LJ: synthesized and characterized the peptide,

VM and CBV: obtained funding

BLB and OBB: critical revision of the manuscript for important intellectual content

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Figures captions:

Figure 1: TSP-HepI enhances FGF2-induced angiogenesis *in vivo* in a mouse model of Matrigel plug assay. **(A-D):** Representative photos of plugs excised on day 14 containing **(A)** PBS, **(B)** TSP-HepI alone (200µg/ml), **(C)** FGF-2 (350ng/ml) alone or **(D)** FGF-2 (350ng/ml) + TSP-HepI (200µg/ml). **(E)** Haemoglobin quantification. Values are means ± SEM of 10 determinations per group. ** $p<0.01$ and *** $p<0.001$ versus PBS; # $p<0.05$, ### $p<0.001$ versus TSP-HepI alone and £ $p<0.05$ versus FGF-2.

Figure 2: TSP-HepI stimulates ECFC chemotaxis. Migration assays were performed with chemotaxis chambers towards control media (control), FGF-2 (10ng/ml, positive control), TSP-HepI (20µg/ml) or S/TSP-HepI peptide (20µg/ml) in the lower chamber. ECFC ($7x10^4$) were seeded in the upper chamber and incubated for 6 hours at 37°C. Data are expressed as percentages of the control. Values are means ± SEM of six determinations. ** $p<0.01$ and *** $p<0.001$ versus control, £££ $p<0.001$ versus FGF-2 and ### $p<0.001$ versus TSP-HepI.

Figure 3: TSP-HepI-HSPG interaction mediates vascular tube formation by ECFC. ECFC treated with a cocktail of heparinases and chondroitinases (GAG-) or untreated (GAG+) were seeded ($1x10^5$ cells/cm^2) in Matrigel containing 10µg/ml TSP-HepI. ECFC ($1x10^5$ cells/cm^2) untreated (GAG+) were seeded in Matrigel mixed with TSP-1 (10µg/ml) or S/TSP-HepI peptide (10µg/ml). **(A)** Quantification of tubular structures in Matrigel. Data are expressed as a percentage of control. **(B)** Morphological aspect of Matrigel after 6h. Untreated (GAG+) and treated ECFC (GAG-) in presence and absence of TSP-HepI. **(C)** Flow cytometry of heparan sulphate surface labelling. Values are means ± SEM of three determinations. *** $p<0.001$ versus control, ### $p<0.001$ versus TSP-HepI.

Figure 4: TSP-HepI-pretreated ECFC showed enhanced adhesion to activated endothelium at a shear rate of $50s^{-1}$. Before flow perfusion, ECFC were incubated for 18h with control medium, TSP-HepI peptide (20µg/ml) or TSP-1 (10µg/ml). SDF-1 stimulation (100ng/ml) for 30 min was used as a positive control. **(A)** Kinetic of ECFC adhesion. **(B)** Total number of cells adherent to activated HUVEC. **(C)** Resistance to detachment in incremented shear stress conditions from 50 to 5000s-1 **(D-G)**: Fluorescents micrographs of endothelium (gray) after ECFC perfusion (white arrows heads) - **(D)** control, **(E)** SDF-1, **(F)** TSP-1 and **(G)** TSP-HepI peptide. The scale bar represents 30µm in the photomicrographs. Control (●), SDF-1 (■), TSP-1 (◇) and TSP-HepI (▲). Data are expressed as a percentage of control. Values are means ± SEM of four determinations. *** $p<0.001$ versus control; £££ p0.001 versus SDF-1, ### $p<0.001$ versus TSP-1.

Figure 5: A monoclonal antibody directed against the ectodomain of syndecan-4 (SDN4) inhibits TSP-HepI-pretreated ECFC adhesion to activated endothelium at $50s^{-1}$. Before flow perfusion, ECFC were incubated with an anti-SDN4 antibody and exposed to TSP-HepI (20µg/ml) for 18h. **(A)**: Evaluation by flow cytometry for surface labelling of SDN4 surface labelling by in comparison with HUVEC. **(B)** Kinetics of ECFC adhesion in each experimental group: Control (●), TSP-HepI (▲), TSP-HepI+anti-SDN4 (✱) and TSP-

HepI+anti-IgG2a (▽). (**C**) Total number of cells adherent to activated HUVEC. Data are expressed as a percentage of control, 100% of CTRL. Values are means ± SEM of four determinations. *** $p<0.001$ versus CTRL; ### $p<0.001$ versus TSP-HepI

Figure 6: TSP-HepI pretreatment leads to alpha6 subunit over-expression on ECFC: ECFC were incubated for 18h with control medium (control) or TSP-HepI peptide (20µg/ml) and then analyzed by flow cytometry for surface expression of the alpha6 subunit. Data are expressed as a percentage of the mean control fluorescence intensity. Values are means ± SEM of four determinations. *** $p<0.001$ versus control; ### $p<0.001$ versus TSP-HepI.

Figure 7: TSP-HepI pretreatment enhances ECFC migration. Before the migration assay, ECFC were incubated for 18h with control medium (control), TSP-HepI peptide (20µg/ml), S/TSP-HepI peptide (20µg/ml) or VEGF (10ng/ml - positive control). Migration was assayed in a chemotaxis chamber towards to control medium (control). ECFC ($7x10^4$) were seeded in the upper chamber and incubated for 6 hours at 37°C. Data are expressed as a percentage of VEGF conditions. Values are means ± SEM of three determinations. *** $p<0.001$ statistically different from control; £££ $p<0.001$ versus VEGF , ### $p<0.001$ versus TSP-HepI.

Figure 1 in TIFF format

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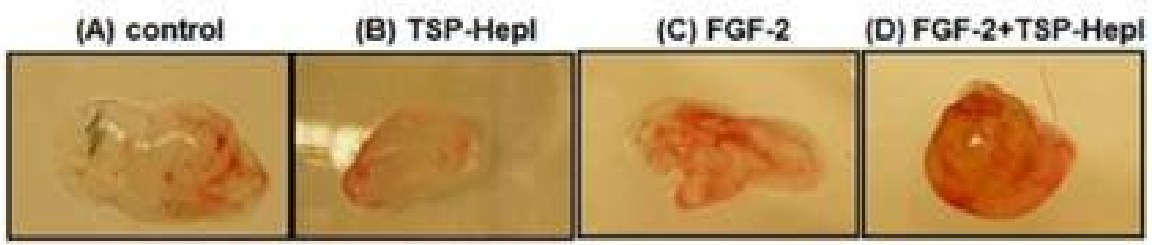

