Faculdade de Medicina de São José do Rio Preto
Programa de Pós-graduação em Ciências da Saúde

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EFEITO DAS CÉLULAS DERIVADAS DA MEDULA ÓSSEA NO TRATAMENTO DA INSUFICIÊNCIA RENAL CRÔNICA EXPERIMENTAL

São José do Rio Preto
2011

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Efeito das Células Derivadas da Medula Óssea no Tratamento da Insuficiência Renal Crônica Experimental

Tese apresentada à Faculdade de Medicina de São José do Rio Preto para obtenção do Título de Doutor no Curso de Pós-graduação em Ciências da Saúde, Área de Concentração: Medicina e Ciências Correlatas.

Orientador: Prof. Dr. Mario Abbud Filho

São José do Rio Preto

2011

Caldas, Heloisa Cristina
Efeito das células derivadas da medula óssea no tratamento da insuficiência renal crônica experimental / Heloisa Cristina Caldas
São José do Rio Preto, 2011
59 p.

Tese (Doutorado) – Faculdade de Medicina de São José do Rio Preto – FAMERP
Eixo Temático: Medicina e Ciências Correlatas

Orientador: Prof. Dr. Mário Abbud Filho

1. Terapia celular; 2. Células-tronco; 3. Engenharia tecidual; 4. Insuficiência renal crônica; 5. Sangue de cordão umbilical.

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Dedicatória

*Em primeiro lugar agradeço a **Deus**, que sempre iluminou meu caminho e me deu forças mesmo nos momentos de maior dificuldade, não me deixando desistir nunca dos meus sonhos.*

Aos meus pais, Benedito e Linda

Que sempre me apoiaram, estiveram presentes e acreditaram em meu potencial. Obrigada pelo incentivo, pela força e principalmente pelo carinho. Sem vocês eu não teria chegado até aqui!

Aos meus irmãos Adriana, Flávio Augusto e Flávio Luis

Pela amizade, cumplicidade e pelo incentivo diante das dificuldades. Amo vocês!

Aos meus sobrinhos Lucas e Pedro

Meus dois tesouros, vocês são um presente de Deus, em minha vida. Obrigada pelo carinho.

Ao meu filho Rafael

Motivo de entusiasmo e paixão de minha pela vida. Por tornar minha vida mais leve e me fazer ver o mundo com novos olhos, todos os dias. Cada dia mais eu quero dizer: Amo Você!

Ao Adriano Tonin

Que me incentivou durante toda a realização desta tese, com seu apoio incondicional, presença, paciência e amor!

Aos amigos

Pela amizade valiosa e pelos conselhos. Jamais esquecerei a paciência as palavras de apoio e a torcida recebida.

Obrigada, por vocês existirem e serem a minha felicidade, a razão sublime do meu viver.

Agradecimentos

- ✓ Em especial, agradeço ao meu orientador Prof. Dr. **Mário Abbud Filho**, por transmitir seu entusiasmo e paixão pela ciência, por partilhar seu conhecimento científico e por desempenhar um papel importante na minha formação e no aprendizado da ciência e da vida.

- ✓ À Dra. **Ida M. M. Fernandes,** com quem tive o prazer de trabalhar lado a lado durante toda realização deste trabalho. Obrigada por seu dinamismo, criatividade e confiança, indispensáveis ao bom andamento dos experimentos e pelos ensinamentos da experimentação animal.

- ✓ À Dra. **Maria Alice S. F. Baptista**, pelo apoio e amizade sempre presente e pelo importante auxílio na execução deste trabalho.

- ✓ À **Carla R. Graça** minha grande amiga de toda a vida, por seu tempo precioso disponibilidade e atenção por mim, pelas palavras de carinho nos momentos difíceis. Eu quero que saiba que a nossa amizade será sempre um amor que nunca irá morrer.

- ✓ À Profa. Dra. **Érika C. Pavarino Bertelli,** pela amizade, colaboração e disponibilidade.

- ✓ À Profa. Dra. **Eny M. Goloni Bertollo,** por acreditar em mim e me proporcionar esta experiência enriquecedora, tanto profissional como pessoal.

- ✓ À Profa. Dra. **Rosa S. Kawasaki Oyama**, pela amizade e estímulo constante, pela paciência e atenção. Pela disponibilidade em participar

deste estudo, sem os quais ele não poderia ter sido realizado. Todo meu reconhecimento e gratidão.

- ✓ A querida amiga **Camila R. Gauch**, pelo carinho, compreensão e pela mão sempre disposta em ajudar.

- ✓ À grande amiga **Mariângela T. Ruiz**, tenho muito orgulho de você, e sei que terá sucesso na sua nova carreira. Sinto falta das conversas no corredor, dos conselhos, das reclamações. Pela amizade e apoio muito obrigado.

- ✓ À amiga **Flávia Graça** por me receber em sua casa para a realização de parte desse trabalho e pela grande amizade.

- ✓ À Profa **Terezila M. Coimbra** pela atenção e cooperação nos experimentos.

- ✓ Ao amigo **Alessandro Catelan** agradeço pela amizade.

- ✓ Ao grande amigo **Celso P. Reis Filho e Fábio Oliveira**, por todo carinho, amizade e apoio muito obrigada.

- ✓ Ás amigas **Marcela A. Pinhel e Greiciane M. da Silva Florim e Glória Elisa F. Mendes,** pela amizade e convívio durante este período. Obrigada amigas!

- ✓ Ás amigas **Zélia C Regis e Patrícia Caldeira,** pela amizade e carinho.

- ✓ Á amiga **Rosana P. Derêncio**, pela sua dedicação e amizade. Obrigada pelo carinho que você tem por mim.

- ✓ Aos colegas do **LITEX**, obrigada pelo apoio, pela amizade, pelo excelente ambiente de trabalho e pela contribuição inestimável de cada um, pequena ou grande, para a realização desse trabalho.

- ✓ Á todos os integrantes da **UPGEM** por manterem sempre a amizade e o profissionalismo e por permitirem a utilização do laboratório para a realização de parte desse trabalho.

- ✓ À Faculdade de Medicina da São José do Rio Preto – FAMERP, na pessoa do Diretor **Prof. Dr. Humberto Liedtke Junior**, pelo apoio sempre presente.

- ✓ À **Fundação Faculdade Regional de Medicina de São José do Rio Preto – FUNFARME,** na pessoa do Diretor Administrativo **Dr. Jorge Fares** e do **Diretor Executivo Dr. Horácio J Ramalho**, pela cooperação.

- ✓ Ao Curso de Pós-graduação da FAMERP, na pessoa do Prof. Dr. **Domingo Marcolino Braile**, que proporcionou a realização deste trabalho.

"É melhor tentar e falhar,
que preocupar-se e ver a vida passar;
é melhor tentar, ainda que em vão,
que sentar-se fazendo nada até o final.
Eu prefiro na chuva caminhar,
que em dias tristes em casa me esconder.
Prefiro ser feliz, embora louco,
que em conformidade viver ..."

Martin Luther King

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Lista de abreviaturas e símbolos

α-SM-actin	*Alfa smooth muscle actin*
AP	*Acid phosphatase*
BM	Biomaterial
BMDC	*Bone marrow derived cells*
BP	*Bovine pericardium*
BPMO	*Bovine pericardium and Mononuclear cells*
BPMSC	*Bovine pericardium and Mesenchymal stem cell*
CaSo4	*Calcium sulfate*
Clcr	*Creatinine clearance*
CMO	Célula mononuclear
CO_2	Dióxido de carbono
Cols	Colaboradores
CRF	*Chronic renal failure*
CT	Células-tronco
CTDMO	Células-tronco derivadas da medula óssea
CTM	Células-tronco mesenquimal
CTMs	Células-tronco mesenquimais
DMEM	*Dulbecco´s Modified Eagle´s Medium*
EDTA	*Ethylenediamine tetraacetic acid*
FACS	*Flow cytometry cell sorter analysis*
FBS	*Fetal bovine serum*

GS	*Glomerular sclerosis*
HES	*Hydroxyethyl starch gel*
HGF	Fator de crescimento de hepatócitos
IF	*Interstitial fibrosis*
IGF-1	Fator de crescimento semelhante à insulina tipo1
IL	*Interstitial lymphocytic infiltration*
IRC	Insuficiência renal crônica
IQSC	*Chemical Institute of Sao Carlos*
KCl	*Potassium Chloride*
LITEX	Laboratório de Imunologia e Transplante Experimental *(Laboratory of Immunology and Experimental Transplantation)*
Lin-	Células da linhagem negativa
MCP-1	Proteína quimiotática de monócitos-1
ME	Matriz extracelular
MoSC	*Mononuclear cell*
MSC	*Mesenchymal cell*
Na2SO4	*Sodium Sulphate*
NaCl	*Sodium chloride*
PAMS	*Methenamine silver*
PAS	*Periodic acid-Schiff*
PBS	*Phosphate buffer saline* (salina de tampão fosfato)
PCNA	*Proliferating cell nuclear antigen*
PDGF	*Platelet-derived growth factor*

pH	Potencial hidrogeniônico
p-JNK	*Jun-N-terminal kinase*
PT-24h	*24h proteinuria*
RBC	*Red blood cells*
SCF system	*Stem cell collection filter system*
SCH	*Hematopoietic stem cells*
sCr	*Serum creatinine*
SCU	Sangue de cordão umbilical
TA	*Tubular atrophy*
TEM	Transição epitélio-mesenquimal
TEndM	Transição endotélio-mesenquimal
TGF	Fator de crescimento transformador beta
UCB	*Umblical cord blood*
v/v	Volume por volume
VEGF	Fator de crescimento endotelial vascular *(Vascular endothelial growth factor)*

Introdução: A insuficiência renal crônica (IRC) é caracterizada pela perda progressiva e irreversível da função renal e seu tratamento gera um gasto público significativo para manutenção de pacientes em tratamento dialítico. A terapia com células-tronco (CT), pelo seu potencial de tratamento das doenças crônicas, pode ser uma estratégia promissora para reparar ou retardar a progressão da IRC. Existem dúvidas sobre o tipo celular, a quantidade de células, o método e local ideal para implantação das CT e o papel por elas desempenhado na reparação do parênquima renal. **Objetivos:** 1) avaliar o efeito da infusão de células derivadas da medula óssea (CDMO) no tratamento da IRC experimental; 2) avaliar o efeito combinado das CT e biomaterial (BM) na progressão da IRC e estudar o efeito dessa terapia em diferentes estágios da IRC; 3) Avaliar o desenvolvimento de técnicas de isolamento e cultivo de células mesenquimais do sangue de cordão umbilical humano (SCU). **Métodos:** ***artigo 1***: usamos o modelo de redução de massa 5/6 para induzir a IRC experimental. Função renal foi medida no início do experimento e 60 e 120 dias depois da cirurgia; ***artigo 2***: animais foram subdivididos conforme a quantidade de parênquima renal lesado (5/6 ou 2/3), o uso de BM como arcabouço para o implante celular e o tipo de células utilizado (célula mononuclear ou mesenquimal). A função renal foi avaliada nos dias 0, 45 e 90 após cirurgia. Análise histológica e imunohistoquimica foram realizadas em todos os grupos ao final do estudo; ***artigo 3:*** Foram utilizadas dez amostras de SCU e testados dois diferentes procedimentos para cultivo de células-tronco mesenquimal (CTM): sem gradiente de densidade Ficoll-Paque, para obtenção de células nucleadas; por gradiente de densidade Ficoll-Paque, para obtenção de células mononucleares. **Resultados:** ***artigo 1:*** Análises da progressão da IRC mostraram que o tratamento com CDMO reduziu significativamente a taxa de declínio do clearance (Clcr) quando comparados

com o grupo controle; ***artigo 2:*** animais tratados apresentaram aumentos significativamente menores de creatinina sérica, proteinúria e Clcr maiores após 90 dias, quando comparado aos animais controles em ambos os modelos de IRC; ***artigo 3:*** as CTM em cultura provenientes do método sem gradiente de densidade Ficoll- Paque mantiveram o crescimento formando focos confluentes de células. **Conclusões:** ***artigo 1:*** a progressão da IRC pode ser retardada pela injeção de CDMO no parênquima renal; ***artigo 2:*** a) utilização da CT combinada com o BM pode ser uma via alternativa para administrar a CTMO; b) terapia celular parece ser mais eficaz quando administrada em estágios menos graves da IRC; ***artigo 3:*** As células nucleadas sem uso do gradiente de densidade Ficoll-Paque mostraram mais eficiente para o cultivo de CTM do SCU quando comparado ao procedimento com gradiente de densidade Ficoll-Paque.

Palavras-chave: 1. Terapia celular. **2.** Células-tronco. **3.** Engenharia Tecidual. **4.** Insuficiência renal crônica. **5.** Sangue de cordão umbilical.

Abstract

Introduction: Chronic renal failure (CRF) is characterized by progressive and irreversible loss of renal function and its treatment generates significant public spending for maintenance and care of patients on dialysis. **S**tem cell (SC) therapy, in its potential for treatment of chronic diseases, may be a promising strategy for repairing the damage from or slowing the progression of CRF. There are questions about cell type, quantity of cells, method and ideal place for deployment of SC and the role it plays in the repair of renal parenchyma. **Objective: 1)** To evaluate the effect of infusion of bone marrow derived cells (BMDC) in the treatment of experimental CRF; **2)** Evaluate the combined effect of SC and biomaterial (BM) in the progression of CRF and study the effect of this therapy in different stages of CRF; **3)** Evaluate the development of techniques for isolation and cultivation of human umbilical cord blood (HUCB) mesenchymal cells. **Methods: Article 1:** We used the 5/6 mass reduction model to induce experimental CRF. Kidney function was measured at the beginning of the experiment and 60 and 120 days after the surgery; **Article 2:** Animals were subdivided as to the amount of renal parenchyma injured (5/6 or 2/3), the use of BM as a scaffold to cell implantation, and cell type used (mononuclear or mesenchymal cells). Renal function was evaluated on days 0, 45, and 90 after surgery. Histological and immunohistochemical analyses were done in all groups at the end of the study; **Article 3:** Ten samples of HUCB were used and two different procedures for cultivation of mesenchymal stem cells (MSC) were tested: without Ficoll-Paque density gradient, to obtain nucleated cells; with Ficoll-Paque density gradient, for obtaining mononuclear cells. **Results: Article 1:** CRF progression analysis showed that treatment with BMDC significantly reduced the rate of decline of creatinine clearance (Clcr) when compared with the control group; **Article 2:**

Treated animals showed significantly lower increases in serum creatinine and 24 hour proteinuria, and higher increases in Clcr after 90 days when compared to control animals in both models of CRF; **Article 3:** The MSC in culture from the method without Ficoll-Paque density gradient maintained growth forming confluent cell foci. **Conclusions: Article 1:** Progression of CRF can be delayed by injection of BMDC in the renal parenchyma; **Article 2:** a) Use of SC combined with BM can be an alternative way to administer BMDC; b) Cell therapy seems to be most effective when administered in less severe stages of CRF; **Article 3**: Nucleated cells without using Ficoll-Paque density gradient showed more efficiency in the cultivation of MSC from HUCB when compared with the procedure employing Ficoll-Paque density gradient.

Keywords: 1. Cellular Therapy. **2.** Stem cells. **3.** Tissue engineering. **4.** Chronic renal failure. **5.** Umbilical cord blood.

1. INTRODUÇÃO

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1.1. Insuficiência renal crônica

A insuficiência renal crônica (IRC) é um importante problema de saúde pública e por essa razão a comunidade nefrológica internacional tem se mobilizado para tentar prevenir e diagnosticar precocemente a doença, além de buscar estratégias visando retardar a progressão da IRC. (1)

Após uma lesão renal inicial o processo que leva a IRC se caracteriza pela perda lenta, progressiva e irreversível da função dos rins. Na fase final da doença predominam os sintomas e sinais de uremia e torna-se necessária a diálise ou o transplante renal. (2)

Embora existam essas duas alternativas terapêuticas, ambas possuem limitações significativas: a diálise é um tratamento temporário, de custo elevado e que não restaura a função endócrina renal; o transplante é limitado pela escassez de doadores de órgãos, complicações da terapia imunossupressora e pela ausência de um tratamento conhecido para a disfunção crônica do transplante. (3)

Os mecanismos que levam à IRC são multifatoriais e a perda da função renal pode ser conseqüência de fatores mecânicos, imunológicos ou tóxicos. Em todos eles o mecanismo final de lesão tecidual envolve proliferação celular, liberação de citocinas inflamatórias, ativação de genes fibrogênicos, aumento na formação de colágeno e fibrose. (4)

As principais causas da IRC são mostradas na (Tabela 1). (3)

Tabela1. Freqüência das principais causas da IRC.

