH and received glucose (5% of body weight) immediately after surgery and glucose (20%) was added in drinking water throughout the experiment as described by Kieling *et al.* (13). Whole bone marrow (WBM) group received $3x10^7$ cells encapsulated in semipermeable alginate beads, whereas control group received empty capsules (EC). Microcapsules were implanted in the peritoneum immediately after 90% partial hepatectomy. Cells were isolated flushing donor rat's bone marrow with DMEM (Dulbecco's Modified Eagle Medium –LGC® – Brasil) supplemented with 10% fetal calf serum (GIBCO® – Grand 5 Island, NY, EUA) and 1% Penicilin/Streptomycin (GIBCO® – Grand Island, NY, EUA). Cells were encapsulated as described by Lagranha *et al.* (2008) without prior culture (14).

Animals were killed at 6, 12, 24, 48, and 72 hours post-surgery (n=5 animals/time/group). This study was approved by the Ethics Committee on Animal Research at Hospital de Clíncas de Porto Alegre (GPPG-12-0114). Part of the remaining liver was snap frozen in liquid nitrogen then stored at -80ºC.

ERK 1/2 activation was analyzed in liver extracts (0.2 g) macerated in 1 mL lysis buffer (HEPES 0,25 mM, Tris 10 mM, EDTA 1 mM, Na3VO 1mM e NaF 10 mM em ph 7,4) containing protease inhibitors (1%) and centrifuged for 30 minutes at 15000 g at 4°C. Protein quantity was determined by Bradford assay (15). After 5 minutes at 100 °C samples were separated on PAGE 10% and transferred to PVDF membrane (MiliPore) by wet blotting. Membranes were blocked in solution containing 5% powdered milk diluted in PBS-Tween (0.05%) for 30 minutes and then hybridized with antibodies for phospho-ERK 1/2 (Cell Signaling Technologies, USA) at 4 ºC for 12 hours. Membranes were exposed to horse-raddish peroxidase conjugated secondary antibody (Sigma-Aldrich, USA) for 1 hour at room temperature. Membranes were revealed with ECL kit (BioRad). The density of the specific bands was quantified with imaging density software (Image J, USA).

Statistical analysis was performed on PASW Statistic 18. Results are expressed as mean with SD and analyzed using One Way ANOVA, P values $p<0.05$ were considered significant.

Results:

The expression pattern of phosphorylated ERK 1/2 at different time points is shown in figure 1. An increase in activated (phosphorylated) ERK 1/2 at 12 hours was observed for EC group compared to 6, 24 and 72 hours after 90% PH ($P<0.05$); whereas WBM group showed an increase at 12 hours when compared at 6 and 72 hours only ($p<0.05$). No difference was found on ERK 1/2 at any time point between both groups, although there was a trend for increase in phosphorylated ERK 1/2 at 24 hours after 90% PH in WBM group ($p=0.07$).

Discussion

The liver has an impressive regenerative capacity due to the ability of mature hepatocytes to enter cell cycle upon damaging stimuli. In a previous work we observed that bone marrow cells entrapped in alginate microcapsules increase 10-day survival in a murine model of 90% partial hepatectomy (11). The present study was conducted to investigate if the beneficial effect of bone marrow cells could be due to increased ERK 1/2 activity.

ERK 1/2 signaling pathway is activated by extracellular stimuli, such as growth factors and cytokines. The consequences of such activation include the progression of cell cycle, induction of survival pathways and stress response (16). Murata *et al*. (17) showed a progressive decrease in ERK 1/2 2 and 6 hours after 70% PH in animals with thrombocytosis, suggesting that this is a protective effect mediated by increased platelet levels. On the other hand, Ninomiya *et al*. (12) showed that blocking ERK 1/2 activation with a specific drug results in decreased hepatocyte proliferation and increased survival. In the present study there was no difference between groups regarding ERK1/2 activation, although there was a trend for increase in phosphorylated ERK 1/2 at 24 hours after 90% PH in the group receiving encapsulated bone marrow cells. This result was not unexpected, since no difference in the number of mitosis was observed between groups in our previously published study, although a steady increase in the number of dividing cells has been observed along the studied time points (11). In a previous study using free BMMC, Baldo *et al.* showed that an increase in survival rate was correlated to an increase in cell proliferation (7). These differences may be due to the ALF models used, as Baldo *et al*. studied Carbon Tetrachloride-induced ALF and Murata *et al*. performed 70%PH, whereas in this study we used 90% PH. Therefore it is possible that responses elicited by bone marrow cells vary according to the type of lesion, or even that increased proliferation is dependent on cell-cell contact, which is impossible in our model as cells are encapsulated (18).

In conclusion, the present study showed that other mechanisms must be investigated in order to explain this difference.

Declared conflict of interest of all authors: none

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LEGEND

Fig. 1 — (A) Western Blot showing ERK 1/2 activation in WBM and EC groups at 6, 12, 24 and 72 hours after 90% PH. (B) Relative intensity of ERK 1/2 bands (normalized by Actin bands) of both groups in the same time points. # $p<0.05$ 72 vs 6 and 12 hours EC group and * $p<0.05$ 72 vs 6 and 12 hours WBM group (one-way ANOVA).

FIGURE

Figure 1