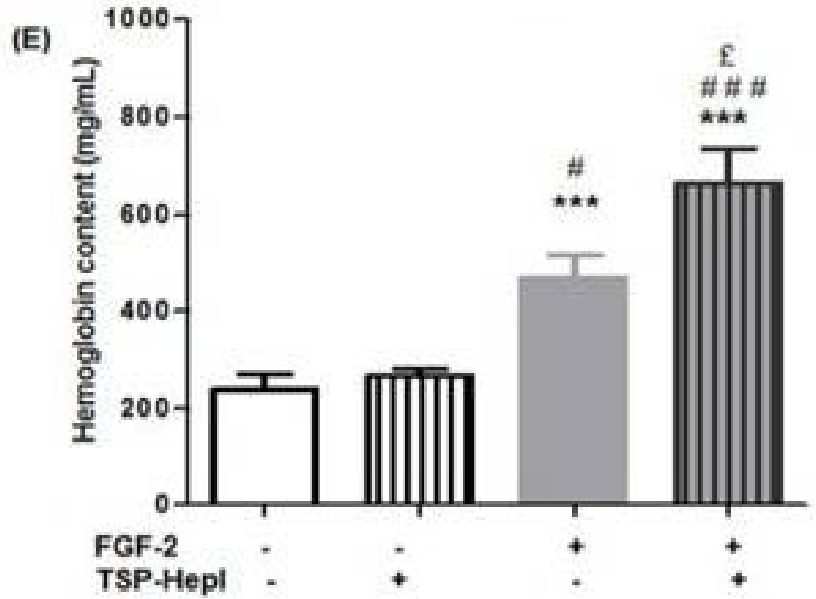

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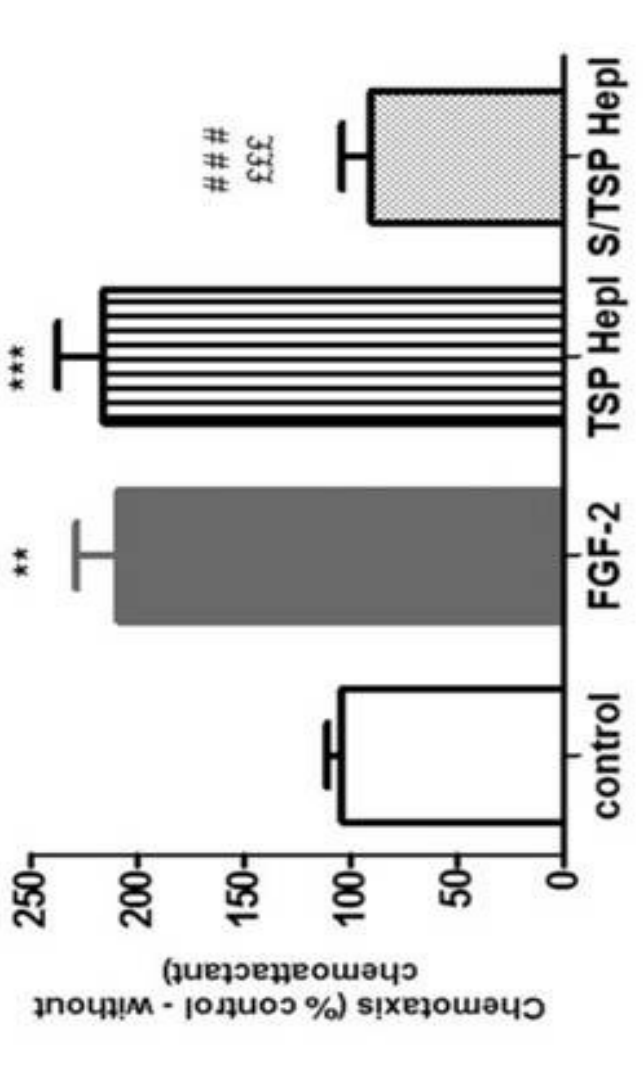

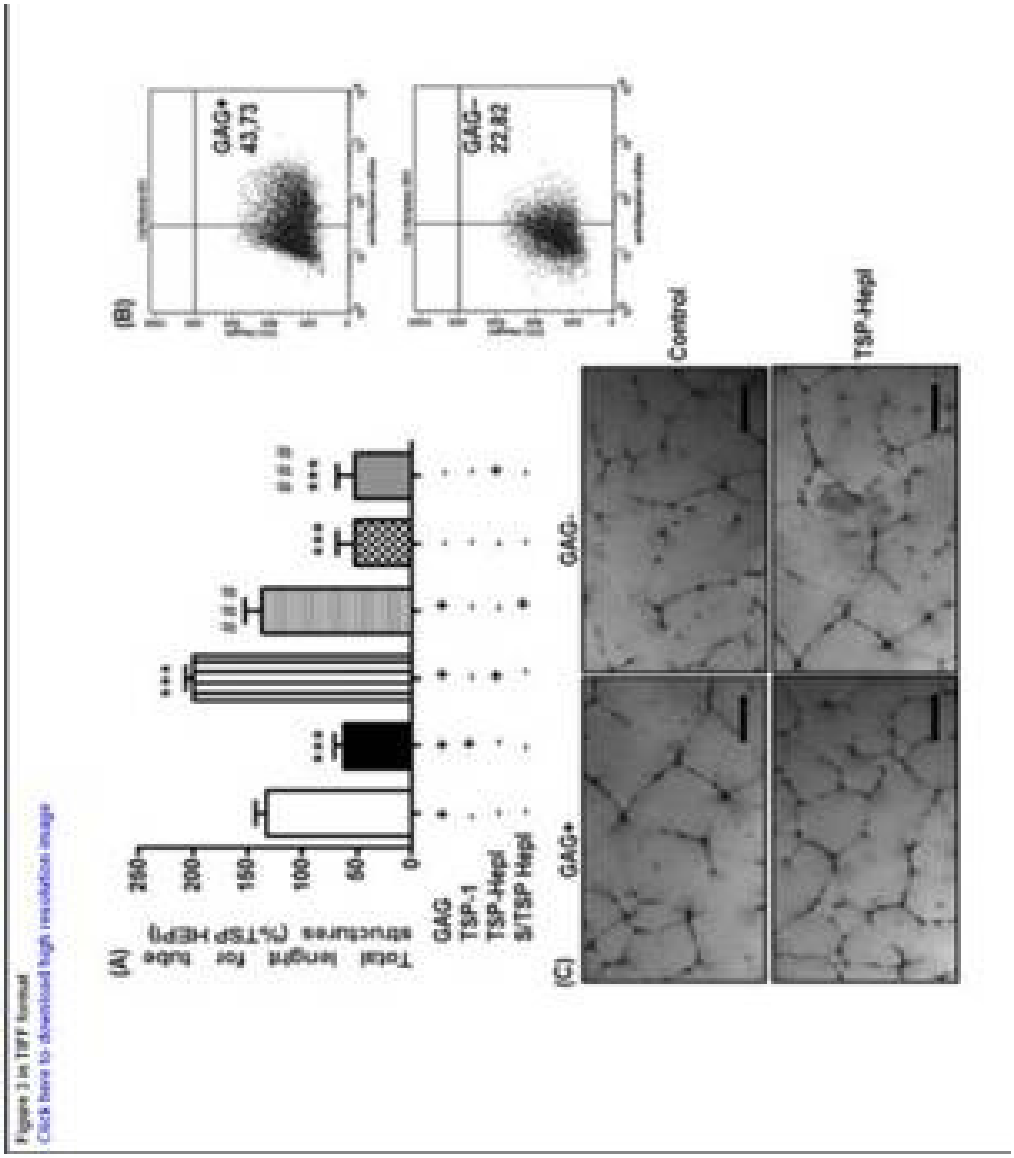
Figure 3 in TIFF format
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(A)
Total length for tube structures (%TSP HEPI)
250
200
150
100
50
0
GAG
TSP-1
TSP-Hepi
STSP Hepi
(B)
GAG+
43.73
GAG-
22.82
(C)
GAG+
GAG-
Control
TSP-Hepi