CAUSA	%
Glomerulonefrite	23,5
Hipertensão	24,1
Diabetes Tipo 1	2,5
Diabetes Tipo 2	14,1
Nefrite intersticial	2,9
Rins policísticos	2,7
Uropatia obstrutiva	3,0
Outros	11,3
Desconhecida	16,0
TOTAL	100,0

No Brasil, segundo dados da Sociedade Brasileira de Nefrologia, cerca de 87.000 (468 pacientes por milhão de habitantes) atualmente recebem tratamento dialítico, mas o número de pessoas da população acometidas por IRC em estágios mais precoces da doença é desconhecido. [5] Além disso, o crescente número de pacientes que anualmente iniciam esse tratamento constitui um grande ônus para qualquer orçamento governamental destinado à saúde. Portanto, estratégias para prevenir, retardar ou reparar a progressão da lesão renal crônica são fundamentais para tentar reduzir o impacto econômico do tratamento dialítico e de suas comorbidades, além de oferecer uma alternativa para o transplante renal.

1.2. Células-tronco

As células-tronco (CT) são células indiferenciadas, encontradas em tecidos embrionários e também em tecidos adultos, responsáveis pela formação do embrião e pela manutenção dos tecidos na vida adulta, respectivamente. [6,7]

As CT *embrionárias* caracterizam-se pela capacidade ilimitada de auto-renovação e diferenciação em todos os tipos celulares. Além dos problemas metodológicos e técnicos relacionados ao controle da multiplicação e diferenciação das CT embrionárias, um importante obstáculo para a utilização desse tipo celular são as questões éticas e religiosas. [8,9]

A CT *adulta* é uma célula indiferenciada que é encontrada em tecidos diferenciados e se caracteriza por apresentarem ciclo celular lento e capacidade de proliferação *in vitro*. Essas células são isoladas em grande parte da medula óssea e podem se diferenciar na linhagem *hematopoética,* que originará as células do sangue ou na linhagem *mesenquimal*, derivada do estroma da medula óssea, que poderá originar outros tipos celulares. [10]

A) Células-tronco mesenquimais

As células-tronco mesenquimais (CTMs) caracterizam-se por ser uma população de células multipotentes, capazes de se diferenciarem e produzirem tipos celulares indispensáveis para a reparação e manutenção tecidual. [10,11] *In vitro*, as CTMs exibem morfologia fibroblastóide, aderem ao substrato plástico têm capacidade de auto-renovação e diferenciação em osteócitos, condrócitos e adipócitos. Essas células expressam várias moléculas características em suas membranas, entre elas CD105, CD73 e CD90 e não possuem os marcadores típicos das células da linhagem hematopoética. [12]

Recentes estudos demonstraram que as CTMs estão presentes na parede vascular de arteríolas pré-capilares e nas pequenas artérias e veias (pericitos) e essa associação

com a vasculatura permite que funcionem como fonte constante de reposição celular e estejam disponíveis para a reparação ou regeneração de lesões locais. [13]

B) Células-tronco de cordão umbilical

Embora a medula óssea seja considerada uma das principais fontes disponíveis de CT há muitas restrições para seu uso, entre elas a sua capacidade de proliferação e diferenciação limitada. [7] Além disso, as dificuldades de encontrar doadores compatíveis e as complicações imunológicas relacionadas à rejeição têm restringido seu uso em transplantes alogênicos. [15] Essas limitações motivaram a busca de fontes alternativas de CT, como o sangue do cordão umbilical (SCU).

O SCU é rico em células-tronco com alta capacidade proliferativa. Dentre as vantagens do transplante a partir do cordão umbilical estão a ausência de risco para o doador, facilidade de coleta e fácil obtenção. Outra vantagem é que o SCU pode ser classificado de acordo com os antígenos leucocitários humanos (HLA) e armazenado sob congelamento, estando sempre disponível para uso. [14,16]

Vários estudos confirmaram o potencial de proliferação das CTs mesenquimais do SCU e sua diferenciação em diversas linhagens como osso, adipócitos, células neurais e hepatócitos. [15-18]

Entretanto, ainda é necessário o aprimoramento das técnicas de isolamento e caracterização dessas células e determinar quais fatores de crescimento, proliferação e diferenciação são mais adequadas para esses tipos celulares.

1.3. Mecanismos de ação das células-tronco na reparação renal

Os mecanismos usados pelas CT para regeneração tecidual, tanto renal como dos outros órgãos ainda são desconhecidos. Algumas hipóteses formuladas sobre esses mecanismos incluem: 1) *transdiferenciação*, processo pelo qual uma célula-tronco se diferencia em uma célula adulta de outro tecido; 2) *fusão celular*, entre as células-tronco da medula óssea com as células do órgão afetado, gerando uma célula híbrida com o fenótipo do órgão lesado; 3) *ação parácrina* modulatória das células-tronco sobre tecido remanescente, onde as CT secretam uma grande variedade de citocinas pró e anti-inflamatórias, além de fatores de crescimento que modulam a resposta inflamatória. Além disso, as CT interagem com as células residentes dos nichos podendo induzi-las, por mecanismo parácrino, a se diferenciarem em linhagens celulares distintas, conforme o tipo de estímulo. Ainda, é possível que o papel desempenhado pelas CT na regeneração tecidual renal envolva a ação concomitante de dois ou mais desses e/ou de outros processos. [19] (Figura 1).

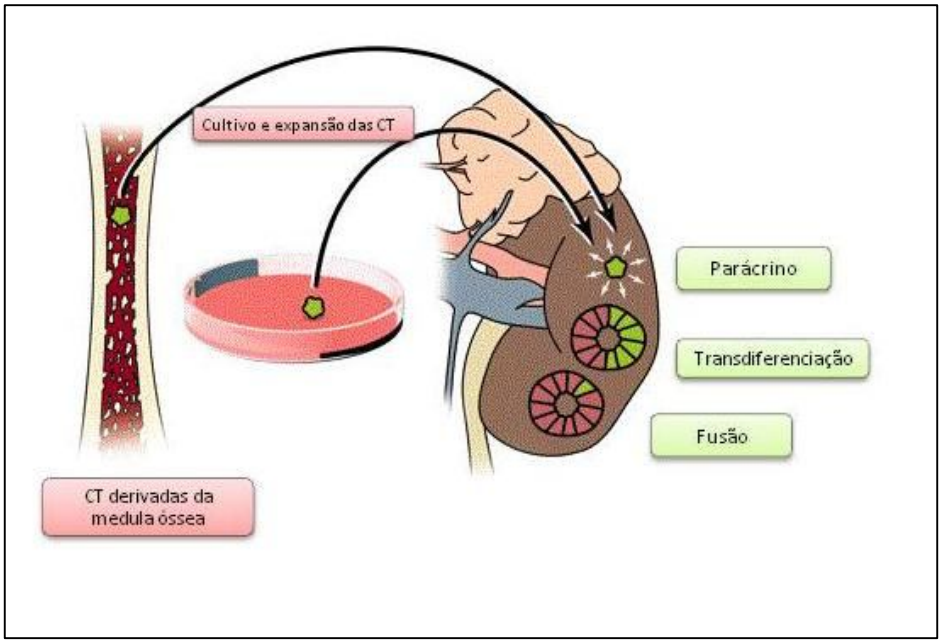

Figura 1. As células-tronco podem ser recrutadas da medula óssea para o rim lesado ou serem expandidas *ex vivo*. Essas células podem contribuir para a reparação renal através da produção de citocinas específicas ou quimiocinas e fatores de crescimento pela transdiferenciação em tipos específico de células renais, ou através da fusão celular. [20]

A fibrose túbulo-intersticial é um evento constante da IRC não sendo apenas uma resposta à lesão tubular, mas um fator determinante contribuindo para a perda do néfron. O mecanismo que leva à fibrogênese renal decorre de uma produção excessiva de componentes da matriz extracelular (ME) para auxiliar no reparo do tecido renal e a persistência da inflamação mantém a síntese de ME elevada agravando a formação de fibrose. [21]

A produção de ME presente no processo fibrótico é produzida por células denominadas miofibroblastos que podem ser originados da medula óssea (15% - fibrócitos) ou pela proliferação local de fibroblastos intersticiais (15%), enquanto 70% origina-se de células epiteliais e endoteliais geradas pela transição epitélio-mesenquimal

(TEM) e pelo processo de transição do endotélio-mesenquimal (TEndM) respectivamente. [22]

A TEM renal é um fenômeno no qual as células do epitélio perdem suas características fenotípicas epiteliais e adquirem as características das células mesenquimais, proporcionando uma fonte renovável de miofibroblastos. Essa célula de origem mesenquimal tem características de fibroblastos e de células musculares lisas e são regulados positivamente pelas moléculas TGF-β (fator de crescimento transformador beta), angiotensina II e PDGF (fator de crescimento derivados de plaquetas). [23]

Recentes estudos sugerem que o bloqueio da TEM e consequentemente da transformação em miofibroblastos, pode representar um tratamento promissor para retardar a progressão da doença renal. [23,24]

O papel da CTM no processo de fibrogênese renal ainda é controverso. É possível que as CTMs possam reverter o processo de fibrose gerando fatores de crescimento como VEGF (fator de crescimento endotelial vascular), HGF (fator de crescimento de hepatócitos) e IGF-1 (Fator de crescimento semelhante à insulina tipo1). Esses fatores inibiriam as ações pró-fibróticas do TGF-β reduzindo a inflamação e dessa forma promoveriam a regeneração do tecido renal lesado (Figura 2). [25]

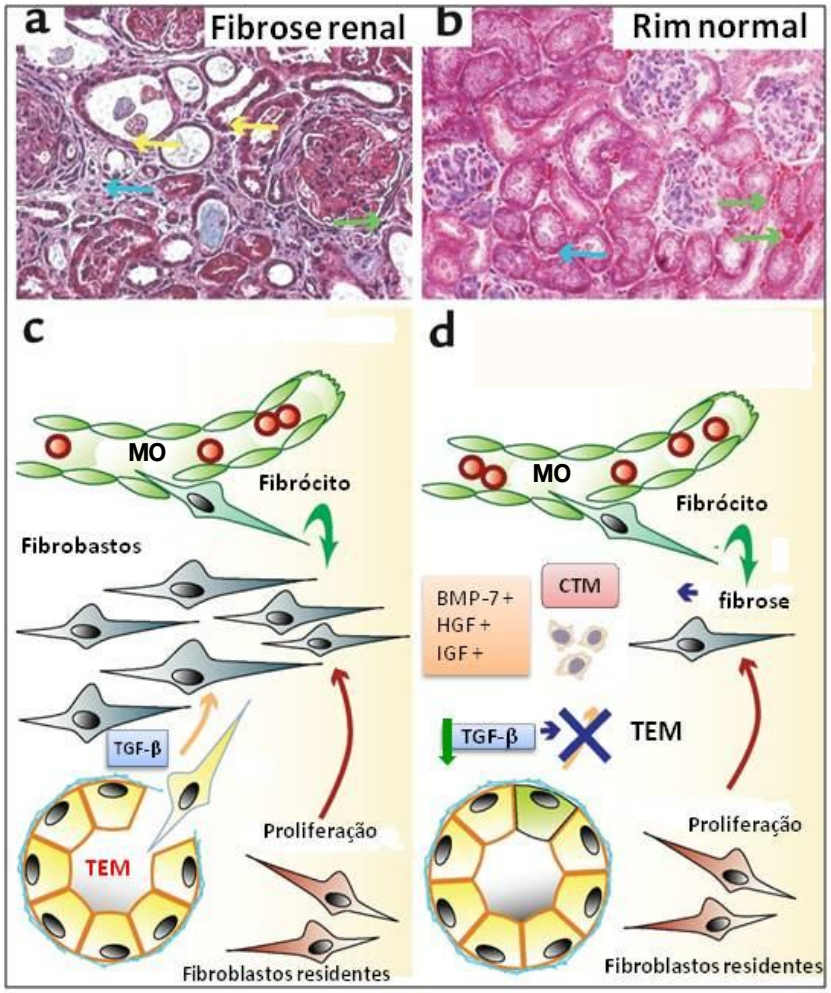

Figura 2. Ação das células-tronco mesenquimais (CTM) no processo de fibrose renal.

Ação das células-tronco mesenquimais (CTM) no processo de fibrose renal. a) Fibrose renal: acúmulo de fibroblastos (seta azul), fibrose túbulo intersticial (seta amarela), fibrose intimal dos vasos (seta verde), b) Rim normal: sem alterações, c) após lesão renal a medula óssea (MO) e a transição epitélio-mesenquimal (TEM), associados ao TGF-β aumentam o número de fibroblastos levando a fibrose; d) as CTM, através da liberação de fatores de crescimento e citocinas reduzem a ação pró-fibróticas do TGF-β bloqueando a TEM e diminuindo a fibrose renal. [24]

1.4. Terapia celular no modelo de IRC experimental

A maioria das doenças renais crônicas se caracteriza por uma lesão inicial, seguida da progressão dessas lesões para formação de fibrose tecidual que gradativamente substitui o parênquima renal. (26)

Experimentalmente, o modelo classicamente utilizado para estudos da IRC é o de redução de 5/6 da massa renal de ratos. Nesses modelos, a lesão renal é induzida pela retirada cirúrgica e/ou infarto de partes do parênquima renal e os animais evoluem com alterações bioquímicas e sinais clínicos semelhantes aos da IRC em humanos. (27-29)

Independente da técnica usada, os autores sugerem que os mecanismos compensatórios tornam-se patológicos e contribuem para o desenvolvimento da progressão da lesão renal. (26-29)

Por esse motivo, intervenções terapêuticas farmacológicas têm sido utilizadas nesses animais e recentemente esses modelos também passaram a servir para os estudos com terapia celular com o objetivo de tentar reparar ou retardar a IRC. (30)

Três hipóteses podem explicar a mobilização das CTMs para o local da lesão e iniciar a reparação renal: 1) após a lesão tecidual ou inflamação renal as CTM *provenientes* da *medula óssea* migram para o local da lesão; 2) CTM *residentes* no tecido renal afluem para o local da lesão e 3) CTM *presentes* na região perivascular migram para a corrente sanguínea e se dirigem para o local da lesão onde exercerão efeitos parácrinos.(13)

O primeiro relato de terapia celular em modelo de IRC experimental, associada à glomerulonefrite induzida por anti-Thy-1, mostrou que as CTM contribuíram para a regeneração de células endoteliais e mesangiais. Entretanto, Zerbine *e cols* não

obtiveram sucesso em retardar a progressão da doença renal utilizando células da medula óssea. [31,32]

Recentemente demonstramos que o uso de células derivadas da medula óssea, quando implantadas no *parênquima* renal de ratos com IRC induzida por redução de 5/6 da massa renal, reduziu ou estabilizou significativamente a taxa de declínio da depuração da creatinina desses animais, após 120 dias de tratamento. [33]

Cavaglieri e *cols*, também estudando o mesmo modelo experimental de IRC, concluíram que, após a inoculação de CTMs na região *subcapsular* renal, havia um efeito renoprotetor com melhora da hipertensão arterial, proteinúria, albuminúria, diminuição da creatinina sérica, além de melhora nos parâmetros histológicos. [34]

Semedo e *cols* demonstraram que funcional e estruturalmente o tratamento com CTMs, em modelo de nefrectomia 5/6, produzia efeitos benéficos. O tratamento com CTMs no parênquima renal também resultou em níveis menos elevados de TGF-β e diminuição da proteinúria. [20]

Choi e *cols* observaram que *injeções* de CTMs na *veia caudal* de ratos com IRC, embora não modificasse de forma significativa a função renal, melhorava a esclerose glomerular e proteinúria nos animais tratados com CTMs, após 120 dias. [35]

Também usando a via intravenosa, Alexandre *et al* infundiram células de linhagem negativa (Lin-) em animais com doença renal crônica e observaram melhora nos níveis de creatinina e de uréia, bem como da proteinúria. [36]

Em resumo, esses resultados sugerem que as CTMs podem ajudar a preservar a estrutura renal ou estabilizar a IRC e reforçam a idéia de que o uso da terapia celular pode ser uma importante arma da medicina regenerativa para reparar as lesões da IRC.

1.5. Medicina regenerativa aplicada à doença renal crônica

Após Macchiarini e *cols* terem transplantado, em 2008, uma traquéia humana acelular revestida por CTMs a comunidade de transplantadores comprovou que a terapia celular havia definitivamente deixado as bancadas de laboratório para ingressar na prática clínica da medicina regenerativa. [37]

Embora vários tipos de tecidos houvessem sido artificialmente construídos ex-vivo, esse foi o primeiro relato de um órgão revascularizado produzido a partir de CT implantadas com sucesso.