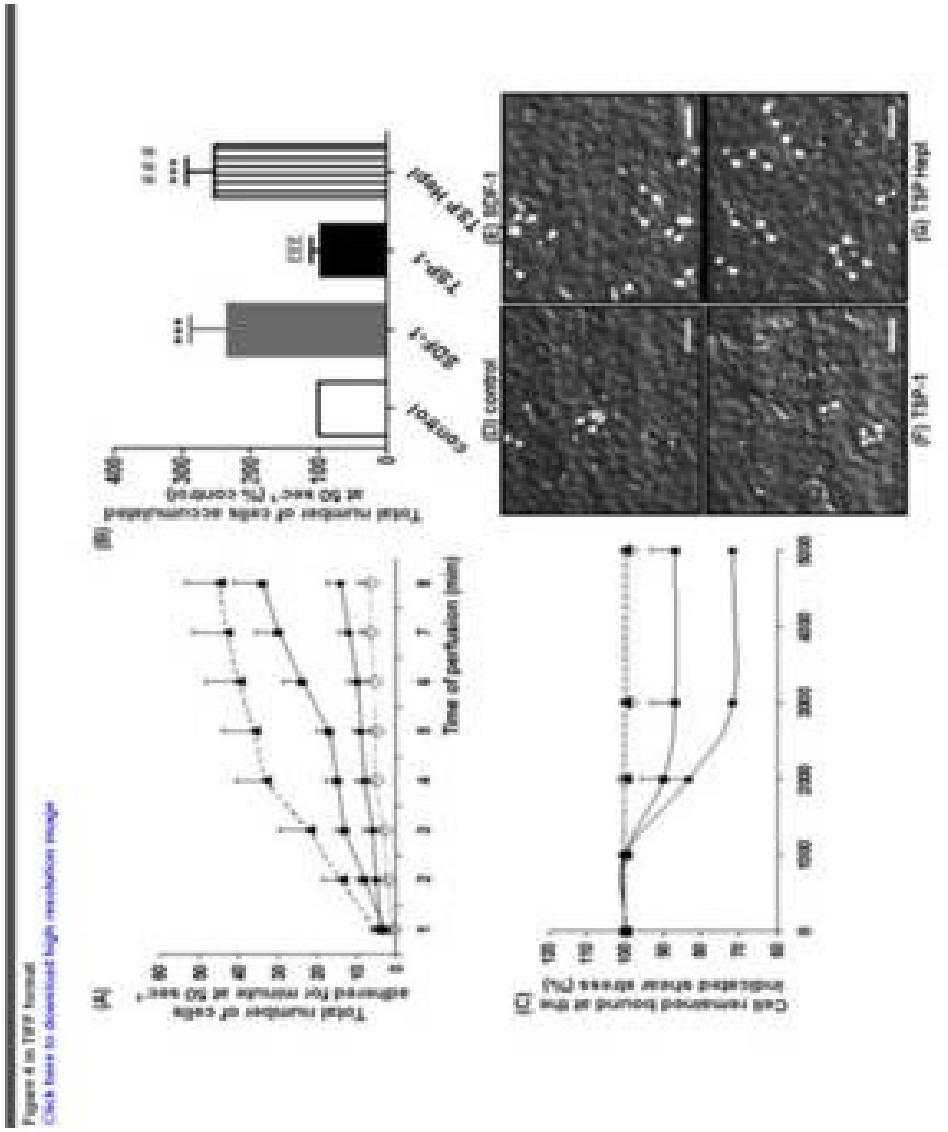
Figure 4 in TIFF format
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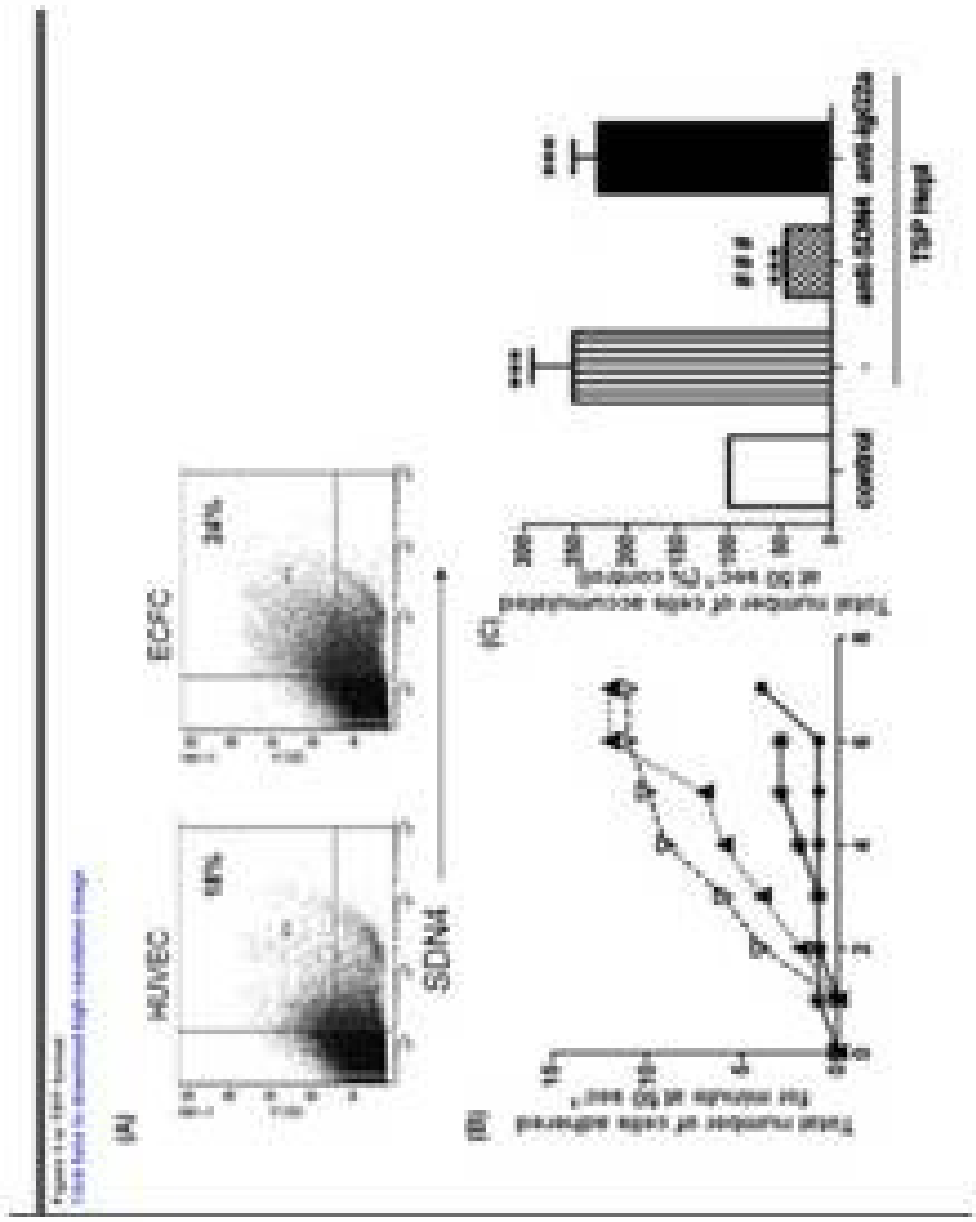
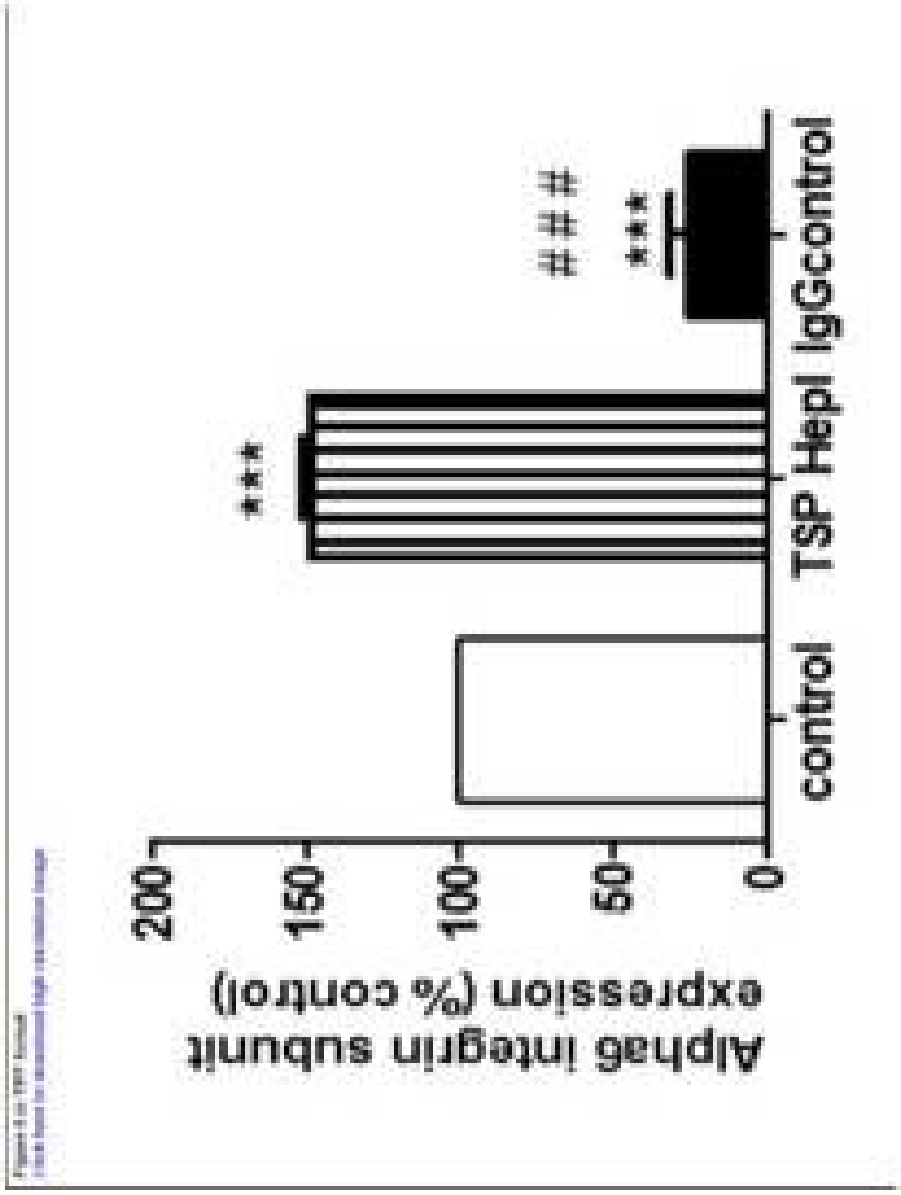
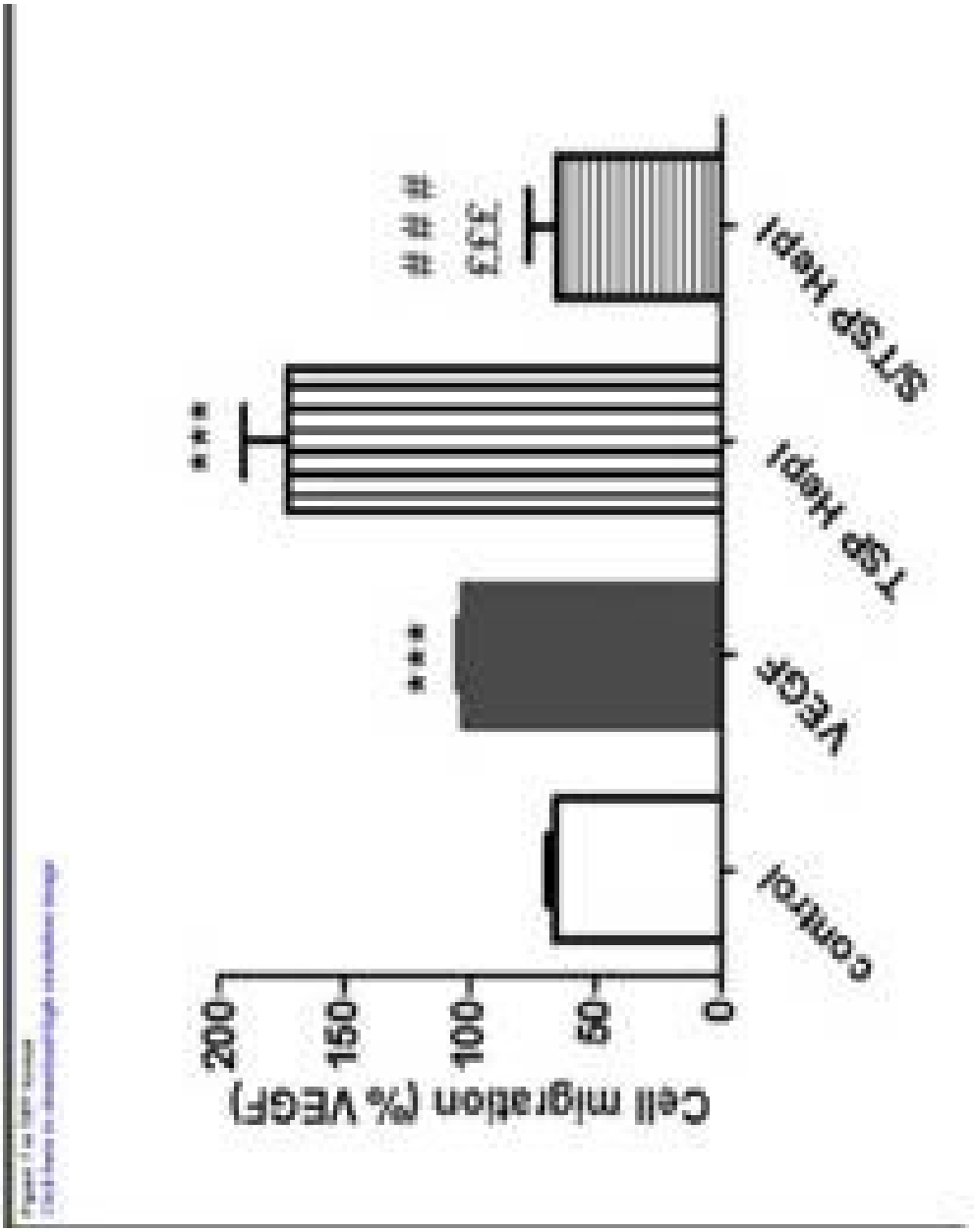
(A)
Total number of cells adhered for rotation at 50 sec-1
Time of perfusion (min)
(B)
Total number of cells accumulated at 50 sec-1 (% control)
control
SDF-1
TSP-1
TSP Hepl
(C)
Cell remained bound at the indicated shear stress (%)
(D) control
(E) SDF-1
(F) TSP-1
(G) TSP Hepl


(A)
HUVEC
ECFC
SDM4
(B)
(C)
control
anti-IgG2a
anti-ADM4
anti-IgG2a
TSP Heparin


Alpha6 integrin subunit
expression (% control)
200
150
100
50
0
control
TSP
Hepl
IgGcontrol
***
***
###


Cell migration (% VEGF)
200
150
100
50
0
Control
VEGF
T5P Hep1
ST5P Hep1
###
***
***

4 **DISCUSSÃO GERAL**

O avanço da área de tecnologias celulares tem impulsionado o desenvolvimento de novas terapias, com o propósito de restaurar e reparar as funções teciduais, através do uso de populações celulares auto-renováveis e com grande potencial de diferenciação.

No domínio da terapia de reconstrução vascular, as células progenitoras endoteliais (EPCs) são as escolhidas para o desenvolvimento de terapias pró-angiogênicas visando ao restabelecimento da irrigação sanguínea de regiões isquêmicas atingidas (35). As EPCs foram inicialmente descritas por Asahara et al, (1997) e Shi et al, (1998), como células isoladas a partir da fração mononuclear da medula óssea do sangue periférico capazes de se diferenciar em células endoteliais maduras (EC) e de iniciar a neovascularização em ensaios in vitro e in vivo (7,8). Desde então, as EPCs têm sido propostas como um interessante alvo terapêutico para uso em situações de isquemia crítica como na doença coronariana e na doença arterial periférica (DAP) (36–38). No entanto, alguns obstáculos têm sido encontrados para obter o transplante eficaz com EPCs autólogas. A rara quantidade de células encontradas na medula óssea (menos de 0,05%) e na circulação periférica (aproximadamente 0,01%), além da baixa incorporação nos tecidos lesionados após a administração intravenosa, gera algumas limitações para a aplicação clínica, sendo necessárias novas abordagens que melhorem a expansão ex vivo das EPCs, bem como a capacidade de essas células participarem da neovascularização pós-natal.

Para obter a quantidade suficiente de EPCs circulantes potencialmente funcionais, muitas estratégias foram estudadas (injeção de fármacos capazes de mobilizar as células para o sangue periférico, o pré-tratamento das células por fatores pró-angiogênicos, terapia gênica e outros) com o objetivo de aumentar a concentração das células antes da extração e/ou para melhorar a capacidade de adesão, melhorando consequentemente a eficácia do transplante. Diversos estudos experimentais e clínicos utilizam moléculas que regulam estritamente os mecanismos pro e antiangiogênicos, que podem ser administrados na corrente sanguínea ou diretamente no tecido isquêmico, como produto da terapia de neovascularização (35,151). No entanto, o uso de fatores solúveis tais como VEGF, FGF e SDF-1α para mobilizar as EPCs in vivo têm também apresentado resultados equivocados. A terapia gênica tem também produzido resultados inexpressivos (152).

Dadas essas limitações, o grupo de Boisson-Vidal (48) tentou uma nova abordagem para melhorar a funcionalidade das poucas células progenitoras disponíveis, através do pré-tratamento ex vivo com fatores angiogênicos solúveis com a finalidade de ativá-las a nível

proteico (priming). Nesse estudo, o grupo utilizou o SDF-1α, uma quimiocina conhecida, capaz de mobilizar e recrutar as células da medula óssea para a corrente sanguínea. O SDF-1 foi capaz de estimular/ativar o potencial angiogênico das EPCs isoladas do sangue de cordão umbilical humano, aumentando a neoangiogênese, a densidade capilar e a perfusão in vivo no modelo murino de isquemia crítica de membros inferiores. Porque as EPCs podem ser ativadas por outros fatores, tais como VEGF, angiopoietina e G-CSF, muitas variantes desta abordagem podem ser utilizadas.

A trombospondina-1 (TSP-1) é uma molécula complexa, agindo, às vezes, como proteína pró- ou antiangiogênica graças a sua capacidade de realizar múltiplas interações que conduzem à regulação do comportamento das células endoteliais, durante o processo de neovascularização (153). Os diferentes domínios da TSP-1 podem agir como proteínas bioativas, desempenhando o papel de fatores de crescimento ou citocinas (75). A TSP-1 foi inicialmente descrita como um inibidor endógeno da angiogênese (154,155), mas atualmente chama a atenção da comunidade científica pela capacidade de regular uma variedade de funções como a adesão, migração, proliferação e diferenciação das células endoteliais (156). Devido a esse papel multifuncional na angiogênese, a TSP-1 tem sido proposta como uma fonte estratégica para o desenvolvimento de terapias que necessitem a amplificação de respostas pró- ou antiangiogênicas, previamente iniciadas no microambiente tecidual.

Por ser uma glicoproteína extremamente complexa ao nível modular e funcional, os estudos da TSP-1 são frequentemente realizados, utilizando seus fragmentos ou domínios. A capacidade multifuncional é atribuída aos seus diferentes domínios que podem estar presentes tanto como fragmentos solúveis no fluido corporal quanto ligados a proteínas da MEC ou a receptores de superfície celular (153). Uma variedade de enzimas tais como trombina, plasmina, catepsinas e ADAMTS1 são capazes capaz de clivar a TSP-1 (75,109,157), liberando os domínios N-terminal e C-terminal. A ideia de que domínios/fragmentos possam ser liberados da TSP-1 por proteólise e, que suas atividades são diferentes da molécula nativa, se tornou um eixo interessante para a discussão dos efeitos fisiológicos dessa proteína (75).

Os primeiros fragmentos da TSP-1 identificados, regiões TSR e domínio pró-colágeno, foram descritos como inibidores da quimiotaxia e indutores de apoptose em células endoteliais microvasculares através da ligação com o receptor CD36 (113,158)). O domínio C-terminal desta glicoproteína inibe a adesão de células endoteliais e de células musculares lisas, interagindo com o receptor CD47 (159). Tem sido demonstrado que a interação de sequências específicas dentro do domínio C-terminal da TSP-1 com CD47/IAP (integrin-associated protein) reduz a ativação de óxido nítrico (NO), limitando a vasodilatação pelas

células musculares lisas. O papel biológico dessa interação é relevante para os processos isquêmicos, visto que o NO é um dos maiores reguladores do sistema cardiovascular, induzindo o relaxamento das células musculares lisas, aumentando assim o diâmetro vascular e o fluxo sanguíneo (160). De fato, Isenberg e colegas (2008) demonstraram que camundongos modificados geneticamente para não expressarem TSP-1 e CD47 apresentaram completa restauração da perfusão vascular após serem submetidos à oclusão da artéria femoral (160). A caracterização de receptores para os diferentes fragmentos da TSP-1 faz parte do desenvolvimento de novas estratégias terapêuticas como o desenvolvimento de peptídeos miméticos para inibir a angiogênese (156,160).