Esse fato mostrou que a medicina regenerativa pode ser uma alternativa concreta para reduzir a falta de órgãos para transplantes e acelerou as pesquisas direcionadas para a aplicação clínica do uso de CT em transplantes de órgãos e tecidos cronicamente lesados. [38]

O princípio geral de reconstrução tecidual ou bioengenharia tecidual corresponde ao transplante de células sobre materiais biocompatíveis ou a indução do crescimento celular sobre esses materiais. [39]

O termo biomaterial pode ser aplicado a qualquer substância ou combinação de substâncias, de origem natural ou sintética, que seja biocompatível e possa ser implantado onde houver perda de tecido biológico ou de sua função. [39,40]

A infusão direta de células no órgão lesado, sem contar com uma estrutura de apoio, dificulta o controle, localização e organização das células injetadas. Por outro lado, os biomateriais além de fornecerem uma superfície para a orientação do crescimento e organização das células implantadas, também promovem sinais biológicos necessários para a fixação do neo-tecido. [41,42]

Os biomateriais utilizados na área da bioengenharia tecidual podem ser confeccionados a partir de materiais naturais ou sintéticos. Dentre os naturais, o colágeno tem sido amplamente empregado na regeneração tecidual devido à mimetização de diferentes funções da matriz extracelular. [43]

Além de seu papel estrutural nos tecidos o colágeno apresenta baixa antigenicidade e tem biocompatibilidade superior à de outros polímeros naturais, fato que favorece seu uso nos biomateriais. [44]

1.6. Biomaterial como suporte para o cultivo das CTMs

O pericárdio bovino é composto predominantemente por colágeno do tipo I que é o principal componente dos tecidos conectivos. O material tem baixo custo, apresenta baixa antigenicidade e toxicidade, boa elasticidade e é biocompatível. [45]

Por esses motivos esse material tem sido usado com sucesso para diversos tipos de cirurgias. [45]

O colágeno também pode ser usado como suporte (*scaffold)* para a cultura de células-tronco na aplicação da engenharia de tecidos. Um estudo demonstrou que quando as células-tronco embrionárias humanas foram cultivadas dentro de biomateriais de colágeno elas se diferenciaram e formaram vasos sanguíneos. [46] Uma abordagem similar, utilizando colágeno cultivado com CT embrionárias, foi usada para gerar hepatócitos humanos. [45]

Além disso, outros tipos de células-tronco têm sido usados em conjunto com biomateriais para a produção de tecidos artificiais, como por exemplo, as células-tronco neurais. [48] Nesse estudo, as células se diferenciaram em neurônios e formaram circuitos neurais dentro do biomaterial. [48] A cultura de CTMs nos biomateriais tem

servido para uma variedade de aplicações, incluindo regeneração óssea de ligamentos e de cartilagens. [49,50]

A perspectiva de construir "bioenxertos" para a reparação de tecidos lesados permite o uso de pequeno número de células que podem ser expandidas *in vitro* e semeadas sobre biomateriais.

1.7. Vias de inoculação das CT no tecido renal lesado

A maioria dos estudos utilizou as vias endovenosa ou intra-arterial por serem consideradas as mais fáceis. Porém, a principal desvantagem, é que somente 8-12% das células infundidas consegue atingir o local da lesão. [50,51]

As vias subcapsular e intraparenquimatosa são também utilizadas para a infusão das CT e a grande vantagem que oferecem no tratamento da IRC é a possibilidade de depositar as células diretamente dentro do tecido renal. Contudo, com esse método, as células podem formar pequenas ilhas e limitar a interação entre as células transplantadas e as células renais. [33,34]

Roessger e *cols* foram os pioneiros na utilização de biomaterial associado às células-tronco visando à criação de um suporte para o desenvolvimento dos túbulos renais. [51]

Entretanto nenhum trabalho, até o momento, utilizou biomateriais como suporte (*scaffold*) para as CT no tratamento da IRC, sendo nosso estudo o primeiro a demonstrar a possibilidade de uso dessa via alternativa.

1.8. OBJETIVOS

Os objetivos dos estudos foram:

1. Avaliar o efeito da infusão de células-tronco derivadas da medula óssea em ratos com IRC experimental num período de 120 dias;

2. Avaliar o efeito combinado das células-tronco e biomaterial na progressão da IRC e estudar o efeito dessa terapia em diferentes estágios da doença;

3. Investigar a implantação de técnicas de isolamento e cultivo de células-tronco mesenquimais do sangue de cordão umbilical humano, com e sem uso de gradiente de densidade Ficoll-Paque (d=1,077g/ml).

4. Revisar as possibilidades de uso da terapia celular no tratamento da doença renal crônica.

2. ARTIGOS CIENTÍFICOS

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Os resultados referentes aos objetivos desta dissertação estão apresentados na forma de artigo:

ARTIGO 1

Tílulo: Effect of whole bone marrow cell infusion in the progression of experimental chronic renal failure.

Autores: Caldas HC, Fernandes IM, Gerbi F, Souza AC, Baptista MA, Ramalho HJ, Kawasaki-Oyama RS, Goloni-Bertollo EM, Pavarino-Bertelli EC, Braile DM, Abbud-Filho M.

Periódico: Transplant Proc. 2008; 40(3):853-855.

ARTIGO 2

Título: Effect of stem cells seeded onto biomaterial on the progression of experimental chronic kidney disease.

Autores: Caldas HC, Fernandes IM, Kawasaki-Oyama RS, Baptista MA, Plepis AMG, Martins VA, Coimbra TM, Goloni-Bertollo EM, Braile DM, Abbud-Filho M.

Periódico: Experimental Biology and Medicine. 2011, 00:1-9.

ARTIGO 3

Título: Cultivo de células mesenquimais do sangue de cordão umbilical com e sem uso do gradiente de densidade Ficoll-Paque.

Autores: Kawasaki-Oyama RS, Braile DM, Caldas HC, Leal JC, Goloni-Bertollo EM, Pavarino-Bertelli EC, Abbud Filho M, Santos ID.

Periódico: Rev Bras Cir Cardiovasc. 2008; 23(1):29-34.

ARTIGO 4

Título: Perspectivas do uso da terapia celular no tratamento da doença renal crônica

Autores: Caldas HC, Abbud-Filho M.

Periódico: Série publicada pela Sociedade Brasileira de Nefrologia "Atualidades em Nefrologia 11".

Caldas HC, Abbud-Filho M. Perspectivas do uso da terapia celular no tratamento da doença renal crônica. In: Jenner Cruz [et al.], editores. Atualidades em Nefrologia 11. São Paulo: Sarvier; 2010. p. 42-45.

ARTIGO 1

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ELSEVIER

Effect of Whole Bone Marrow Cell Infusion in the Progression of Experimental Chronic Renal Failure

H.C. Caldas, I.M.M. Fernandes, F. Gerbi, A.C. Souza, M.A.S.F. Baptista, H.J. Ramalho, R.S. Kawasaki-Oyama, E.M. Goloni-Bertollo, E.C. Pavarino-Bertelli, D.M. Braile, and M. Abbud-Filho

ABSTRACT

Introduction. The therapeutic potential of adult stem cells for the treatment of chronic diseases is becoming increasingly evident over the last few years. In the present study, we sought to assess whether the infusion of bone marrow–derived mononuclear cells (MoSCs) and mesenchymal cells (MSCs) could reduce/stabilize the rate of progression of chronic renal failure (CRF) in rats.

Methods. We used the 5/6 renal mass reduction model to induce chronic renal failure in male Wistar rats. Renal function was assessed by measurements of serum creatinine (sCr), creatinine clearance (Clcr), and 24-hour proteinuria at baseline as well as 60 and 120 days after surgery. MoSCs and MSCs obtained from bone marrow aspirates were separated by the Ficoll-Hypaque method. After a 12- to 14-day culture, 1.5×10^6 MSCs and the same number of MoSCs were injected into the renal parenchyma of the remanant kidney of rats with CRF on the day of surgery.

Results. Among the control group, at day 120, the results were sCr = 1.31 ± 0.5 mg/dL, Clcr = 0.64 ± 0.35 mL/min, and proteinuria = 140.0 ± 57.7 mg/24 h. Rats treated with MoSCs at day 120 had sCr = 0.81 ± 0.20 mg/dL, Clcr = 1.05 ± 0.26 mL/min, and proteinuria = 61 ± 46.5 mg/24 h, while rats injected with MSCs had sCr = 0.95 ± 0.1 mg/dL, Clcr = 0.68 ± 0.24 mL/min, and proteinuria = 119.2 ± 50.0 mg/24 h. Analysis of the progression to CRF showed that the treatment significantly reduced the rate of decline in Clcr after treatment with MoSc: control: −0.0049 ± 0.0024 mL/min/d versus MSC: − 0.0013 ± 0.0017 mL/min/d versus MoSC: +0.0002 ± 0.0016 mL/min/d (P = .017). Proteinuria tended to be lower among the treated groups. Histological scores of chronic damage were not different, but distinct patterns of chronic lesions were observed among treated rats.

Conclusion. Our results showed that progression of CRF in rats could be slowed/stabilized by intrarenal parenchymal injection of MoSCs. A trend toward reduction in the progression rate of CRF was also observed with injection of MSCs.

THE USE OF BONE MARROW is a promising approach to improve the function of damaged kidneys.[1] However, data concerning the possibility of restoring the chronically damaged kidney by using stem cells are still lacking. Bone marrow–derived stem cells have been shown to improve the outcomes of acute renal injury models, but the effects of these cells on the progression of chronic kidney disease are unknown.[2] The aim of this study was to examine

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This work was supported in part by the FAMERP-BAP/FUNFARME and Braile Biomédica.

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0041-1345/08/$–see front matter
doi:10.1016/j.transproceed.2008.03.009

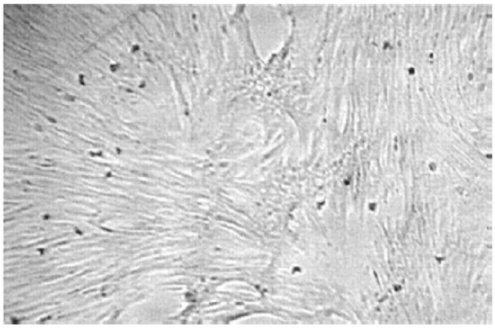

Fig 1. Morphological characteristics of MSCs from rat bone marrow. Photomicrograph showing MSCs on day 14 of culture, with attached cells demonstrating typical fibroblast-like morphology.

whether intraparenchymal injection of bone marrow–derived mononuclear as well as of mesenchymal cells modified the progression of chronic renal failure (CRF) in rats.

MATERIALS AND METHODS

Animal procedures were performed in accordance with protocols approved by our Animal Care and Use Committee. Fifteen adult male Wistar rats (250 to 300 g) underwent 5/6 renal mass reduction as previously described.[3] Renal function was assessed by measurements of serum creatinine (sCr), creatinine clearance (Clcr), and 24-hour proteinuria at baseline, as well as 60 and 120 days after surgery. Bone marrow cells isolated from femoral and tibial bones of Wistar rats were separated into mononuclear elements cells (MoSCs) by Ficoll-Hypaque (d = 1.077) density gradient centrifugation. Cells plated in culture in DMEM medium (Gibco) with 20% fetal bovine serum and penicillin/streptomycin/amphotericin (Gibco), at a density of 1.5×10^6 mononuclear cells/cm^2 were incubated at 37°C with 5% CO_2. Mesenchymal cells or mesenchymal-like cells (MSCs) were characterized by morphological and cytochemical criteria as previously described and according to the statement of the International Society for Cellular Therapy.[4–6] Bone marrow MoSC cells plated for 24-hour adherence in plastic and after 12 to 14 days in culture were evaluated for morphology. MSCs had a fibroblast-like pattern, with spindle-shaped or polygonal cell bodies, that differentiated to osteclast, adipocyte, and chondrocytes (Figure 1). The cytochemical markers acid phosphatase and periodic *acid-Schiff* were used to detect MSC positive for PAS and negative for AP activity. After surgery rats were divided into the three groups that underwent intrarenal parenchymal injections of 0.15 mL medium (control group); 1.5×10^6 MoSCs in 0.15 mL of medium; or MSCs at the same cell concentration. Histological analysis of kidneys was performed using a chronicity score. Statistical analyses were performed with the software Graphpad Instat 3.0; P values $< .05$ were considered signicant.

RESULTS

sCr at baseline was 0.43 ± 0.04 mg/dL in the control group, 0.43 ± 0.05 mg/dL in the MoSC group, and 0.5 ± 0.1 mg/dL in the MSC group (Table 1). At 120 days, sCr values were 1.31 ± 0.5 mg/dL (control), 0.81 ± 0.2 mg/dL (MoSC), and 0.95 ± 0.1 mg/dL (MSC) (P = NS). The percentages of increase in sCr at day 120 versus the baseline levels were: 205% (control), 81% (MoSC), and 97.5% (MSC). Baseline values of Clcr did not vary significantly among the groups (control = 1.23 ± 0.33 mL/min; MoSC = 1.14 ± 0.34 mL/min; MSC = 0.85 ± 0.11 (mL/min), but after 120 days

Table 1. Serum Creatinine (mg/dL) and Creatinine Clearance (mL/min) at Baseline and After 120 Days

Rat	Creatinine baseline	Creatinine clearance baseline	Creatinine 120 d	Clearance 120 d
Control group rats				
1	0.46	1.49	0.83	1.04
2	0.44	1.50	0.93	0.32
3	0.38	1.30	1.35	0.81
4	0.41	0.69	2.09	0.22
5	0.48	1.19	1.34	0.80
Mean	0.43	1.23	1.31	0.64
SD	0.04	0.33	0.50	0.35
MSC group rats				
6	0.41	0.80	0.83	0.72
7	0.52	0.76	0.90	0.50
8	0.60	0.78	0.94	0.40
9	0.59	0.85	1.00	1.00
10	0.40	1.04	1.10	0.80
Mean	0.50	0.85	0.95	0.68
SD	0.10	0.11	0.10	0.24
MoSC group rats				
11	0.50	1.09	0.97	0.90
12	0.41	0.90		
13	0.38	1.09	0.59	1.36
14	0.40	1.73		
15	0.45	0.90	0.88	0.90
Mean	0.43	1.14	0.81	1.05
SD	0.05	0.34	0.20	0.26

Clcr was 0.64 ± 0.35 mL/min in the control group, 1.05 ± 0.26 mL/min in the MoSC group, and 0.68 ± 0.24 mL/min in the MSC group. Analysis of progression to CRF showed that treatment with MoSCs significantly reduced the rate of decline in Clcr when compared to the control group: +0.0002 ± 0.0016 mL/min/d versus −0.0049 ± 0.0024 mL/min/d, respectively (P = .017). A trend toward slower progression of CRF was also observed after treatment with MSC (MSC = −0.0013 ± 0.0017 mL/min/d versus −0.0049 ± 0.0024 mL/min/d, respectively; P = NS). Proteinuria at day 120 was lower among animals injected with MSCs and MoSCs (MSC = 119 ± 50 mg/24 h and 61 ± 46.5 mg/24 h, respectively) than the control group (140 ± 58 mg/24 h; P = .09). Histological evaluation did not show differences in the chronicity score among the three groups (control: 31%, MSC: 26%, and MoSC: 24%; P = NS). Interestingly, the control group rats showed chronic lesions with a scattered distribution, while treated animals revealed chronic damage limited only to the region next to the infarction area.

DISCUSSION

Stem cell therapy has been used to repair injuries in liver, heart, and brain models. Reports have shown that bone marrow stem cells contribute to the repair of acutely damaged kidneys.[7] Our results showed that injection of MoSCs or MSCs may have an effect on the CRF induced in rats because after 120 days treated animals showed a increase in sCr at least two times lower than untreated rats. In addition, the rate of progression of CRF, measured by the decline in Clcr, was significantly reduced or stabilized with MoSC treatment when compared to the control group. Despite not reaching statistical significance, progression of CRF was also retarded with the injection of MSCs suggested by an almost fourfold slower decline in Clcr among these animals. The proteinuria results displayed similar behavior, decreasing in the group receiving cell therapy. The histological features of chronicity did not differ among groups, but it seemed that the system score did not reflect the various patterns of observed lesions.

In conclusion, our preliminary results suggested some benefits could be achieved in rats with CRF; bone marrow–derived cells, especially MoScs, reduced/stabilized the rate of decline in Clcr of these animals.