A porção N-terminal (NH_2) da TSP-1 é atualmente conhecida como responsável pelos efeitos pró-angiogênicos da molécula, porém a importância biológica no contexto da neovascularização ainda resta ser compreendida. Nesse cenário, o foco do nosso trabalho foi estudar o papel do domínio NH_2 da TSP-1 na modulação das células progenitoras endoteliais, como uma molécula potencial para aplicação terapêutica. Em nosso estudo inicial desenvolvemos um protocolo de isolamento de EPCs a partir do sangue periférico de indivíduos saudáveis, visando otimizar a expansão e o aperfeiçoamento funcional de células autólogas para uma futura aplicação terapêutica, sem risco de provocar rejeição.

Para estabelecimento das condições iniciais de obtenção de colônias de EPCs, decidimos comparar o desenvolvimento das culturas na presença de meio condicionado de HUVECs confluentes, em cultura primária, cultivadas rotineiramente em nosso laboratório, uma vez que o aparecimento de colônias se mostrou um evento raro, quando as células mononucleares obtidas pelo fracionamento em Histopaque eram plaqueadas unicamente no meio-padrão de base para progenitores (EGM-2 com suplementos comerciais). O meio desenvolvido para avaliar a otimização foi denominado EGM-2/ENR, uma mistura na proporção de 1:1 do meio condicionado de HUVECs com o meio clássico EGM-2. Nossos resultados mostraram que o meio EGM-2/ENR contém fatores de crescimento necessários para estimular o surgimento de colônias endoteliais dentro de 20 dias, enquanto com o uso do meio EGM-2 sozinho não observamos a presença destas colônias.

Demonstramos ainda que essas células expressam CD34/CD31/CD146/CD177/KDR, não expressam CD133 e CD45 e são capazes de formar redes vasculares in vitro em Matrigel. Mais ainda, nossos resultados sugerem que as ECFCs isoladas do sangue periférico possuem um comportamento menos adesivo, diferente das células maduras (HUVECs). Essa hipótese é respaldada pela quantidade de fibronectina (FN) em níveis cerca de 3 vezes menores que os encontrados em endotélio de fenótipo maduro (HUVECs), tanto na matriz extracelular (MEC)

como no sobrenadante das ECFCs. Este fato parece também se refletir na organização dos filamentos de actina das células progenitoras, que exibem um grande número de estruturas como lamelipódios e filopódios em abundância, sugestivos de um comportamento migratório acentuado. Esse resultado surpreendente, demonstrado pela primeira vez, sugere uma nova ferramenta para caracterizar as ECFCs isoladas e expandidas ex vivo, visto que não há um conceito na literatura quanto à diferença desses progenitores endoteliais em relação às células maduras. Nossos achados são consistentes com outros, relatando a propriedade migratória e proliferativa de células circulantes: Hynes & Yamada (1982) relataram que diversas células proliferativas/migratórias de caráter tumoral expressam menos FN (161); Schmidt e colaboradores (2007) propuseram que angioblastos e ECFCs possuem uma capacidade de migrar muito maior que células endoteliais mais maduras (22).

Após ter estabelecido um protocolo eficaz de isolamento de ECFCs do sangue periférico e ter caracterizado essas células quanto à morfologia, o fenótipo e a funcionalidade, nós nos interessamos em determinar se a TSP-1 e os peptídeos pró-angiogênicos derivados da região NH_2, poderiam modular as atividades angiogênicas das ECFCs. O domínio NH_2-terminal (ou HBD, de heparin-binding domain) da TSP-1 foi descrito inicialmente como um fragmento de ligação à heparina, capaz de modular a adesão e a migração das células endoteliais. No entanto, na última década, a estrutura e funções do HBD têm sido estudadas por alguns grupos, que demonstraram o papel desse domínio na indução da angiogênese.

Lawler, em 1981, identificou um fragmento de 25 kDa retido na coluna de heparina-sepharose que se liga com alta afinidade à heparina(81). As sequências de aminoácidos deste fragmento foram isoladas e caracterizadas pelo grupo de Frazier em (162). Os primeiros indícios do papel biológico do HBD, relevantes para a angiogênese, foram demonstrados por Taraboletti et al (1990) (89). Nesse trabalho o grupo mostrou que um anticorpo monoclonal solúvel, dirigido contra o HBD (A2. 5) reduziu a adesão e a quimiotaxia de células endoteliais da aorta bovina (BAEC). Além disso, o acréscimo de heparina ou fucoidan causou o mesmo efeito inibitório, confirmando a interação deste domínio à heparina e seus derivados. Outros demonstraram o papel do HBD na quimiotaxia e adesão (94,163). O HBD foi capaz de desestabilizar adesões focais em células firmemente aderidas, resultando em uma transição para um estado intermediário de adesão. Em 1993, o grupo de Murphy-Ullrich rebuscou ainda mais o estudo do domínio NH_2-terminal da TSP-1, identificando um peptídeo de 19 aminoácidos, denominado hepI, capaz de interferir nas adesões focais de células endoteliais (163). Outros demonstraram que o peptídeo hepI induziu a migração de células endoteliais e fibroblastos (88,164). O isolamento de sequências específicas permitiu confirmar que os

fragmentos ligantes de heparina suportavam a adesão de células tumorais através da ligação aos glicosaminoglicanos (GAG) (165). De fato, a ligação da TSP-1 intacta aos proteoglicanos é descrita ser mediada pela interação de sequências específicas do HBD. A interação com sindecan-1, -3, e -4, dermatan sulfato e perlecan tem sido relatada (166). O HBD ainda induz proliferação e migração de neutrófilos, monócitos, fibroblastos e células de músculo liso (88).

Durante o desenvolvimento dos estudos aqui apresentados (Capítulos 1, 2 e 3), demonstramos o efeito positivo de peptídeos derivados do HBD da TSP-1 nas etapas-chave do processo angiogênico, em por ECFCs humanas isoladas do sangue periférico e do sangue de cordão umbilical. Os peptídeos sintéticos, TSP-HepI (aa 17-35) e TSP-HepII (aa 78-94), representativos dos domínios ligantes de heparina da TSP-1: (i) atraem fortemente as ECFCs em níveis comparáveis aos obtidos com o FGF-2, no ensaio de quimiotaxia; (ii) induzem a adesão das ECFCs em condições estáticas quando na forma imobilizada nos suportes de adesão; esses efeitos são equivalentes àqueles observados com a TSP-1 íntegra; (iii) estimulam fortemente a diferenciação das ECFCs em redes vasculares in vitro quando são misturados ao Matrigel, sendo que o efeito oposto é observado com a TSP-1; e por fim (iv) não afetam a proliferação celular, sendo que a TSP-1 inibe a proliferação das mesmas células.

Estes resultados corroboram os trabalhos precedentes do nosso grupo que demonstraram o efeito positivo na adesão em condições estáticas e na tubulogênese em Matrigel de células endoteliais maduras isoladas da veia umbilical humana (HUVEC) e atribuído ao fragmento de 18 kDa do HBD da TSP-1 (TSP18, uma sequência que compreende os aminoácidos 1-174), quanto aos peptídeos derivados desta região da proteína (TSP HepI/A1 ou TSP HepII/A2), que possuem motivos estruturais de caráter básico de ligação de alta afinidade a glicosaminoglicanos.

Somente três grupos de pesquisa observaram e descreveram os efeitos pro-angiogênicos do domínio HBD in vivo, no modelo da membrana corioalantóide de frango (CAM) e de implante de Matrigel (116,118,167). Para avaliar o papel in vivo do peptídeo mais ativo durante os experimentos TSP-HepI, nosso grupo escolheu o ensaio de implante de Matrigel, descrito como o modelo capaz de mimetizar as condições presentes na maioria das doenças vasculares (168). Observamos que o peptídeo TSP-HepI induziu angiogênese quando ele estava associado ao fator de crescimento angiogênico FGF-2, sugerindo a existência de um efeito sinérgico entre este fator de crescimento e o domínio NH_2-terminal da TSP-1. No entanto, TSP-HepI, sozinho, não teve nenhum efeito sobre a vascularização da matriz extracelular. Estes dados estão em contradição com os resultados de outros grupos que demonstraram o efeito pró-angiogênico do domínio NH2 sozinho in vivo:

•Taraboletti et al (1997) relatou que a TSP-1 se liga ao FGF-2, através da seu domínio C-terminal (fragmento de 140kDa). Esta interação conduz à inibição da proliferação e da quimiotaxia induzida pelo FGF-2 em células endoteliais maduras (78). Outro trabalho do mesmo autor demonstrou que o efeito inibitório da TSP-1 in vivo no modelo de córnea de coelho – no qual implantes contendo fatores são implantados nas córneas - não parece ser associado à capacidade de a TSP-1 sequestrar o FGF-2 na matriz, visto que a quantidade deste fator foi liberado/difundido no meio sem alteração da quantidade (116). Em contrapartida, o fragmento de 25kDa presente no domínio HBD foi mais eficaz que a TSP-1 em promover a angiogênese tanto quando administrado sozinho no implante ou em combinação com o FGF-2.

•Staniszewska e colaboradores (2007) demonstraram as propriedades pró-angiogênicas in vivo no modelo de implante de Matrigel, do peptídeo recombinante NoC1, representando a sequência 1-356 do domínio NH2 da TSP-1 (118).

As propriedades angiogênicas observadas nestes estudos poderiam ser relacionadas à presença de diversos motivos no domínio HBD que interagem com diferentes receptores, como as integrinas α3β1, α4β1 e α6β. Estas interações estão implicadas na modulação da adesão e da quimiotaxia das células endoteliais micro e macrovasculares pela TSP-1 (85–87). Nosso peptídeo TSP-HepI contém a sequência 17-35 que não se liga às integrinas, mas apresentam forte afinidade pelo HSPG. O sítio de interação do TSP-HepI com as células não está totalmente elucidado, mas envolve o sindecan-4, de acordo com nossos estudos.

Estudos experimentais e clínicos têm demonstrado que a administração combinada de moléculas pró-angiogênicas parece ser a estratégia mais recomendada para a terapia vascular, visto que a estimulação com um único fator angiogênico pode ser insuficiente para a indução de vasos sanguíneos funcionais (169). A utilização do VEGF-2 sozinho através da terapia gênica ou via injeção intramiocárdica demonstrou modestos benefícios em pacientes com doença cardiovascular (170). Em concordância, nenhum efeito significativo foi observado na densidade vascular em diversos modelos animais e em protocolos clínicos, após a administração coronariana do FGF-2 (171). Em contrapartida, a injeção intramuscular de VEGF-2 e FGF-2 na pata de coelhos submetidos à excisão da artéria femoral aumentou a quantidade de vasos recém-formados e da densidade vascular, efeito que foi superior ao observado com a injeção das citocinas sozinhas (172,173). Este efeito sinérgico entre duas citocinas já foi demonstrado por outros grupos (169). Estes resultados estão em concordância com nossos achados, que sugerem que uma sinergia entre o fator pro-angiogênico FGF-2 e nosso peptídeo TSP-HepI leva a uma melhor vascularização dos implantes experimentais.

A TSP-1 é encontrada em concentrações relativamente baixas no plasma em condições basais (180- 250ng/ml), podendo ter a sua expressão aumentada em sítios de lesão vascular (174). A principal fonte de TSP-1 no reparo vascular e isquemia são os grânulos-α de plaquetas ativadas, porém as células (93,175,176) e macrófagos (177) já foram relatados secretarem TSP-1 in vitro. Altos níveis da expressão do RNAm da TSP-1 e da proteína são encontrados em células endoteliais semiconfluentes, enquanto a expressão é diminuída na confluência e na angiogênese (93).

A superexpressão de TSP-1 in vivo foi encontrada em seções histológicas de tecidos isquêmicos de humanos e murinos, em estudos de isquemia de membros inferiores. Esse resultado foi associado com a diminuição da densidade de capilares nos músculos afetados (98). Camundongos transgênicos superexpressando TSP-1 apresentam pouca neovascularização após a lesão tecidual (178). O papel da TSP-1 na isquemia critica de membros inferiores camundongos knockout para TSP-1 apresentaram aumentada regeneração tecidual e diminuição da necrose (179).

Em nossos achados (Capítulo 2), observamos elevados níveis de TSP-1 no plasma de pacientes com doença arterial periférica e que provavelmente a TSP-1 estaria associada com a perda do potencial angiogênico das EPC. Mais ainda, apesar das propriedades antiangiogênicas da TSP-1, nosso estudo mostrou que ela foi fortemente aumentada em vasos recém-formados de regiões que receberam a injeção local de BM-MNCs. Neste trabalho, nós confirmamos o duplo papel da TSP-1 no processo angiogênico. Como relatado anteriormente, o domínio HBD, através do nosso peptídeo TSP-HepI (denominado A1 neste artigo), foi responsável pelas propriedades pró-angiogênicas (proliferação e tubulogênese), enquanto a proteína íntegra foi inibidora do efeito. Estes resultados nos permitiram compreender melhor o mecanismo de ação da TSP-1 na angiogênese e a importância fisiológica do domínio HBD N-terminal. Além disso, com nossos indícios comprovando que a TSP-1 é expressa em microambientes isquêmicos, é razoável imaginar que os fragmentos clivados dessa proteína poderiam modular a angiogênese local, contribuindo dessa forma para a resolução do quadro isquêmico tecidual.

Como relatado anteriormente, a TSP-1 é rapidamente clivada por proteases locais (trombina, plasmina, catepsinas e metaloprotease ADAMTs1), que são capazes de liberar fragmentos N-terminais de tamanhos diversos. Fisiologicamente, é concebível que tais fragmentos possam ficar em solução, em fluidos fisiológicos, ou se associar à matriz extracelular, realizando interações com proteínas locais (109,157,180,181). Esse domínio pode ser também clivado/liberado da matriz extracelular durante o seu remodelamento após a

injúria e durante o desenvolvimento, podendo atuar independentemente do restante da molécula (88). Desse modo, em situações como reparo de lesão e neovascularização em situações isquêmicas é plausível que a concentração local de HBD sofra um aumento significativo, suficiente para promover efeitos não exibidos pela TSP-1 íntegra. A sequência entre o HBD globular e a região de trimerização da TSP-1 foi denominada linker, e parece ser uma sequência flexível de aminoácidos, que permite o domínio HBD adotarem diferentes orientações: por exemplo, interações com proteoglicanos podem ser facilitadas com este domínio, através de uma parte do HBD, deixando o resto da molécula livre para se ligar a outros receptores (104). O domínio linker possui sítios específicos de clivagem que levam à liberação do HBD por diversas proteases relevantes no microambiente vascular (182), sugerindo que os fragmentos gerados dessa região podem diferir funcionalmente uns dos outros e também da proteína íntegra, como na ação dupla na angiogênese.

Mais recentemente, o grupo do Dr. Jack Lawler (104) estudou a estrutura cristalizada do domínio HBD, demonstrando que o resíduo R29 do peptídeo TSP-HepI (aa 17-35) se encontra exposto e disponível para interações. Nesse sentido, este peptídeo poderia efetivamente mimetizar a interação sem nenhuma mudança conformacional do HBD com receptores como o sindecan-4 (106,107) e calreticulina (163).

Nesse contexto, nossa hipótese de estudo é que os peptídeos derivados do domínio HBD sejam partes acessíveis no HBD nativo. Visando a aplicação do peptídeo em abordagens terapêuticas, dispomo-nos a avaliar o papel dos peptídeos na forma solúvel nas propriedades funcionais das ECFCs. Com esse objetivo, nós pré-incubamos as ECFCs isoladas do sangue de cordão umbilical com nosso peptídeo TSP-HepI, a fim de simular o efeito do pré-tratamento na mobilização das células-tronco adultas e sua interação com o endotélio vascular ativado em condições fisiológicas similares àquelas encontradas in vivo. Demonstramos que a estratégia do pré-tratamento (ou pré-condicionamento) aumentou a adesão das ECFCs isoladas do sangue de cordão umbilical ao endotélio ativo em condições dinâmicas, além da motilidade.

A atividade pró-angiogênica do domínio NH_2 é exercida pela ligação a diversos receptores na superfície celular, exercendo uma vasta gama de efeitos biológicos e dificultando as tentativas de determinar o preciso papel da TSP-1 na doença vascular (85–87,153,163). No presente estudo, identificamos o sindecan-4 (SDN4) como o receptor envolvido no efeito pró-angiogênicos. O uso de um anticorpo dirigido contra o SDN4 ou o estudo de uma versão modificada do peptídeo TSP-HepI (peptídeo S/TSP-HepI, que não possui o sítio de ligação a GAGs), antes do pré-tratamento, mostrou a redução das

propriedades pró-angiogênicas observadas. Além disso, a deleção de GAGs na superfície das ECFCs reduziu a tubulogênese. O SDN4 é fortemente expresso em tecidos isquêmicos e nos sítios de lesão vascular (145). É provável que estes efeitos sejam ligados principalmente às suas propriedades estabilizadoras na formação dos contatos focais. Além disso, recentemente, foi demonstrado que o SDN4 é essencial para a regeneração da neoíntima em um processo que envolve o recrutamento de células progenitoras vasculares (148). A potencial utilização destes HSPG e de seus ligantes para gerar novas estratégias de revascularização de sítios isquêmicos resta ainda um domínio largamente inexplorado.