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ARTIGO 2

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Periódico: Experimental Biology and Medicine. 2011, 00:1-9

EBM-1101-RM-24

Original Research

Effect of stem cells seeded onto biomaterial on the progression of experimental chronic kidney disease

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Abstract

Different routes for the administration of bone marrow-derived cells (BMDC) have been proposed to treat the progression of chronic renal failure (CRF). We investigated whether (1) the use of bovine pericardium (BP) as a scaffold for cell therapy would retard the progression of CRF and (2) the efficacy of cell therapy differently impacts distinct degrees of CRF. We used 2/3 and 5/6 models of renal mass reduction to simulate different stages of chronicity. Treatments consisted of BP seeded with either mesenchymal or mononuclear cells implanted in the parenchyma of remnant kidney. Renal function and proteinuria were measured at days 45 and 90 after cell implantation. BMDC treatment reduced glomerulosclerosis, interstitial fibrosis and lymphocytic infiltration. Immunohistochemistry showed decreased macrophage accumulation, proliferative activity and the expression of fibronectin and α-smooth muscle-actin. Our results demonstrate: (1) biomaterial combined with BMDC did retard the progression of experimental CRF; (2) cellular therapy stabilized serum creatinine (sCr), improved creatinine clearance and 1/sCr slope when administered during the less severe stages of CRF; (3) treatment with combined therapy decreased glomerulosclerosis, fibrosis and the expression of fibrogenic molecules; and (4) biomaterials seeded with BMDC can be an alternative route of cellular therapy.

Keywords: collagen, fibrosis, mesenchymal stem cell, scaffold, stem cell

Experimental Biology and Medicine 2011; 00: 1–9. DOI: 10.1258/ebm.2011.011024

Introduction

The progressive deterioration observed in chronic renal disease is caused by a series of inflammatory events leading to glomerulosclerosis, interstitial cell infiltration, tubular atrophy, activation of fibroblasts, scar formation or fibrosis.[1]

Since the process of renal repair depends on the severity and extent of kidney injury and the number of resident stem cells, the recent development in cell-based therapies aiming to restore or replace chronically injured tissues may bring hope for the treatment of many chronic diseases.[2,3]

However, there is a paucity of reports using bone marrow-derived cells (BMDC) as a regenerative therapy for chronic renal failure (CRF). Comparisons among the results are therefore jeopardized by study differences such as the use of different models of renal damage, the amount of administered cells and differences in the routes of cell administration.[4–9]

Generally, 5/6 nephrectomized rats are used as a model to mimic human CRF in the majority of published papers because the procedure causes a significant reduction in nephron number, resulting in severe CRF.[10,11] We reasoned that with the reduction of a large amount of renal mass, the regenerative capacity of the organ could be compromised; this fact would impact the result of cell therapy.[12] To address this issue, we investigated the effect of treatment with BMDC on a 2/3 nephrectomized rat (CRF2/3) with less renal damage and a lesser degree of CRF, in addition to the usual 5/6 model (CRF5/6).

In this study, we also introduced a basic concept of tissue engineering that includes the use of a scaffold to provide an

ISSN: 1535-3702

EBM-1101-RM-24

architecture upon which seeded cells can organize and develop to promote tissue repair.[13] Using this combination as opposed to traditional methods of cell administration, we could evaluate whether a biomaterial seeded with BMDC could be an alternative route for cell therapy.

Considering that in both models of renal mass reduction, chronic deterioration of the remnant kidney is due to the participation of cellular and inflammatory mechanisms that lead to fibrosis, we hypothesized that BMDC treatment would reduce the inflammatory activity and expression of profibrotic molecules and fibrosis.

In the present report, we sought to evaluate whether (1) biomaterial seeded with mesenchymal stem cells (BPMSC) or mononuclear cells (BPMO) could retard the progression of experimental CRF; (2) the amount of remnant renal mass would impact the efficacy of cell therapy; (3) the expression of profibrotic molecules would be affected by BMDC treatment; and (4) biomaterial combined with BMDC could be an alternative route of cellular therapy.

Materials and methods

Animal models

Animal procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (No. 3310/2008). Female Wistar rats weighing 250–350 g that underwent a reduction of 5/6 or 2/3 renal mass reduction were used for this study. All animals were provided standard rat chow and water *ad libitum*.

CRF models

The 5/6 renal mass reduction procedure was performed as previously described to experimentally induce severe CRF.[14] To create a less severe CRF model, we reduced the renal mass by only 2/3. Briefly, female rats were administered anesthesia in the form of ketamine hydrochloride (50 mg/kg) and xylazine (10 mg/kg). Infarction of approximately one-third of the left kidney was performed by microsurgical ligation of one branch of the left renal artery, followed by right nephrectomy.

Experimental groups

Animals ($n = 45$) were divided into groups according to the amount of renal mass reduction and the severity of CRF (2/3 and 5/6 models). They were further subdivided according to treatment received: (1) sham (S, $n = 5$); (2) animals with 2/3 or 5/6 mass reduction alone (CRF2/3, $n = 5$ or CRF5/6, $n = 5$); (3) animals implanted with bovine pericardium (BP) alone (CRF2/3 + BP, $n = 5$ or CRF5/6 + BP, $n = 5$); (4) animals treated with BP seeded with mesenchymal stem cells (BPMSC2/3, $n = 5$ or BPMSC5/6, $n = 5$); and (5) animals treated with BP seeded with mononuclear cells (BPMO2/3, $n = 5$ or BPMO5/6, $n = 5$). Renal function was assessed by measurements of serum creatinine (sCr), creatinine clearance (Clcr), and 24-h proteinuria (PT-24 h) at the baseline, at 45 and 90 d after surgery. Progression of CRF was measured by the 1/sCr slopes and decline in the Clcr rate (mL/min/d). At the end of 90 d, the animals were weighed and sacrificed.

Isolation and characterization of BMDC

BMDC were isolated from the femur and tibia of male Wistar rats. Mononuclear cells (MO) were separated by Ficoll-Hypaque ($d = 1.077$) density gradient centrifugation and cultured in Dulbecco's modified Eagle's medium (Gibco, Invitrogen Co, New York, NY, USA), 20% fetal bovine serum (Cultilab, Campinas, Brazil) and penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) for 14 d. Mesenchymal stem cells (MSC) were recovered using their tendency to adhere to plastic; non-adherent cells were removed by washing. Flow cytometry analyses (FACSCanto, Becton Dickinson, San Jose, CA, USA) were performed with antibodies for the cell-surface markers CD31, CD44, CD90, CD45 and CD34 (Caltag Laboratories, Carlsbad, CA, USA) kindly provided by Dr Irene Noronha. Cell differentiation potential was tested by adipogenic and osteogenic differentiation as previously described.[15]

Preparation of acellular BP

BP was prepared by the Biomaterials Group of the Chemical Institute of Sao Carlos – University of Sao Paulo. Briefly, fresh BP to be used as a collagen source was treated at 20°C for periods of 0–48 h with an alkaline solution (3 mL/g of tissue) containing 6 vol% of dimethyl sulfoxide, salts (chlorides and sulfate) and bases of alkaline (K^+, 1.19 mol/L, and Na^+, 1.74 mol/L), and alkaline earth metals (Ca^{2+}, 0.86 mol/L). The resulting materials were equilibrated with a solution containing Na_2SO_4, NaCl, KCl and $CaSO_4$ (6 mL of solution/g of tissue) for a period of 12 h, and the excess salts were removed by washes with the following solutions: 3% w/w of boric acid solution (3 × 2 h, 250 mL), deionized water (3 × 6 h, 250 mL), 0.3% w/w ethylenediamine tetraacetic acid (EDTA) solution (3 × 2 h, 150 mL), pH 11.0 and (6 × 2 h, 250 mL) washes with deionized water.

The resulting material is basically acellular polyanionic collagen matrices associated with an elastin content and triple helical structure. Previous studies showed that the biomaterial used is biocompatible, causing only mild and progressive chronic inflammation.[16,17]

MSC and MO seeding in BP

After rehydration in phosphate-buffered saline (PBS), 0.5 × 0.5 cm segments of BP were placed in six-well Petri dishes and MSC resuspended at a concentration of 2.5×10^6 cells/mL were seeded onto the BP. The plates were kept in a humidified 5% CO_2 incubator at 37°C during 14 d of culture. After this period, the segments of BPMSC were fixed in 10% phosphate-buffered formalin and samples were subjected to histological evaluation after embedding in paraffin and stained with hematoxylin and eosin (H&E) and Giemsa (Gibco, Invitrogen Co., New York, NY, USA) (Figures 1a and b). BP was seeded

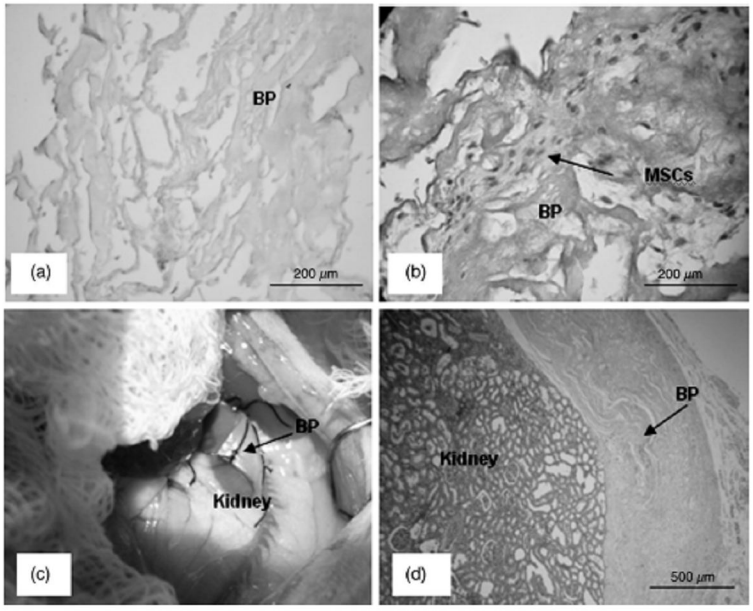

Figure 1 (a) Photograph of biomaterial adhered to renal parenchyma after renal mass reduction surgery. (b) Histological appearance of the biomaterial attached to renal parenchyma at 90 d postsurgery. (c and d) Hematoxylin/eosin staining of unseeded or MSC-seeded collagen scaffolds cultured for 14 d in culture medium. Bars = 100 μm. BP, bovine pericardium; MSC, mesenchymal stem cells. (A color version of this figure is available in the online journal)

with MO at a concentration of 5×10^6 cells/mL 3 h before implantation.

Implantation of BP in renal tissue

Immediately after renal mass reduction surgery, the renal capsule was removed from the interface region of renal infarction/healthy and segments of biomaterial alone (BP) or seeded with BPMSC or BPMO were sutured with 8.0 mononylon adjacent to the renal surface (Figures 1c and d).

Histological and immunohistochemical analysis

Sections (3-μm) of renal tissue were stained with H&E and methenamine silver (PAMS) for histological analysis. A semi-quantitative evaluation based on percentages was performed by two blinded renal pathologists (+1 = <25% damage, +2 = 25–50% damage, +3 = 51–75% damage, +4 = >75%).

The criteria for the semi-quantitative analysis were used for the following histopathological indicators: glomerulosclerosis (GS), tubular atrophy (TA), interstitial fibrosis (IF) and interstitial lymphocytic infiltration (IL).[18]

Immunohistochemical analysis was performed as previously described.[19] Briefly, the sections were incubated with the anti-α-smooth muscle (SM)-actin (Dako, Glostrup, Denmark), antifibronectin (Chemicon International, Temecula, CA, USA) or anti-Jun-N-terminal kinase (p-JNK; Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibody at 4°C, or the anti-proliferating cell nuclear antigen (PCNA) (Sigma, St Louis, MO, USA) or anti-ED1 antibody at room temperature for 30 min. The reaction product was detected with an avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA, USA). The material was counterstained with methyl green, dehydrated and mounted. Counterstaining of the p-JNK slides was performed with Harris hematoxylin. Non-specific protein binding was blocked by incubation with 20% goat serum in PBS for 20 min. Negative controls consisted of replacement of the primary antibody with normal rabbit IgG and mouse IgG for the polyclonal and monoclonal antibodies, respectively, at equivalent concentrations.

Each grid field was semi-quantitatively graded, and the mean score per kidney was calculated to evaluate the immunoperoxidase staining of fibronectin and α-SM-actin. Each score mainly reflected changes in the extent rather than the intensity of staining and depended on the percentage of the grid field showing positive staining: 0 = absent or less than 5%; I = 5–25%; II = 25–50%; III = 50–75%; IV > 75%.

The number of ED1-positive (macrophage cells) and PCNA-positive cells in each section was calculated by counting the number of positive cells in 30 sequential (0.245 mm^2) grid fields from the renal cortex.[19]

Statistical analysis

Data were expressed as mean ± standard deviation. Analyses were performed using GraphPad Prism software (San Diego, CA, USA), with the critical level set at $P < 0.05$. Comparisons among multiple groups were made using analyses of variance (ANOVA). When F values were

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significant, differences between the groups were specified with Tukey's multiple comparison post-tests. When comparing data, a two-sided Student's *t*-test and Mann-Whitney *U* test were performed.

Results

Functional studies

Comparing severity of the CRF2/3 and 5/6 models

As expected, the 2/3 nephrectomized rats presented a pattern of less severe CRF when compared with the 5/6 mass reduction model. This result was demonstrated by the significantly lower increase in sCr level observed at day 90 in CRF2/3 animals compared with CRF5/6 rats (CRF2/3 = 48.2% versus CRF5/6 = 128%; $P = 0.006$). Interestingly, the 5/6 but not 2/3 nephrectomized rats treated with unseeded BP showed the greatest increases in sCr after 90 d, suggesting a worsening of chronic inflammation. In addition, progression of disease as measured by the slope of 1/sCr was significantly slower in the CRF2/3 model (CRF2/3 = −0.24 ± 0.03 versus CRF5/6 = −0.49 ± 0.04; $P = 0.008$), demonstrating a lesser degree of chronic deterioration in these animals.

Effects of treatment in the CRF2/3 model

After 90 d, the increase in sCr level in CRF2/3 rats treated with BPMSC was blocked in a way similar to that of rats in the Sham group (S = 5.2% versus BPMSC = 7.6%; P = NS), while sCr in untreated CRF2/3 animals continued to rise (CRF2/3 = 48.2% versus BPMSC = 7.6%; $P < 0.05$) (Figure 2a). In this model, the greatest effect of treatment was evident after day 45, when the use of BPMSC and BPMO stabilized the increase in sCr (CRF2/3 versus BPMSC and BPMO, $P < 0.05$; BP versus BPMSC and BPMO, $P < 0.001$) (Figure 2a).

Treatment with BPMSC and BPMO also resulted in improved Clcr (S = 1.05 ± 0.2 mL/min versus CRF2/3 = 0.4 ± 0.12 versus BP = 0.34 ± 0.09 versus BPMSC = 0.65 ± 0.22 versus BPMO = 0.5 ± 0.07; $P < 0.0001$) (Figure 2b). Chronic deterioration of renal function in the CRF2/3 model as measured by 1/sCr slope significantly slowed with BPMSC treatment (S = 0.88 ± 0.91 versus CRF2/3 = −0.24 ± 0.03 versus BP = −0.20 ± 0.09 versus BPMSC = 0.78 ± 0.81 versus BPMO = 0.09 ± 0.71; P = 0.034 (Figure 2c). The excretion of PT-24 h was very similar to that of sham animals when the rats were treated with BPMSC or BPMO (S = 5.4 ± 1.1 mg/24 h versus BPMSC = 4.9 ± 2.4 versus BPMO = 7.6 ± 1.6; P = NS), while the untreated groups retained elevated rates of PT-24 h excretion (CRF2/3 = 27 ± 16.8 mg/24 h and BP = 20.2 ± 4.7; $P < 0.01$) (Figure 2d).