Estes resultados corroboram fortemente os do nosso grupo que previamente demonstrou que a inibição da porção extracelular do sindecan-4 inibiu em cerca de 70% a ligação do fragmento TSP18 aos extratos celulares, indicando que o efeito pró-angiogênico do TSP18 foi provavelmente devido à interação com o sindecan-4 (106). Em outro trabalho, caracterizamos duas sequências dentro do domínio HBD da TSP-1 capazes de interagir com HUVECs (TSP HepI, aa 17-35 and TSP HepII, aa 78-94) e induzir a formação de tubos no ensaio de Matrigel. Ambos os fragmentos demonstrados possuir a maior atividade pró-angiogênica da TSP-1, contém afinidade por glicasaminoglicanos (GAG) e interagem com sindecan-4 (107).

Durante este trabalho de pesquisa fundamental, relatamos um novo papel dos peptídeos do domínio NH2 da TSP-1 na estimulação in vitro das etapas-chave da angiogênese, revelando uma ferramenta na aplicação farmacológica. No entanto, trabalhos que explorem o potencial pró-angiogênico de tais peptídeos para a terapia de revascularização ainda não estão bem estabelecidos. Trabalhos in vitro demonstraram que o domínio NH2 pode ser detectado no sobrenadante de agregados plaquetários assim como no meio condicionado de células endoteliais (180,183). Nesse contexto, seria razoável imaginar que a TSP-1, clivada por proteases secretadas por plaquetas e células endoteliais durante o processo de angiogênese, poderia liberar seus diversos fragmentos. Portanto, propomos que as células recrutadas durante o processo angiogênico, como as células progenitoras da medula óssea e os progenitores endoteliais, poderiam ser estimuladas neste microambiente, ligando-se a fragmentos solúveis do domínio NH2, estimulando assim o processo de neovascularização.

O uso de peptídeos angiogênicos sintéticos para a revascularização in vivo de processos isquêmicos tem sido relatado. Uma única injeção intramuscular do peptídeo Roy (sequência de 12 aa, presente no banco de dados das proteínas localizadas em células endoteliais) melhora a perfusão e alivia a isquemia da pata de camundongos submetidos à oclusão da artéria femoral (184). Além disso, tanto Roy quanto AdoPep1 (sequenciado pela

mesma técnica) aumentam o fluxo sanguíneo e estimulam o crescimento de capilares em modelo murino de diabetes (185).

As terapias celulares utilizando células-tronco da medula óssea, tais como células mesenquimatosas ou a fração mononuclear foram bem documentadas em diversas doenças, tais como infarto do miocárdio e isquemia (186,187). Para a otimização do processo angiogênico, é necessário obter uma janela terapêutica em função da dose e da periodicidade de administração das células (188). Devido à limitada capacidade que os grupos têm encontrado para estabelecer o número de células a serem injetadas ou, no caso das EPCs, obtemos poucas células ativas, a possibilidade da utilização de peptídeos e fatores de crescimento, representaria certas vantagens: (i) a possibilidade de regular a dose da proteína determinando uma janela terapêutica entre a eficácia e a toxicidade e (ii) a capacidade de definir claramente um perfil de toxicidade que permitirá a suspensão do tratamento quando necessário.

Como relatado nos artigos 4 e 5 alguns ensaios clínicos de fase 1 foram realizados na Universidades Federal do Rio de Janeiro, que confirmaram a simplicidade e a reprodutibilidade da técnica utilizada para injetar células mononucleares autólogas, extraídas da medula óssea, de pacientes com cirrose hepática e AVC. Com o conhecimento dessas técnicas e a disponibilidade destas linhas de pesquisa, seria interessante poder avaliar o efeito de nossos peptídeos sobre as células–tronco adultas. No presente estudo nós demonstramos que TSP-HepI estimulou a revascularização in vivo induzida pelo fator pro-angiogênico FGF-2, sugerindo a existência de um efeito sinérgico no processo angiogênico. Nesse contexto, a administração de TSP-HepI associado à outra molécula pro-angiogênica, tal como VEGF ou FGF-2, diretamente no sítio da lesão poderia estimular a revascularização local. Ou ainda a ativação das células mononucleares autólogas através do pré-tratamento ex vivo com TSP-HepI poderia ser uma estratégia interessante para melhorar a eficiência do transplante, visto que observamos esse efeito quando as ECFCs foram pré-condicionadas.

5 CONCLUSÕES E PERSPECTIVAS

Nosso estudo revelou, pela primeira vez, que o domínio N-terminal da TSP-1 estimulou o fenótipo angiogênico das ECFCs humanas derivadas da cultura do sangue periférico e do sangue de cordão umbilical. Mais especificamente nós identificamos que:

(1) os dois peptídeos miméticos TSP-HepI (aa 17-35) TSP-HepII (aa 78-94), estimularam fortemente a adesão estática de ambas as fontes de ECFCs, efeito equivalente ao observado com a TSP-1, quando utilizados como proteínas imobilizadas;

(2) quando imobilizados no Matrigel, os peptídeos induziram a diferenciação angiogênica *in vitro* de ambas as fontes de ECFCs, efeito que foi inibido quando utilizando a TSP-1;

(3) a proliferação de ECFCs derivadas do sangue de cordão umbilical foi inibida pela TSP-1, enquanto os peptídeos não demonstraram efeito, quando utilizados como suporte;

(4) os dois peptídeos foram capazes de induzir a quimiotaxia de ambas as fontes de ECFCs, em níveis comparáveis aos obtidos com o FGF-2;

(5) a exposição das ECFCs derivadas do sangue de cordão umbilical ao peptídeo TSP-HepI favoreceu a interação dessas células imaturas com o endotélio ativado (HUVECs) no ensaio de adesão em condições dinâmicas e aumentou a motilidade;

(6) apesar de a exposição das ECFCs derivadas do sangue de cordão umbilical ao peptídeo TSP-HepII ter sido capaz de estimular a migração celular, esse efeito foi considerando menos significativo em relação ao peptídeo TSP-HepI. Além disso, não observamos efeito na adesão ao endotélio ativado em condições dinâmicas utilizando TSP-HepII. Esse último dado nos permitiu concluir que TSP-HepI é mais relevante para as aplicações previstas neste estudo;

(7) o peptídeo TSP-HepI foi capaz de estimular a neovascularização induzida pelo FGF-2, no ensaio de Matrigel plug *in vivo*;

(8) por fim, esses dados (1-7) confirmam que o efeito promotor da angiogênese da TSP-1, presente no domínio N-terminal, também é reprodutível com células progenitoras.

Nossos resultados abrem novas perspectivas para o entendimento do significado clínico da TSP-1 em sítios de angiogênese. No entanto, existem inúmeras questões em nosso

estudo que merecem uma investigação mais profunda. Para completar o nosso trabalho, seria interessante estudar esses peptídeos em estratégias experimentais complementares, descritas a seguir:

1-Utilizar os peptídeos nos modelos murino de IMI e de infarto do miocárdio, a fim de validar as possíveis aplicações terapêuticas destes motivos estruturais derivados da TSP-1;

2-Utilizar células progenitoras silenciadas para o sindecan-4, para confirmar a importância da interação dos HPSG e as propriedades angiogênicas dos peptídeos TSP HepI e TSP HepII.

Este trabalho sugere ainda novos mecanismos para manter, expandir e diferenciar as células progenitoras *in vitro*, ideias que resultarão em novas ferramentas para manipular células-tronco e precursores *in vivo* e prover novas propostas para melhorar a terapia baseada em células.

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APÊNDICE A - Caracterização do estado de ativação endotelial em condições dinâmicas

Este modelo utiliza câmaras de perfusão e permite mimetizar as forças de cisalhamento sofridas *in vivo* pelas células progenitoras recrutadas quando estas aderem à parede dos vasos. As ECFCs marcadas com calceína e pré-tratadas ou não com os peptídeos TSP HepI ou TSP HepII foram perfundidas em tampão de aderência, sobre o endotélio (HUVECs) a uma força de cisalhamento de 50s-1 (0,1ml/min), durante 30 min sob a ação do fluxo. Nessas condições, as HUVECs passam a expressar ICAM-1, VCAM-1 e P-selectina (Figura A).