Effects of treatment in the CRF5/6 model

In contrast to the CRF2/3 model, 5/6 nephrectomized rats demonstrated a better response to treatment during the initial 45-day period. During this period of time, both types of cells significantly reduced sCr (Figure 3a). At the end of the 90-day period, the treatment effectively prevented increases in sCr in the CRF5/6 model (S = 5.2% versus CRF5/6 = 128% versus BP = 168% versus BPMSC =

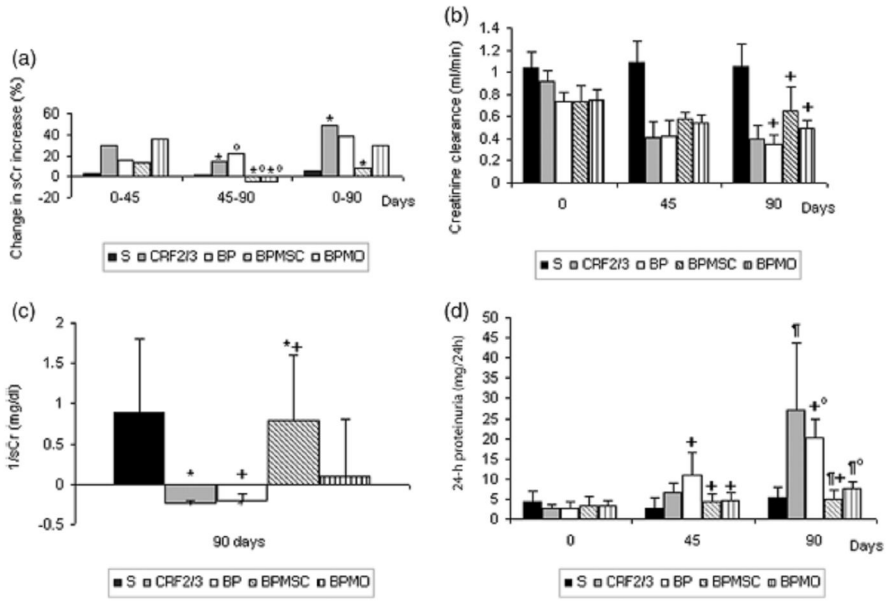

Figure 2 Renal function studies at days 0, 45 and 90 in the 2/3 renal mass reduction model. (a) Percentage increase in sCr, (b) creatinine clearance, (c) slopes of the reciprocal serum creatinine plot (1/sCr), (d) 24-h proteinuria (PT-24 h). Values are expressed as means ± SD in b, c and d (*$P < 0.05$, °$P < 0.05$, +$P < 0.01$, ¶$P < 0.01$). sCr, serum creatinine; S, sham; CRF, chronic renal failure; BP, bovine pericardium; BPMSC, BP seeded with mesenchymal stem cells; BPMO, BP seeded with mononuclear cells

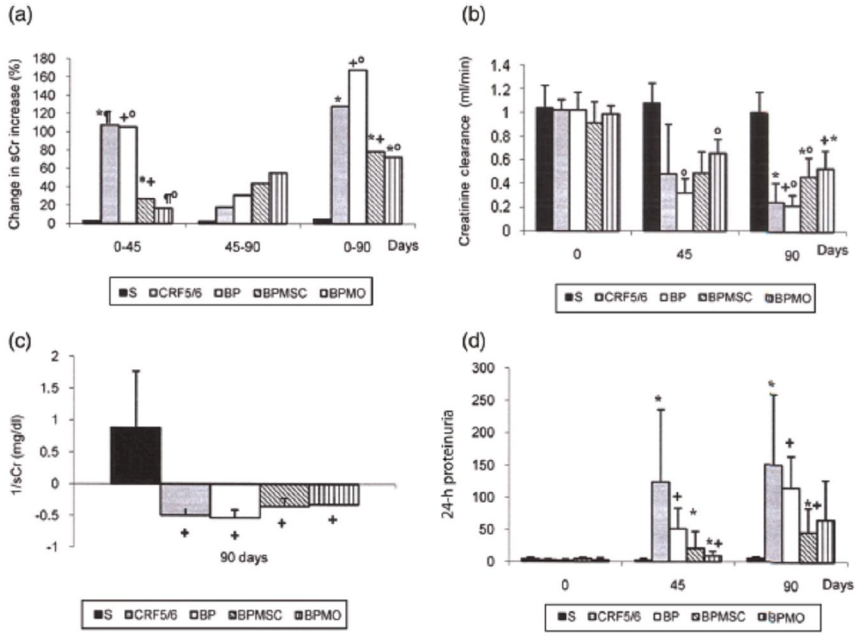

Figure 3 Renal function studies at days 0, 45 and 90 in the 5/6 renal mass reduction model. (a) Percentage increase in sCr, (b) creatinine clearance, (c) slopes of the reciprocal serum creatinine plot (1/sCr), (d) 24-hour proteinuria (PT-24 h). Values are expressed as means ± SD in b, c and d (*$P < 0.05$, °$P < 0.05$, +$P < 0.01$, ¶$P < 0.01$). sCr, serum creatinine; S, sham; CRF, chronic renal failure; BP, bovine pericardium; BPMSC, BP seeded with mesenchymal stem cells; BPMO, BP seeded with mononuclear cells

78.6% versus BPMO = 72.4%; $P < 0.01$) (Figure 3a). Profound decreases in Clcr were observed in the untreated groups, while groups treated with BPMSC and BPMO demonstrated a lower reduction after 90 days (S = 1.05 ± 0.18 mL/min versus CRF5/6 = 0.25 ± 0.17 versus BP = 0.22 ± 0.09 versus BPMSC = 0.46 ± 0.10 versus BPMO = 0.53 ± 0.15; $P < 0.01$) (Figure 3b). The progression of disease in the CRF5/6 model was significantly retarded using both treatments (S = 0.88 ± 0.9 versus CRF5/6 = −0.49 ± 0.04 versus BP = −0.53 ± 0.10 versus BPMSC = −0.35 ± 0.12 versus BPMO = −0.32 ± 0.13; $P < 0.01$) (Figure 3c), although to a lesser degree than observed for the CRF2/3 model.

PT-24 h decreased significantly in treated groups after the 45-day period (S = 2.8 ± 1.64 mg/24 h versus CRF5/6 = 124 ± 112 versus BP = 51.6 ± 32 versus BPMSC = 20.8 ± 26.4 versus BPMO = 9.6 ± 6.6; $P = 0.012$); this effect persisted until day 90 (Figure 3d).

Renal histology

GS, TA, IF and IL were significantly less severe in CRF2/3 rats. In spite of the significantly less severe morphological chronic injury observed in the CRF2/3 model, treatment with BPMSC and BPMO effectively reduced the amount of GS and IF. A trend toward less injury was noted for all other evaluated histological parameters (Table 1).

The effect of treatment with BPMSC or BPMO in the CRF2/3 animals was more striking and significantly reduced GS, TA, IF and IL ($P < 0.05$) by at least two-fold. At the end of the study, the treated CRF5/6 animals presented significantly less renal damage when compared with untreated rats.

Table 1 Histological changes and the effect of treatment with BMDC on the remnant kidneys of 2/3 and 5/6 nephrectomized animals

	CRF2/3				CRF5/6			
	CRF2/3	BP	BPMSC	BPMO	CRF5/6	BP	BPMSC	BPMO
GS	19.4 ± 6*	13.3 ± 8.3	6.4 ± 1.8*	14 ± 6.8	28.5 ± 17*	15.2 ± 9.1	14.7 ± 13.4	9.95 ± 5.2*
TA	8.6 ± 8	9 ± 9.6	3.8 ± 2.7	3.2 ± 3.9	31 ± 16*	15.7 ± 11	7.9 ± 4.3*	9.4 ± 8.1*
IF	8 ± 6.2	13.6 ± 9.5*	2.2 ± 2.2*	3 ± 2.1*	25 ± 15*	18.3 ± 13.7	7.2 ± 4.6*	7.5 ± 7.4*
IL	7 ± 6.6	6 ± 6.5	1 ± 1.4	1.4 ± 0.9	20 ± 11*	16.7 ± 13	6.4 ± 4.5*	5.1 ± 4.4*

CRF, untreated chronic renal failure; BP, chronic renal failure with unseeded biomaterial alone; BPMSC, CRF rats treated with BP seeded with mesenchymal stem cells; BPMO, CRF rats treated with BP seeded with mononuclear cells; GS, glomerular sclerosis; TA, tubular atrophy; IF, interstitial fibrosis; IL, interstitial lymphocytic infiltration

Data are expressed as means ± SD (*$P < 0.05$)

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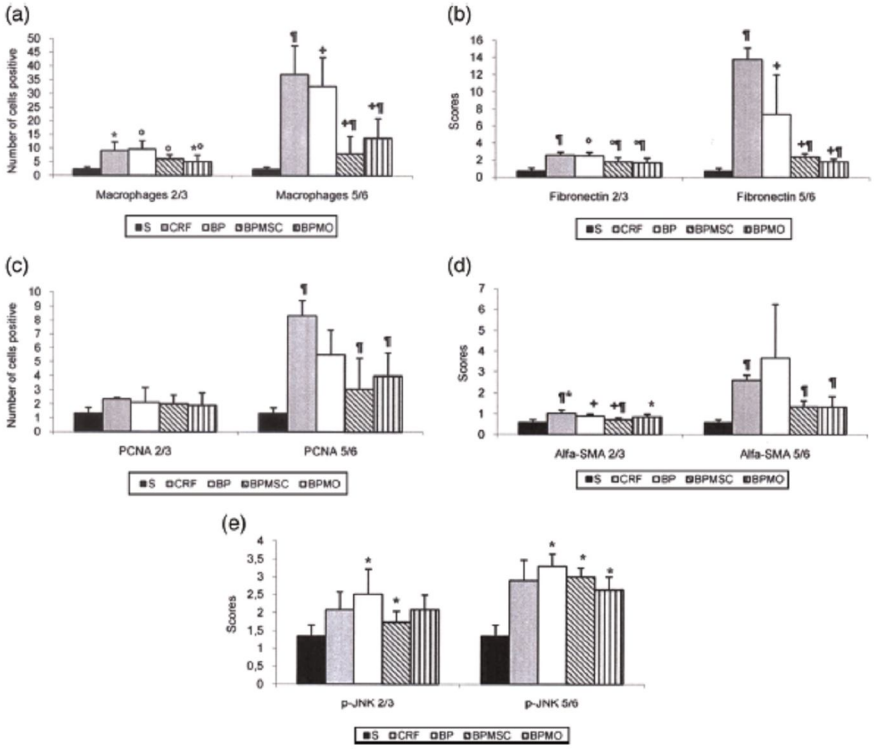

Figure 4 Immunohistochemistry of renal tissue from 2/3 and 5/6 nephrectomized rats. (a) Immunolocalization of ED-1-positive cells; (b) staining for fibronectin; (c) immunolocalization of PCNA; (d) immunostaining of interstitial α-SM-actin; and (e) p-JNK in the renal cortex. Data are expressed as means $\pm$ SD ($^{*}P < 0.05$, $^{\circ}P < 0.05$, $^{+}P < 0.01$, $^{\P}P < 0.01$). S, sham; CRF, chronic renal failure; BP, bovine pericardium; BPMSC, BP seeded with mesenchymal stem cells; BPMO, BP seeded with mononuclear cells; PCNA, proliferating cell nuclear antigen; SMA, smooth muscle actin; JNK, Jun-N-terminal kinase

Immunohistochemical analysis

Monocytes/macrophages and PCNA

The accumulation of macrophages (ED-1-positive cells) was significantly reduced in rats with less severe chronic kidney disease (CRF2/3 = 9.1 $\pm$ 3 versus CRF5/6 = 37 $\pm$ 10.5 ED-1-positive cells; $P = 0.0012$); the same as observed with the proliferative activity as expressed by the number of PCNA-positive cells (CRF2/3 = 2.36 $\pm$ 1 versus CRF5/6 = 8.33 $\pm$ 1.1 PCNA-positive cells; $P = 0.0002$) (Figures 4a and c).

Treatment with BPMSC or BPMO decreased the number of ED-1-positive cells in the CRF2/3 model (S = 2.28 $\pm$ 0.78 versus CRF2/3 = 9.1 $\pm$ 3 versus BP = 9.6 $\pm$ 2.8 versus BPMSC = 6.09 $\pm$ 1.5 versus BPMO = 5.06 $\pm$ 2.4; $P = 0.0024$) but had no effect on PCNA expression (Figure 4c).

In contrast, a significant decrease in the number of ED-1-positive cells and in the expression of PCNA were observed following BPMSC or BPMO treatment in 5/6 nephrectomized rats ($P < 0.001$) (Figures 4a, c and 5).

Fibronectin and α-SM-actin

Staining for both markers matched the degree of severity of CRF. Much lower expression of both molecules occurred in the CRF2/3 model compared with the 5/6 model. Treatments with BPMSC and BPMO were equally effective at reducing fibronectin and α-SM-actin expression in both CRF2/3 and CRF5/6 animals ($P < 0.05$).

Efficacy of treatment was observed in CRF2/3 rats in spite of the low expression of these proteins in this model (Figures 4b, d and 5).

Jun-N-terminal kinase

In contrast to the results with CRF2/3 rats, JNK activity markedly increased after 5/6 nephrectomy (Figure 4e; $P < 0.01$). As observed with the increase in sCr, unseeded BP groups showed the highest expression of JNK activity. Comparisons between the two models showed differences according to the degree of tissue injury. Only BPMSC reduced the expression of JNK in the 2/3 model, while the same effect was observed with both treatments in the 5/6 nephrectomized rats (Figures 4e and 5).

Discussion

We and other groups have previously shown that the administration of BMDC into a chronically damaged kidney improves renal function and retards the progression of chronic kidney disease in 5/6 nephrectomized rats.[5–9]

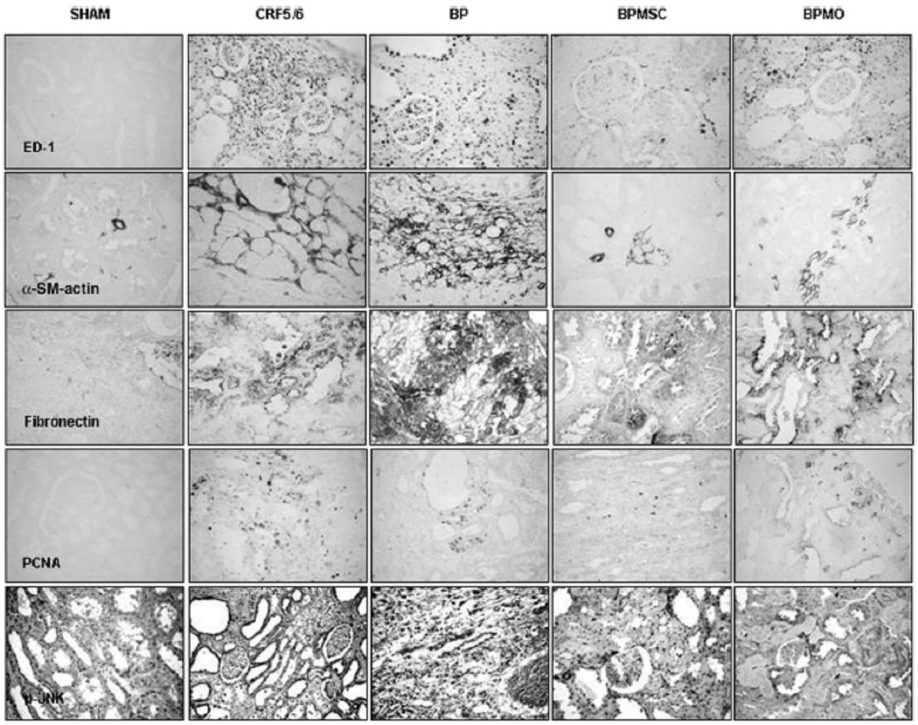

Figure 5 Representative images of immunolocalization of ED-1 cells (macrophages/monocytes), α-SM-actin, fibronectin, PCNA and p-JNK in the renal cortices of the sham, CRF5/6, BP, BPMSC and BPMO groups (CRF5/6 model) on postoperative day 90 (×40). CRF, chronic renal failure; BP, bovine pericardium; BPMSC, BP seeded with mesenchymal stem cells; BPMO, BP seeded with mononuclear cells; PCNA, proliferating cell nuclear antigen; SM, smooth muscle; JNK, Jun-N-terminal kinase

In the present study, our main goals were to elucidate whether scaffolds seeded with BMDC could be an effective alternative to deliver BMDC in rats with CRF and whether the amount of remnant renal mass would impact the efficacy of cellular therapy.

Major considerations that led us to use this approach were the ability of decellularized scaffolds made from biological tissues to retain the properties necessary to interact with the host and the capacity of the scaffolds to favor the process of proliferation and differentiation of precursor cells.[20] Moreover, the inflammatory reaction elicited after scaffold implantation and associated with the plasticity of BMDC would generate a local microenvironment that favors tissue remodeling and repair.[21]

As we hypothesized that cell therapy could be more effective if administered during less severe stages of the disease, we evaluated the effectiveness of treatment in rats sustained by a remnant renal mass two-fold greater than that of the traditional 5/6 model using the 2/3 reduction of renal mass model.

Our study revealed two main findings. First, the approach of using seeded scaffolds was effective in retarding the progression of disease in both models of CRF. Significantly lower increases in sCr and PT-24 h improved CLcr and reduced slopes of the progression of CRF were seen only in animals treated with BPMSC and BPMO (Figures 2a and b). Second, we provide evidence that the amount of remnant renal mass positively did impact the efficacy of cell therapy. After 90 d, renal functional studies showed that in the 2/3 model, treatment with BPMSC and BPMO fully reversed the decline in the 1/sCr slopes and kept the rate of PT-24 h excretion similar to that of the sham animals. Despite the statistically significant results, the progression of CRF was only attenuated in rats with a 5/6 nephrectomy (Figures 3 and 4).

The kinetics of sCr elevation after surgery also differed between the two models. Treatment of CRF2/3 rats with BPMSC or BPMO did not significantly alter the progressive increase in sCr during the initial 45 d after surgery, but the increase in sCr leveled off thereafter. In contrast, both treatments prevented the increase in sCr during the same period in the 5/6-nephrectomized animals, but after 45 d, neither treatment could effectively avoid the increase in sCr (Figures 2a and 3a). These results could be due to differences in the amount of damaged tissue, the capacity of BMDC to promote repair in response to inflammatory cues within the local microenvironment or to the reduced number of host stem cell niches consequent to renal mass reduction.[21,22] Interestingly, the implantation of unseeded BP seems to accelerate the progression of CRF in animals

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with the 5/6 reduction in renal mass, suggesting additional inflammatory stimuli elicited by the biomaterial in the already severely injured kidney tissue.[23]

It has been suggested that BMDC can reduce renal injury and facilitate tissue repair by upregulating the expression of anti-inflammatory cytokines and chemokines by the host infiltrating macrophages, which results in reduced glomerulosclerosis and fibrosis.[21,24–26] In fact, comparisons of the morphological findings between the two models of CRF showed more glomerulosclerosis, tubular atrophy and fibrosis in the CRF5/6 group than in the CRF2/3 group (Table 1). The amelioration of renal function following treatment with BMDC was also associated with a significant improvement in the histological features of both groups of CRF animals. Taking into account the extent of renal damage, the greatest 'renoprotective' effect occurred in the 5/6 nephrectomized animals, with much less GS, IF and LI observed in these remnant kidneys after treatment with BPMSC and BPMO when compared with untreated animals (Table 1). Even more interesting is the observation that even in the presence of smaller amounts of histological lesions, a significant reduction in IF could still be detected in the treated CRF2/3 groups (Table 1).

The accumulation of macrophages in the renal interstitium plays an important role in the process of renal inflammation that occurs during the initiation and progression of chronic kidney injury. This proinflammatory response is mediated in part by activation of the JNK signaling pathway and has been associated with chronic inflammation and tubular apoptosis.[27] Ma *et al.*[28] demonstrated that the inhibition of JNK signaling prevents the development of a proinflammatory response and provides protection against the progression of crescentic glomerulonephritis. Therefore, we assessed the effect of treatment with BMDC on the accumulation of macrophages and the expression of fibrogenic molecules and JNK signaling components in the remnant renal tissue of 2/3 and 5/6 nephrectomized animals. As expected, the number of macrophages/monocytes and the proliferative activity was three- to four-fold higher in CRF5/6 animals than in CRF2/3. Accordingly, the extent of the fibrogenic response was also greater in CRF5/6 animals, which expressed significantly more fibronectin and α-SM-actin. In the same way, JNK staining increased after renal mass reduction and was slightly greater in CRF5/6 rats, suggesting a direct correlation between the activation of this pathway and the amount of tissue damage.

Treatment with BPMSC and BPMO significantly reduced the number of macrophages and the expression of fibronectin, α-SM-actin and JNK in both models. Treatments reduced the proliferative activity only in animals with severe CRF5/6, which was probably a consequence of more inflammation and tissue damage in this model. Interestingly, the reduction of JNK expression in the CRF2/3 model after BPMSC treatment was observed only in rats with unseeded BP. This result corroborates the idea that the biomaterial alone could elicit additional inflammatory stimuli throughout the signaling pathway.

Subcapsular, intraparenchymal or intravenous injection of BMDC and lineage-negative cells reduces renal injury and the infiltration of macrophages and lymphocytes in the 5/6 nephrectomy model, but the mechanisms by which the stem cells induce kidney repair remain controversial.[5–8] Differentiation into different types of renal cells, direct replacement of damaged structures and the release of growth factors and cytokines are some of the mechanisms proposed to promote functional and structural repair.[29]

Despite limited information on the relationship of hyperfiltration and inflammation in this model, it is likely that lymphocyte and macrophage infiltration play a significant role in the development of fibrosis.[30]

Biomaterials could exert their effects in two ways, by serving to deliver cells to the remnant renal parenchyma and by acting as a template for tissue regenerate. In addition, scaffolds could favor cell adhesion and differentiation and enhance stimulatory growth factors; in this way, they could contribute to amplify the regenerative capacity of BMDC. Moreover, it is important to consider the capacity of biomaterials to promote regeneration throughout a series of mechanisms that, when combined with seeded and resident stem cells, provide stimuli favoring regeneration instead of fibrosis.[31]

For the first time, our experiments have shown that biomaterial seeded with BMDC was effective at preventing the progressive deterioration of chronic kidney disease. This effect was associated with a significant reduction in glomerulosclerosis and interstitial fibrosis, probably due to attenuation of macrophage accumulation and reduction of the proliferative activity and expression of myofibroblasts and fibronectin. Moreover, we demonstrated that the amount of remnant renal mass does impact on the efficacy of treatment, which suggests that cellular therapy should be started in less advanced stages of CRF.

Using the approach of combining the biomaterial and BMDC, we have provided a novel and efficient route for cellular therapy of CRF. We speculate that seeded scaffolds could have the same beneficial effect when implanted in other sites beyond renal tissue.

Author contributions: HCC, RSK-O, IMMF: collection and assembly of data, data analysis and interpretation, manuscript writing; MASFB: histological analysis; AMGP, VAM: preparation of the biomaterial; TMC: immunohistochemical analysis; EMG-B, DMB: collection and/or assembly of data; MA-F: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript, administrative support.

ACKNOWLEDGEMENTS

We thank Rita Cavaglieri for her skilled technical assistance. This work received partial financial support from FAPESP, CNPq and FAMERP/FUNFARME and was partially supported by grants from the FAMERP-BAP.

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(Received January 18, 2011, Accepted March 21, 2011)

COMPROVANTE DE ACEITE DA PUBLICAÇÃO

EXPERIMENTAL BIOLOGY AND MEDICINE

ONLINE MANUSCRIPT SUBMISSION AND PEER REVIEW

Home

Detailed Status Information

Manuscript #	1101-RM-24-
Current Revision #	0
Submission Date	2011-01-18 11:34:21
Current Stage	Waiting for Revision
Title	Effect of stem cells seeded onto biomaterial on the progression of experimental chronic kidney disease
Running Title	Stem cells retard the progression of chronic kidney disease
Manuscript Type	Regular Manuscript
Thematic Issues	N/A
Category	Stem Cell Biology
Corresponding Author	Mario Abbud-Filho (Medical School of Sao Jose do Rio Preto)

ARTIGO 3

Título: Cultivo de células mesenquimais do sangue de cordão umbilical com e sem uso do gradiente de densidade Ficoll-Paque.

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Periódico: Rev Bras Cir Cardiovasc. 2008 Mar;23(1):29-34.

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ARTIGO ORIGINAL

Rev Bras Cir Cardiovasc 2008; 23(1): 29-34

Blood mesenchymal stem cell culture from the umbilical cord with and without Ficoll-Paque density gradient method

Cultivo de células mesenquimais do sangue de cordão umbilical com e sem uso do gradiente de densidade Ficoll-Paque

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RBCCV 44205-946

Abstract

Objectives: **Implantation of cell separation and mesenchymal stem cell culture techniques from human umbilical cord blood with and without using the Ficoll-Paque gradient density method (d=1.077g/ml).**

Methods: **Ten samples of the umbilical cord blood obtained from full-term deliveries were submitted to two different procedures of mesenchymal stem cell culture: a) Method without the Ficoll-Paque density gradient, which concentrates all nucleated cells; b) Method with the Ficoll-Paque density gradient, which selects only low-density mononuclear cells. Cells were initially plated into 25 cm^2 cultures flasks at a density of $1x10^7$ nucleated cells/cm^2 and $1x10^6$ mononuclear cells/cm^2.**

Results: **It was obtained 2-13$x10^7$ (median = 2.35$x10^7$) nucleated cells/cm^2 by the method without the Ficoll-Paque gradient density, and 3.7-15.7$x10^6$ (median = 7.2$x10^6$) mononuclear cells/cm^2 by the method with the Ficoll-Paque gradient density. In all cultures adherent cells were observed 24 hours after being cultured. Cells presented fibroblastoid and epithelioid morphology. In most of the cultures, cell proliferation occurred in the first week, but after the second week only some cultures - derived from the method without the Ficoll-Paque gradient density - maintained the growth rate reaching confluence. Those cultures were submitted to trypsinization with 0.25% trypsin/EDTA solution and cultured for two to three months.**

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Article received in December 27th, 2007
Article accepted in February 1st, 2008

Conclusion: **In the samples analyzed, cell separation and mesenchymal stem cell culture techniques from human umbilical cord blood by the method without the Ficoll-Paque density gradient was more efficient than the method with the Ficoll-Paque density gradient.**

Descriptors: **Umbilical cord blood. Mesenchymal stem cells/cytology. Cells, cultured. Cell culture techniques.**

Resumo

Objetivos: **Implantação de técnicas de isolamento e cultivo de células-tronco mesenquimais do sangue de cordão umbilical humano, com e sem uso de gradiente de densidade Ficoll-Paque (d=1,077g/ml).**

Métodos: **Dez amostras de sangue de cordão umbilical humano de gestação a termo foram submetidas a dois procedimentos de cultivo de células-tronco mesenquimais: sem gradiente de densidade Ficoll-Paque e com gradiente de densidade. As células foram semeadas em frascos de 25cm² a uma densidade de 1x10⁷células nucleadas/cm² (sem Ficoll) e 1,0x10⁶ células mononucleares/cm² (com Ficoll). As células aderentes foram submetidas a marcação citoquímica com fosfatase ácida e reativo de Schiff.**

Resultados: **No procedimento sem gradiente de densidade Ficoll, foram obtidas 2,0-13,0x10⁷ células nucleadas (mediana=2,35x10⁷) e, no procedimento com gradiente de densidade Ficoll, foram obtidas 3,7-15,7x10⁶ células mononucleares (mediana=7,2x10⁶). Em todas as culturas foram observadas células aderentes 24 horas após o início de cultivo. As células apresentaram morfologias fibroblastóides ou epitelióides. Na maioria das culturas houve proliferação celular nas primeiras semanas de cultivo, mas após a segunda semana, somente três culturas provenientes do método sem gradiente de densidade Ficoll-Paque mantiveram crescimento celular, formando focos confluentes de células. Essas culturas foram submetidas a várias etapas de tripsinização para espalhamento ou subdivisão e permaneceram em cultivo por períodos que variaram de dois a três meses.**

Conclusão: **Nas amostras estudadas, o isolamento e cultivo de células-tronco mesenquimais do sangue de cordão umbilical humano pelo método sem gradiente de densidade Ficoll-Paque foi mais eficiente do que o método com gradiente de densidade Ficoll-Paque.**

Descritores: **Sangue de cordão umbilical. Células-tronco mesenquimais/citologia. Células cultivadas. Técnicas de cultura de células.**

INTRODUCTION

In the last decade, a considerable number of studies have proven that human umbilical cord blood (UCB) has hematopoietic stem cells (SCH) and a pool of mesenchymal stem cells (MSC). MSCs are capable of multilineage proliferation and differentiation, similarly to those observed in bone marrow cells.

These characteristics create expectations of using cellular therapies to regenerate tissues and organs affected by the so called incurable diseases, such as neurologic, cardiac, and kidney diseases, etc. Nevertheless, as these cells correspond to only a small portion of mononuclear cells present in each sample, it is necessary to isolate and multiply them *in vitro*.

Mesenchymal stem cells different isolation and culture protocols of umbilical cord blood have already been employed successfully, all involving one early stage of mononuclear cell separation using Ficoll-Paque density gradient [1-4]. Despite the positive outcomes observed in these groups, many studies have reported great difficulty to culture and maintain these cells *in vitro*, and others report a total failure in isolating and culture of these same cells [5-8].

All mesenchymal stem cell protocols of umbilical cord blood start with an isolation phase of mononuclear cells with Ficoll-Paque density gradient, which requires several manipulation and centrifugation phases of umbilical cord blood. This procedure increases the risk of contamination of UCB. In attempting to speed up the process making the mononuclear cell isolation processes faster, several methods have already been tested such as the use of poligeline, hydroxyethyl starch gel (HES) and gelatin to deplete red blood cells (RBC). Despite the efficacy of these procedures, the release of these products for clinical practice depends on the approval of the centers for disease control in several countries [9].

Moreover, these procedures also involve several phases of the manipulation process that increase the risk of contamination. Also, easy to handle and closely sealed filtration systems to concentrate mononuclear cells, the so-called SCF SYSTEM (the stem cell collection filter system), have already been tested. This system has proven to be more efficient and faster in separating mononuclear cells when in comparison to the conventional technique which uses Ficoll-Paque density gradient; however, the release of this system for clinical practice yet requires further studies [10].

In face of these difficulties, our group has developed a mesenchymal stem cell culture of UCB from the isolation of nucleated cells present in the buffy coat collected through centrifugation without Ficoll-Paque density gradient. These cells correspond to all nucleated cells present on UCB and not only to the low-density mononuclear cells isolated by

the Ficoll-Paque density gradient. This protocol was compared to the conventional procedure using Ficoll-Paque density gradient centrifugation in order to isolate mononuclear cells.

METHODS

Collection of umbilical cord blood

Ten samples of UCB were collected from full-term placenta of healthy women, nonsmokers, nondrinkers, age ranging from 20 to 40 years, regardless ethnic group at the Obstetric Service of Hospital de Base Regional Medical School Foundation (FUNFARME) from the Medical School of São José do Rio Preto. Free written informed consent was obtained from the mothers or the next of kin following the guidelines of the local Institutional Review Board and Ethics Committee, according to certificate Nº 168/2005. Blood was collected in sterile syringes containing heparin sodium (1000 IU).

Processing of umbilical cord blood

UCB samples were diluted in a proportion of 1:1 in a phosphate-buffered saline solution (PBS) and submitted to two different mesenchymal culture cell procedures: a) Method without Ficoll-Paque density gradient (*d*=1.077 g/mL) (Amershan Pharmacia); b) Method with Ficoll-Paque density gradient. These procedures are described as follows:

a) Method without Ficoll-Paque density gradient: UCB samples were transferred to centrifuge tubes (15 mL) and submitted to centrifugation at 1000 rpm for 10 minutes to obtain buffy coat. The buffy coat containing all nucleated cells present in UCB was transferred to a new centrifuge tube, which was washed out twice with culture medium through centrifugation at 1000 rpm for 8 minutes. The number of nucleated cells was estimated after counting into Neubauer chamber and cellular viability was determined by the Tripan Blue exclusion method.

b) Method with Ficoll-Paque density gradient: UCB samples were transferred to centrifuge tubes (15 mL) containing Ficoll-Paque solution and submitted to centrifugation at 2000 rpm for 30 minutes in order to isolate low-density mononuclear cells. Mononuclear cells were transferred to a new tube and washed twice with culture medium through centrifugation at 2000 rpm for 10 minutes, according the protocol described by Erices et al. [2]. Cell number estimate and viability followed the same procedure described for the nucleated cells.

Culture of mesenchymal cells

Cultures of mesenchymal stem cells were initiated from nucleated and mononuclear cells. Mesenchymal stem cells isolation was possible due to its capacity of adhesion to the flasks, differently from the nonadherent hematopoietic mesenchymal stem cells, which are eliminated from the culture during the procedures of medium change. The culture procedures are described below:

a) Culture of Mesenchymal Stem Cell from nucleated cells: cultures were initiated in cell culture flasks of 25 cm^2 at a density of $1x10^7$ nucleated cells/cm^2. Cells were nurtured with culture medium á-MEM supplemented with 20% fetal bovine serum, 1% antibiotic/antimycotic, and 1% glutamine. Cultures were incubated at 37ºC, humidified atmosphere containing 5% CO2. The first change of culture medium was performed 24-48 hours after initial plating to eliminate nonadherent cells. Posteriorly, the culture medium was changed at every four days and cellular growth assessed daily under an inverted microscope. When the cells reached 50-60% confluence, they were subdivided after trypsin/EDTA (0.025%) (Gibco-BRL).

b) Culture of mesenchymal stem cell from mononuclear cells: cultures were initiated in culture flasks of 25 cm^2 at a density of $1.0x10^6$ mononuclear cells/cm^2, following the same procedures described for nucleated cells

Cytochemistry characterization of mesenchymal cells

Adherent cells were submitted to cytochemistry labeling with acid phosphatase (AP) and periodic acid-Schiff reactive (PAS), according to the protocol (with modifications) described by Erices et al. [3].

RESULTS

Ten samples were submitted to isolation protocols of nucleated and mononuclear cells. In isolation procedures of nucleated cells (without Ficoll) were obtained $2.0\text{-}13.0x10^7$ (median of $2.35x10^7$) cells/cm^2. In isolation procedures of mononuclear cells (with Ficoll) were obtained $3.7\text{-}15.7x10^6$ (median of $7.2x10^7$) cells/cm^2. In all cultured samples were observed adherent cells 24 hours after initial plating. After the second week of plating, the growth rate of the cultures from isolation with Ficoll decreased until complete stagnation. In the cultures from isolation without Ficoll only three samples kept their growth, forming confluent focuses of cells. These cultures were submitted to several phases of trypsinization for dissemination or subdivision and were kept in culture for period varying from two to three months.

Cells from the beginning of culture presented epithelioid and fibroblastoid morphologies (Figure 1A). After the subdivisions, there has been predominance of fibroblastoid cells (Figure 1B) and, in some cases, the presence of a large rounded multinucleated cell (Figure 1C). During trypsinization, fibroblastoid cells detached rapidly from the

flask, whereas the rounded cells needed a longer period of exposition to trypsin to be detached from the flask. The cytochemistry characterization highlighted fibroblastoid cells PAS positive and AP negative and rounded cells AP positive and PAS negative (Figure 2).

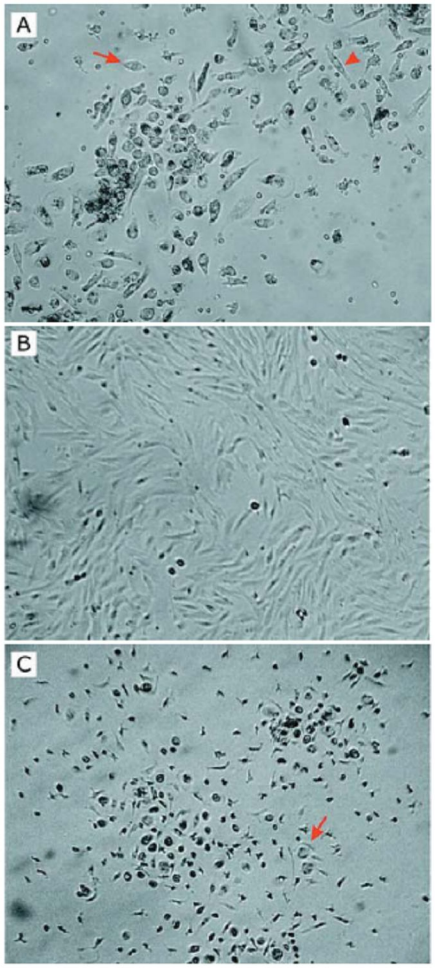

Fig. 1 – Microphotograph showing adherent cells from umbilical. A. Fibroblastoid (arrow) and epithelioid cells (arrow head) observed in initial plating. B. Confluent fibroblastoid cells. C. Rounded cells (arrow). Magnification x200.

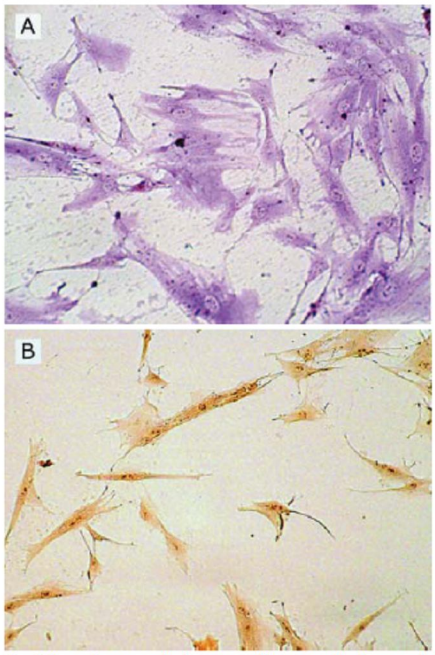

Fig. 2 – Microphotograph showing adherent cells from umbilical Cord blood. A. Cytochemistry analysis showing mesenchymal cells labeled positively by periodic acid-Schiff reactive (PAS). B. Cytochemistry analysis showing mesenchymal cells labeled negatively by acid phosphatase. Magnification x200.

DISCUSSION

Our study examined comparatively the isolation and culture procedure of mesenchymnal cells of umbilical cord blood without Ficoll-Paque density gradient. This procedure, which enables to separate all nucleated cells present in the umbilical cord blood, was compared to the standard technique using Ficoll-Paque density gradient, which allows the selective isolation of mononuclear cells. The results show that both mononuclear cells and nucleated cells isolated from UCB, when cultured in vitro, were able to produce adherent cells with different morphologies. Among the cell types observed, we could see fibroblastoid-shaped elongated cells (*spindle-shaped cells*), egg-shaped cells similar to epithelioid cells, and rounded cells similar to osteoclasts (*osteoclast-like cells*).

Different studies with umbilical cord blood have identified similar cells, whose nature was determined by

means of immunocytochemistry and cytochemistry labeling. Fibroblastoid cells (elongated) proved to be positive for mesenchymal cell markers and the rounded cells were positive for osteoclast markers. The confirmation that fibroblastoid cells corresponded to mesenchymal stem cells was established by their cellular differentiation capacity induced by growth factors [2,4,11-14].

In the present study, adherent cells were characterized cytochemistrically with PAS and acid phosphatase. PAS labels mesenchymal cells (fibroblastoids) positively and the osteoclasts (rounded cells) negatively, whereas acid phosphatase labels the osteoclasts (rounded cells) positively and the mesenchymal cells (fibroblastoids) negatively. The results obtained associated to morphological analysis indicate that the fibroblastoid cells correspond to the mesenchymal cells, thus demonstrating that it is possible isolate and culture these cells from UCB without using Ficoll-Paque density gradient.

Although in the present study specific mesenchymal stem cell markers have not been used and the induction of cellular differentiation to confirm the presence of stem cells, the intense cellular proliferation observed in the three samples is a predictor of the presence of stem cells. That's because, the differentiated cells or senescent cells have a limited life span, characterized by loss of proliferation capacity and morphology alteration, leading to culture stagnation [15].

The success rate in isolating and plating mesenchymal stem cells from UCB observed in our study was of 30% (n=3/10). These data are in accordance with the literature findings [2,4,11-14]. The major difficulty in plating mesenchymal stem cells from UCB results from the small number of mesenchymal stem cells present in each sample. According to Goodwin et al. [1] only about 0.05-2.8x10^6 mononuclear cells planted correspond to a mesenchymal stem cell. Moreover, survival or death of these cells can be strongly affected by changes in sample storage time until the beginning of the plating, quantity of mononuclear cells obtained, the presence of clot, hemolysis, as well as by the own conditions of plating of each laboratory [11.15].

Another factor that hampers the detection of mesenchymal stem cells in vitro is that these cells are normally detected after 2-4 weeks of plating, differently of what is observed in the mesenchymal stem cell cultures from bone marrow or from fatty tissue, in which these cells are identified after 4-5 days of plating [16]. However, once established, the mesenchymal stem cell cultures from UCB are capable of generating much more mother cells than those from bone marrow [15]. This is resultant from the immaturity of the newborn cells when compared to the adult cells. Aging is associated to the reduction of mesenchymal stem cell life span and to its differentiation capacity [17].

These difficulties are responsible for the scepticism of some researchers as to the presence of mesenchymal stem cells in the UCB [5-8]. However, a number of studies have proved the presence of mesenchymal stem cells in the UCB, their potential of cellular differentiation and proliferation, showing that the umbilical cord blood can be an important source of cells for cellular therapeutics for treatment of diseases the so called incurable diseases [1-4,11-20].

CONCLUSION

The procedure to obtain nucleated cells without using Ficoll-Plaque density gradient has shown to be more efficient for the culture of mesenchymal stem cell from UCB when compared to the procedure using Ficoll-Paque density gradient. The possibility of isolating and plating mesenchymal cells without using Ficoll, which is clearly known to be toxic, has become the safest and fastest procedure. These results are preliminary and need further studies to be validated.

ACKNOWLEDGEMENTS

This study was carried out with the financial support of BAP (Researcher and Grant-holders) and Braile Biomédica Indústria, Comércio e Representações SA.

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ARTIGO 4

Título: Perspectivas do uso da terapia celular no tratamento da doença renal crônica

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Periódico: Série publicada pela Sociedade Brasileira de Nefrologia "Atualidades em Nefrologia 11". Caldas HC, Abbud-Filho M. Perspectivas do uso da terapia celular no tratamento da doença renal crônica. In: Jenner Cruz [et al.], editores. Atualidades em Nefrologia 11. São Paulo: Sarvier; 2010. p. 42-45.

7

PERSPECTIVAS DO USO DA TERAPIA CELULAR NO TRATAMENTO DA DOENÇA RENAL CRÔNICA

Heloisa Cristina Caldas
Mario Abbud Filho

INTRODUÇÃO

Embora a nefrologia seja uma especialidade que oferece duas alternativas terapêuticas para a manutenção e tratamento da fase terminal da doença renal crônica, essas terapias têm limitações significativas: a diálise é um tratamento temporário, de elevado custo e que permite a reposição parcial, mas não restaura a função renal, enquanto o transplante é limitado pela escassez de doadores de órgãos, complicações da terapia imunossupressora e pela ausência de tratamento da disfunção crônica do transplante.

Nos últimos anos, o uso de células derivadas da medula óssea (células-tronco, células estromais) abriu perspectivas para a possibilidade da utilização da medicina regenerativa no tratamento de várias doenças crônicas, substituindo células mortas ou não funcionais por células saudáveis.

O rim é um órgão complexo e ainda pouco estudado com relação aos efeitos da terapia celular para a reparação do tecido renal lesado e, embora recentemente, vários estudos tenham mostrado os efeitos positivos dessa terapia no modelo de insuficiência renal aguda, pouco ainda se conhece ou foi publicado em modelos de lesão crônica.

Este capítulo tem como objetivo revisar esses trabalhos e apresentar alternativas da medicina regenerativa com probabilidade de serem aplicadas no tratamento da doença renal crônica.

CÉLULAS-TRONCO ADULTAS

As células-tronco são células com capacidade de autorreplicação e diferenciação em outras linhagens celulares especializadas. Podem ser divididas em dois grupos: embrionárias e adultas[1].

As células-tronco adultas caracterizam-se por apresentarem ciclo celular lento, capacidade de proliferação *in vitro* e localização em tecidos específicos. Essas células são isoladas em grande parte da medula óssea e podem diferenciar-se distintamente: a linhagem *hematopoiética* originará as células do sangue, enquanto a linhagem *mesenquimal*, derivada do estroma da medula óssea, poderá originar outros tipos celulares[2].

As células-tronco mesenquimais caracterizam-se por serem uma população celular multipotente capaz de se diferenciar e produzir tipos celulares indispensáveis para a reparação e manutenção tecidual[3,4]. *In vitro*, as células-tronco mesenquimais exibem morfologia fibroblastoide, adesão em substrato plástico, autorrenovação e diferenciação em osteócitos, condrócitos e adipócitos[5].

Recentes estudos demonstraram que as células-tronco mesenquimais estão presentes na parede vascular de arteríolas pré-capilares e nas pequenas artérias e veias (pericitos) e a associação dessas células com a vasculatura permite que funcionem como fonte de reposição celular e estejam disponíveis para a reparação ou regeneração de lesões locais[6].

Os mecanismos de regeneração empregados pelas células-tronco, não só do tecido renal, mas também dos diferentes órgãos são desconhecidos. Algumas hipóteses têm sido formuladas sobre esses mecanismos: a *transdiferenciação*, o processo pelo qual uma célula-tronco se diferencia em uma célula adulta de outro tecido; *a fusão celular* entre as células-tronco da medula óssea com as células do órgão afetado, gerando uma célula híbrida que assumiria o fenótipo do órgão lesado, com consequente regeneração tecidual e *ação parácrina* modulatória das células-tronco sobre o tecido remanescente, no qual as células-tronco secretam uma grande variedade de

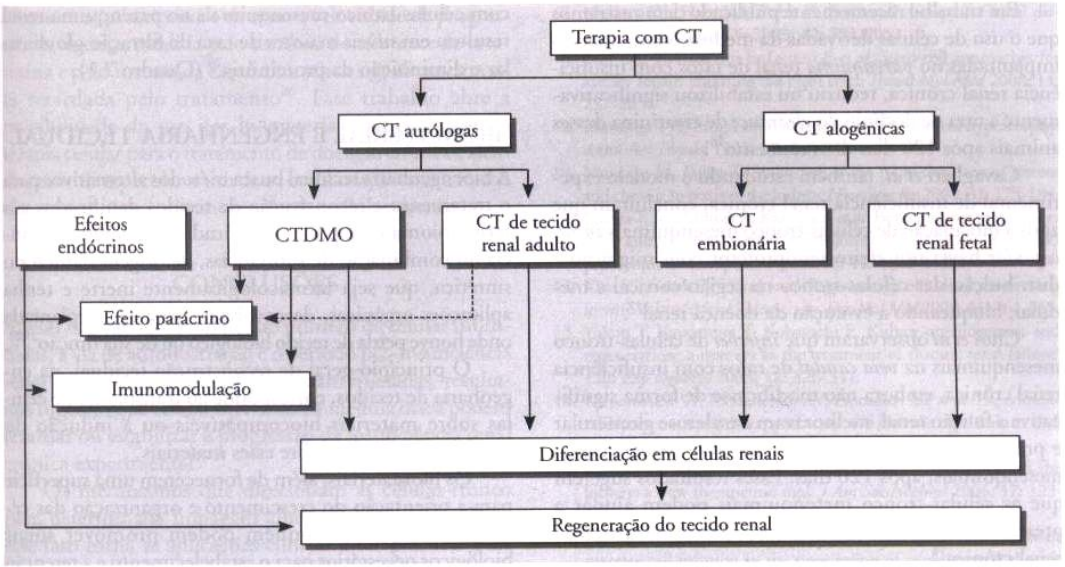

Figura 7.1 – Representação esquemática de diferentes abordagens terapêuticas para o tratamento dos mecanismos múltiplos que representam o potencial regenerativo dos diferentes tipos de células-tronco em lesões do tecido renal (adaptado de Sagrinati[8]). CT = células-tronco; CTDMO = células-tronco derivadas da medula óssea.

quimiocinas, além de expressarem receptores para citocinas e fatores de crescimento. Dessa forma, as células-tronco interagem com as células residentes (nicho) e podem induzi-las a se diferenciar em linhagens celulares distintas, de acordo com esta sinalização, promovendo a restauração do tecido renal.

Na verdade, não há uma explicação definitiva sobre o processo de atuação das células-tronco na regeneração tecidual renal e certamente deve ocorrer ainda ação concomitante de dois ou mais desses e/ou de outros processos[7] (Fig. 7.1).

TERAPIA CELULAR NO MODELO EXPERIMENTAL DE DOENÇA RENAL CRÔNICA

A maioria das doenças renais crônicas caracteriza-se pela lesão inicial seguida da progressão dessas lesões para a destruição completa do parênquima renal e formação de fibrose tecidual.

Os mecanismos envolvidos nesse processo são pouco conhecidos, e modelos experimentais mostram que a redução da quantidade de néfrons serve como estímulo inicial para promover vários eventos moleculares e celulares para compensar a redução de massa renal remanescente. Esse processo compensatório leva a esclerose glomerular, atrofia tubular e fibrose intersticial[9].

Insuficiência renal crônica terminal pode ser induzida pelo modelo de redução de 5/6 da massa renal. Nesse modelo, autores sugerem que os mecanismos compensatórios se tornam patológicos e contribuem para o desenvolvimento da progressão da lesão renal, resultando na síndrome urêmica característica observada em pacientes[9,10].

Devido às observações da melhoria da função renal após o transplante de medula óssea, Drewa *et al* investigaram o efeito de células derivadas da medula óssea retiradas de ratos com insuficiência renal crônica e concluíram que a população da medula óssea com marcador CD34– mostrou uma capacidade reduzida para a proliferação *in vitro*. Esse estudo sugeriu que células da medula óssea obtidas de pacientes com insuficiência renal crônica poderiam não ser úteis para o transplante autólogo de células-tronco[11].

Kim *et al* investigaram se o transplante de células precursoras de rim fetal de rato, isoladas por meio da desagregação de metanéfrons de fetos e injetadas sob a cápsula renal dos animais com redução da massa renal de 5/6, poderia reconstituir o tecido renal. Após cinco semanas, o transplante de células de rim fetal mostrou o potencial para o aumento parcial da estrutura e da função renal no tratamento da insuficiência renal, reduzindo os sintomas urêmicos e promovendo a reconstituição do tecido renal[12].

Yokoo *et al* transplantaram células-tronco mesenquimais na região onde se forma o rim do embrião de rato em desenvolvimento e os rins embrionários resultantes foram então recolhidos após vários dias em cultura e injetados em ratos uninefrectomizados. O neo-órgão quimérico resultante apresentava parênquima renal, vascularização e produção de urina[13].

O primeiro relato da terapia celular em modelo de insuficiência renal crônica experimental demonstrou o efeito terapêutico das células-tronco mesenquimais da medula óssea fetal humana, com melhora e normalização da função renal em ratos[14], enquanto Zerbine *et al* não obtiveram sucesso em retardar a progressão da doença renal utilizando células da medula óssea[15].

Em trabalho recentemente publicado demonstramos que o uso de células derivadas da medula óssea, quando implantadas no *parênquima* renal de ratos com insuficiência renal crônica, reduziu ou estabilizou significativamente a taxa de declínio do *clearance* de creatinina desses animais após 120 dias de tratamento[16].

Cavaglieri *et al*, também estudando o modelo experimental de insuficiência renal crônica, concluíram que após a inoculação de células-tronco mesenquimais *intracapsular* havia um efeito renoprotetor com migração e distribuição das células-tronco na região cortical e medular, bloqueando a evolução da doença renal[17].

Choi *et al* observaram que *injeções* de células-tronco mesenquimais na *veia caudal* de ratos com insuficiência renal crônica, embora não modificasse de forma significativa a função renal, melhoravam a esclerose glomerular e proteinúria nos animais tratados com células-tronco mesenquimais, após 120 dias. Esses resultados sugerem que as células-tronco mesenquimais podem ajudar a preservar a estrutura renal e a estabilizar a insuficiência renal crônica[18].

Em modelo semelhante, Alexandre *et al* infundiram células derivadas da medula óssea na veia caudal de ratos no 15º, 30º e 45º dias de pós-operatório e observaram que após 60 dias houve redução da proteinúria, de diferentes parâmetros histológicos, da infiltração do tecido renal por células imunes e da expressão tecidual de MCP-1 (proteína quimiotática de monócitos-1), P21 (proteína oncogênica – P21) e fator de crescimento do endotélio vascular. Esses dados corroboraram com os trabalhos anteriores e reforça a ideia de que o uso dessas células pode retardar a progressão da insuficiência renal crônica[19].

Semedo *et al* demonstraram melhora funcional e estrutural no tratamento com células-tronco mesenquimais e células de linhagem negativa (Lin–) terapeuticamente em modelo de nefrectomia 5/6. O tratamento com células-tronco mesenquimais no parênquima renal resultou em níveis maiores de taxa de filtração glomerular e diminuição da proteinúria[20] (Quadro 7.1).

BIOMATERIAL E ENGENHARIA TECIDUAL

A bioengenharia tecidual busca métodos alternativos para o tratamento e reconstrução de tecidos danificados e o termo biomaterial pode ser aplicado a qualquer substância ou combinação de substâncias, de origem natural ou sintética, que seja farmacologicamente inerte e tenha aplicações orgânicas, desenvolvidas para ser implantada onde houve perda de tecido biológico ou de sua função[21,22].

O princípio geral de reconstrução tecidual, na engenharia de tecidos, corresponde ao transplante de células sobre materiais biocompatíveis ou à indução do crescimento celular sobre esses materiais[23].

Os biomateriais, além de fornecerem uma superfície para a orientação do crescimento e organização das células implantadas, também podem promover sinais biológicos necessários para o estabelecimento e a retenção daquele tecido em formação. A infusão direta de suspensão celular no órgão lesado, sem contar com uma estrutura de apoio, é de difícil controle para a localização e organização das células transplantadas[23,24].

Roessger *et al* foram os pioneiros na utilização de biomaterial associado às células-tronco visando à criação de um interstício artificial de apoio ao desenvolvimento dos túbulos renais[25]. Recentemente, em nosso laboratório, avaliamos o efeito das células-tronco derivadas da medula óssea semeadas sobre um biomaterial (pericárdio bovino) na progressão da insuficiência renal crônica em ratos. Após um período de observação de 90 dias, observamos que os animais com insuficiência renal crônica que receberam o biomaterial semeado com células da medula óssea apresentaram menores aumentos da creati-

Quadro 7.1 – Resumo dos estudos com terapia celular em doença renal crônica.

Referência	Tipo celular	Efeitos terapêuticos	Via de administração	Efeitos histológicos
Cavaglieri *et al*[17]	CTM	↓ Albuminúria ↓ Creatinina	Subcapsular renal	↓ Glomerulosclerose
Alexandre *et al*[19]	Lin–	↓ Proteinúria	Veia caudal	↓ Glomerulosclerose ↓ Fibrose intersticial ↓ Infiltração
Choi *et al*[18]	CTM	↓ Proteinúria	Veia caudal	↓ Glomerulosclerose
Kirpatovskii *et al*[14]	CT fetal	↓ Creatinina ↓ *Clearance*	Intraparênquima renal	Não reportado
Semedo *et al*[20]	CTM	↓ Creatinina ↓ Ureia	Veia caudal	↓ Regeneração tubular
Zerbine *et al*[15]	CTM	↑ Creatinina	Veia cava	↑ Regeneração tubular
Caldas *et al*[16]	CTM e CMO	↓ Creatinina ↓ Proteinúria	Intraparênquima renal	↓ Glomerulosclerose
Caldas *et al*[26]	CTM e CMO	↓ Creatinina ↓ Proteinúria	Células-tronco + biomaterial no parênquima renal	↓ Glomerulosclerose

CTM = célula-tronco mesenquimal; CMO = célula mononuclear; CT = célula-tronco.

nina sérica, proteinúria e tiveram a velocidade de progressão da doença, medida pelo declínio do *clearance* de creatinina e pelo inverso da creatinina sérica significativamente retardada pelo tratamento[26]. Esse trabalho abre a possibilidade do uso dos biomateriais associado com a terapia celular para o tratamento de doenças crônicas, além de permitir especular o uso dessa combinação com a forma alternativa para a administração das células-tronco.

CONCLUSÕES

Apesar das variações quanto ao número de células infundidas, a via de administração e o período pós-insuficiência renal crônica em que as células foram injetadas, resultados sugerem que células derivadas da medula óssea podem retardar ou estabilizar a progressão da insuficiência renal crônica experimental[16-20].

Os mecanismos que direcionam as células-tronco para determinada linhagem ainda são desconhecidos e esse fato torna as aplicações clínicas e terapêuticas para tratamento da insuficiência renal crônica ainda incertas. As pesquisas com células-tronco abrem novas perspectivas e estimulam a continuidade dos estudos para o entendimento correto de como essas células agem e diferenciam, podendo, no futuro, tratar doenças hoje incuráveis por meio dessa terapia. O uso de biomateriais como suporte (*scaffolds*) para a terapia celular permite expandir as aplicações da medicina regenerativa.

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3. CONCLUSÕES

3. CONCLUSÕES

1- A progressão da IRC pode ser retardada pela injeção de células derivadas da medula óssea.

2- A) biomaterial semeado com células derivadas da medula óssea pode ser uma rota alternativa para a terapia celular. B) a associação de células–tronco mesenquimais com o BM retarda a progressão da IRC experimental. C) ainda a terapia celular parece ser mais eficaz quando administrada em estágios menos graves da IRC.

3- A etapa inicial de isolamento de células nucleadas sem uso do gradiente de densidade Ficoll-Paque mostrou-se mais eficiente para o cultivo de CTM do SCU quando comparado ao procedimento de centrifugação com gradiente de densidade Ficoll-Paque.

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5. ANEXOS

Anexo 1. Aprovação da Comissão de Ética na Experimentação Animal (CEEA).

Comissão de Ética em Experimentação Animal
CEEA
FAMERP

Faculdade de Medicina de São José do Rio Preto
Comissão de Ética na Experimentação Animal CEEA
FAMERP Autarquia Estadual, Av. Brig. Faria Lima 5416 CEP 15090.000 Tel. 3201-5700 S.J.Rio Preto/ SP

COMISSÃO DE ÉTICA NA EXPERIMENTAÇÃO ANIMAL

O projeto de pesquisa intitulado "Efeitos de células mesenquimais da medula óssea cultivadas sobre matriz de colágeno na insuficiência renal crônica experimental" (Protocolo FAMERP n° 3310/2008) sob responsabilidade da Prof. Dr. Mário Abbud Filho, por cumprir com os princípios éticos exigidos em experimentação animal, foi aprovado pela CEEA-FAMERP.

Lembramos ao senhor pesquisador a necessidade de relatório completo ao final do estudo.

São José do Rio Preto, 25 de Julho de 2008.

Cristiane Damas Gil

Profa. Dra. Cristiane Damas Gil
Presidente CEEA - FAMERP

Anexo 2. Aprovação da Comissão de Ética na Experimentação Animal (CEEA).

Comissão de Ética na Experimentação Animal
CEEA
FAMERP

Faculdade de Medicina de São José do Rio Preto

Comissão de Ética na Experimentação Animal - CEEA

FAMERP Autarquia Estadual, Av. Brig. Faria Lima 5416 CEP 15090.000 Tel. 3201-5700 S.J.Rio Preto/ SP

CERTIFICADO

Certificamos que o projeto de pesquisa intitulado **"Efeito de células tronco derivada da medula óssea na insuficiência renal crônica experimental moderada" (Protocolo FAMERP n°3476/2009)**, sob responsabilidade da Profa. Dra. Maria Alice Sperto Ferreira Baptista, está de acordo com os princípios éticos estabelecidos na Lei nº 11.794/2008 e na Resolução nº 714/2002 e foi **aprovado** pela Comissão de Ética na Experimentação Animal da Faculdade de Medicina de São José do Rio Preto (CEEA-FAMERP).

Lembramos ao senhor pesquisador a necessidade de relatório completo ao final do estudo.

We certify that Project entitled "**Effect of the stem cells in moderate chronic renal failure experimental" (Protocol n° 3476/2009**), agrees with the Ethical Principles for Animal Research established by Brazilian law nº11.794/2008 and Resolution nº 714/2002 and was approved by the Institutional Committee for Ethics in Animal Research of Faculty of Medicine from São José do Rio Preto (CEEA – FAMERP) on June 04, 2009.

São José do Rio Preto, 22 de julho de 2009.

Cristiane Damas Gil

Profa. Dra. Cristiane Damas Gil
Presidente Da CEEA – FAMERP

Anexo 3. Aprovação da Comissão de Ética na Experimentação Animal (CEEA).

Comissão de Ética na Experimentação Animal
CEEA
FAMERP

Faculdade de Medicina de São José do Rio Preto

Comissão de Ética na Experimentação Animal - CEEA

FAMERP Autarquia Estadual, Av. Brig. Faria Lima 5416 CEP 15090.000 Tel. 3201-5700 S.J.Rio Preto/ SP

CERTIFICADO

Certificamos que o projeto de pesquisa intitulado **"Efeito de células tronco derivada da medula óssea na insuficiência renal crônica experimental" (Protocolo FAMERP n°3475/2009)**, sob responsabilidade do Prof. Dr. Mario Abbud Filho, está de acordo com os princípios éticos estabelecidos na Lei nº 11.794/2008 e na Resolução nº 714/2002 e foi **aprovado** pela Comissão de Ética na Experimentação Animal da Faculdade de Medicina de São José do Rio Preto (CEEA-FAMERP).

Lembramos ao senhor pesquisador a necessidade de relatório completo ao final do estudo.

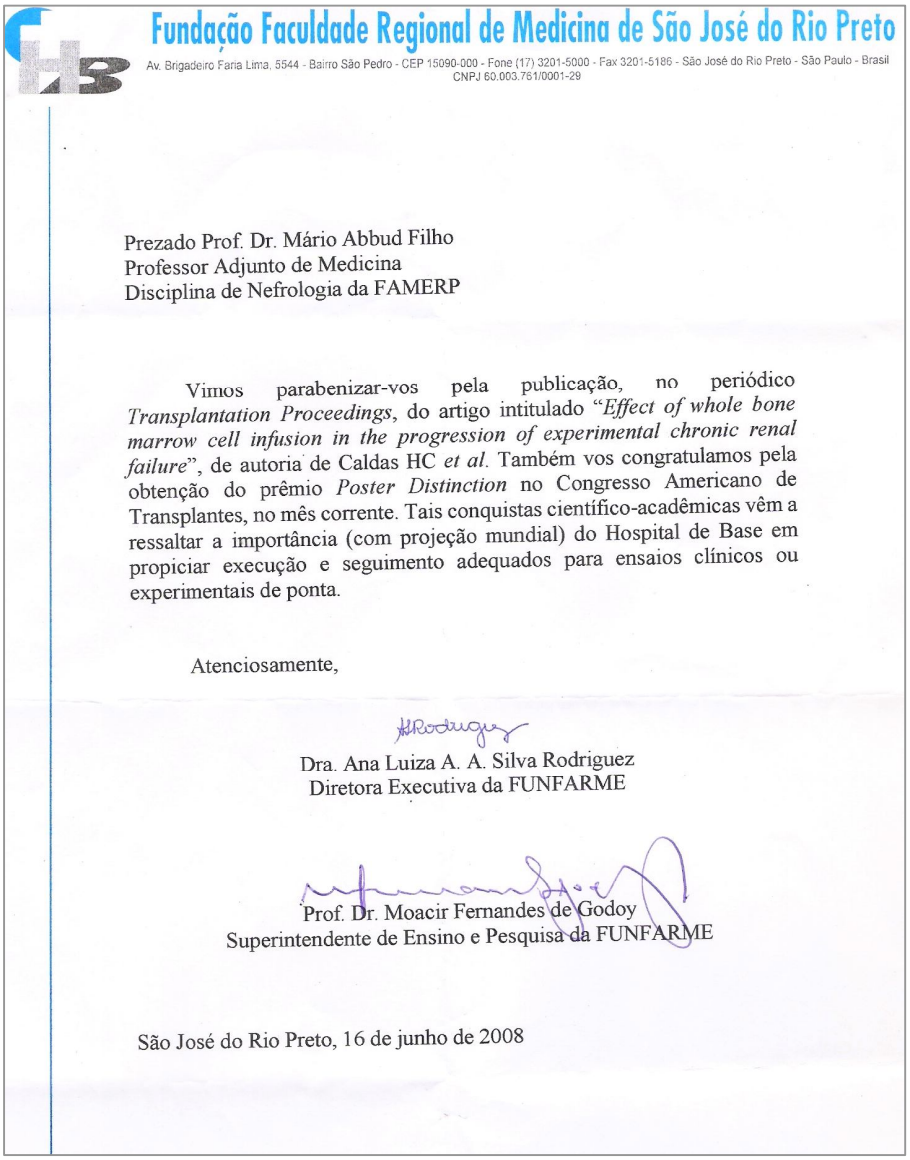
We certify that Project entitled "**Effect of the stem cells in chronic renal failure experimental" (Protocol n° 3475/2009**), agrees with the Ethical Principles for Animal Research established by Brazilian law nº11.794/2008 and Resolution nº 714/2002 and was approved by the Institutional Committee for Ethics in Animal Research of Faculty of Medicine from São José do Rio Preto (CEEA – FAMERP) on June 04, 2009.

São José do Rio Preto, 06 de agosto de 2009.

Cristiane Damas Gil

Profa. Dra. Cristiane Damas Gil
Presidente Da CEEA – FAMERP

Anexo 4. Distinção em congressos.



Fundação Faculdade Regional de Medicina de São José do Rio Preto

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CNPJ 60.003.761/0001-29

Prezado Prof. Dr. Mário Abbud Filho
Professor Adjunto de Medicina
Disciplina de Nefrologia da FAMERP

Vimos parabenizar-vos pela publicação, no periódico *Transplantation Proceedings*, do artigo intitulado "*Effect of whole bone marrow cell infusion in the progression of experimental chronic renal failure*", de autoria de Caldas HC *et al.* Também vos congratulamos pela obtenção do prêmio *Poster Distinction* no Congresso Americano de Transplantes, no mês corrente. Tais conquistas científico-acadêmicas vêm a ressaltar a importância (com projeção mundial) do Hospital de Base em propiciar execução e seguimento adequados para ensaios clínicos ou experimentais de ponta.

Atenciosamente,

Dra. Ana Luiza A. A. Silva Rodriguez
Diretora Executiva da FUNFARME

Prof. Dr. Moacir Fernandes de Godoy
Superintendente de Ensino e Pesquisa da FUNFARME

São José do Rio Preto, 16 de junho de 2008