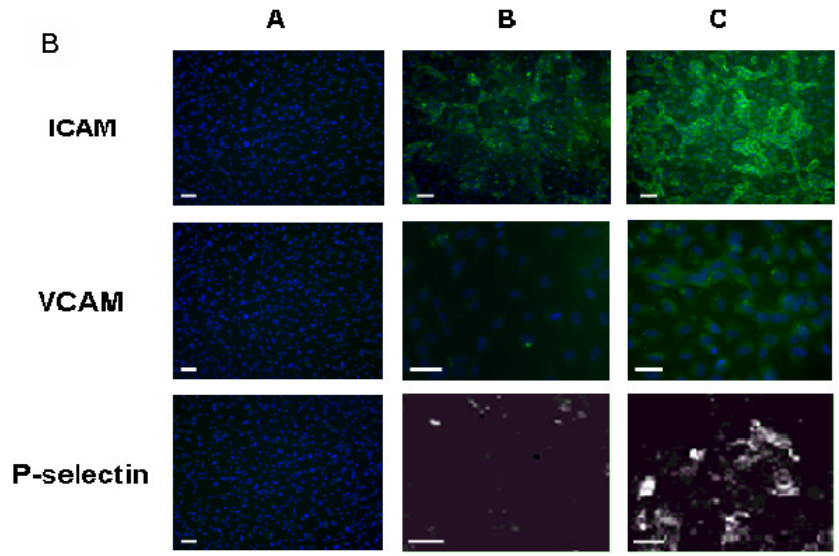

Figura A. 1: Expressão membranar de ICAM-1, VCAM-1 e de P-selectina por HUVECs (A) isotipo controle (B) HUVECs controle (C) HUVEC submetidas a um fluxo de 0,1ml/min correspondendo à $50sec^{-1}$, durante 30 min.

APENDICE B - Comparação entre os peptídeos TSP-HepI e TSP-HepII no efeito de pré-condicionamento de ECFCs, em condições dinâmicas.

As células aderentes foram visualizadas com a ajuda de um microscópio de contraste de fase e quantificadas em uma média de 40 campos (correspondente a uma superfície total de 1cm2). As células aderentes foram, em seguida, submetidas a um fluxo crescente (50sec-1 à 2500sec-1) para avaliar sua resistência ao descolamento. Todos os experimentos foram registrados em tempo real e reavaliados para as análises suplementares. Inicialmente, avaliamos o efeito do pré-tratamento por ambos os peptídeos, TSP-HepI e TSP-HepII, sobre a adesão das ECFCs ao endotélio. Os melhores resultados foram obtidos com o peptídeo TSP-HepI, que resultou em aumento da adesão de ECFCs a HUVECs em níveis comparáveis aos obtidos com o pré-tratamento com SDF-1, levando-nos a focar nosso estudo sobre este peptídeo (Figura B).

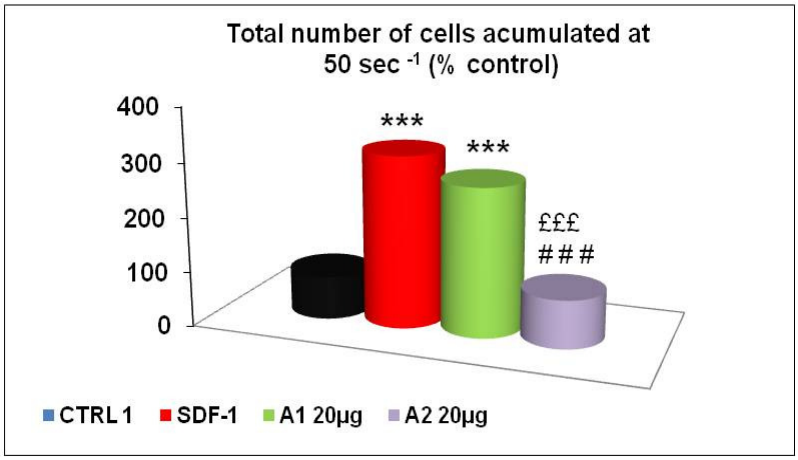

Figura B1: Efeito do pré-tratamento da ECFCs com os peptídeos TSP-HepI (A1) et TSP-HepII (A2) à 20 µg/ml sobre adesão ao endotélio ativado em condições dinâmicas. As ECFCs do sangue de cordão foram pré-tratadas durante 18h com TSP-HepI ou TSP-HepII, depois foram perfundidas sobre o endotélio (HUVECs) a um fluxo de 50 sec^{-1} durante 10 minutos. Os resultados foram comparados àqueles obtidos nas mesmas condições após o pré-tratamento com o SDF-1 (100 ng/ml). Os resultados são expressos como a porcentagem do número total de células aderidas ao endotélio em relação ao controle (sem tratamento, CTRL) após 10 minutos de perfusão. *** $p< 0.001$ em comparação à condição controle (sem tratamento, CTRL), ### $p< 0.001$ em comparação ao pré-tratamento com SDF-1 (SDF-1 controle positivo); £££ $p< 0.001$ em comparação ao TSP-HepI (A1).

Em seguida, testamos o peptídeo TSP-HepI em diferentes concentrações (10, 20 e 40 µg/ml). Os melhores resultados foram obtidos a uma concentração de 20 µg/ml (Figura B2). Portanto, esta concentração foi escolhida para os testes realizados em seguida.

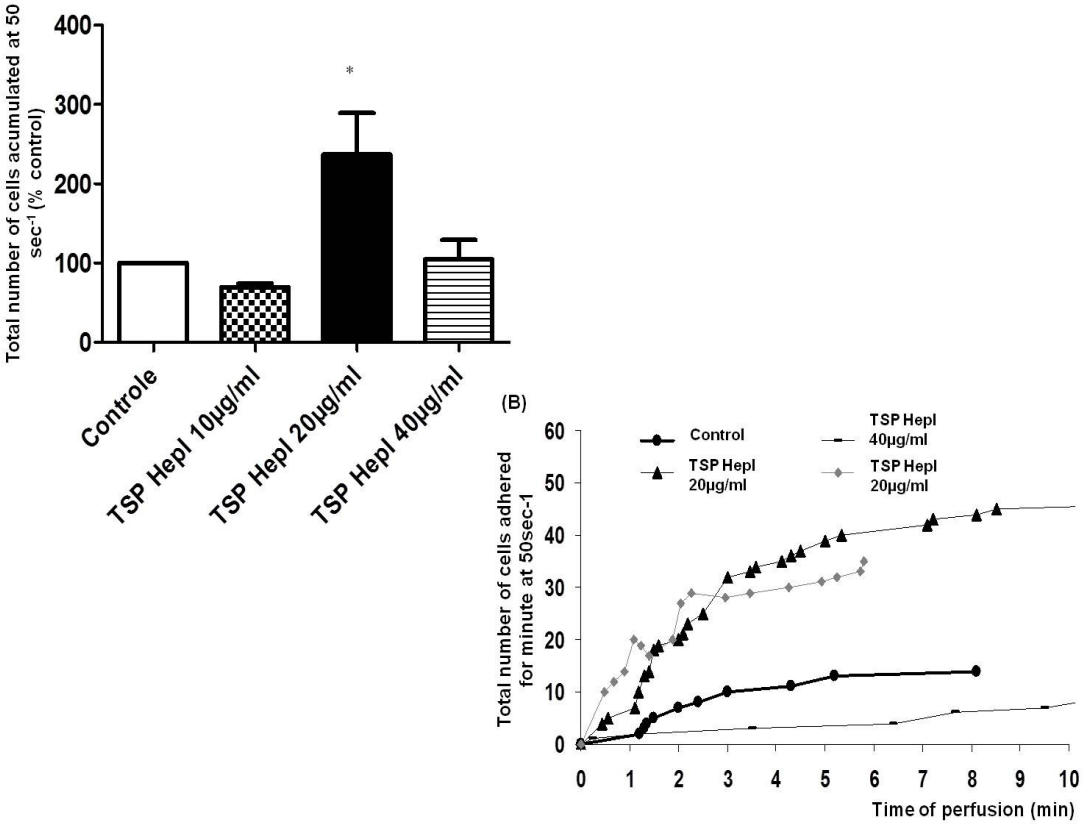

Figura B2: Efeito do pré-tratamento das ECFCs com diferentes concentrações do peptídeo TSP-HepI sobre adesão ao endotélio ativado, em condições dinâmicas. As ECFCs do sangue de cordão foram pré-tratadas, durante 18h, com TSP-HepI (10, 20 ou 40µg/ml), depois foram perfundidas sobre o endotélio (HUVECs) a um fluxo de 50 sec^{-1} durante 10 minutos. (A) Número total de células aderidas ao endotélio expressa em porcentagem em relação ao controle (ECFCs não tratadas) após 10 minutos de perfusão (B) Cinética de adesão das ECFCs quantificadas a cada minuto durante 10 minutos de perfusão. Os resultados são expressos como. * $p< 0.05$ em comparação à condição controle (sem tratamento, CTRL).

APÊNDICE C – Artigo 4: Bone marrow mononuclear cell therapy for patients with cirrhosis:a Phase 1 study

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CLINICAL STUDIES

Bone marrow mononuclear cell therapy for patients with cirrhosis: a Phase 1 study

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Abstract

Background: Bone marrow-derived cell therapy has been investigated in patients with severe liver disease. *Aims:* To assess the feasibility, safety and cell kinetics of autologous bone marrow-derived mononuclear cells (BMMCs) infusion in cirrhotic patients. *Methods:* BMMCs were isolated from autologous bone marrow and 10% of the cells were labelled with ^{99m}Tc-$SnCl_2$. Whole body scintigraphy (WBS) was performed 3 and 24 h after infusion via the hepatic artery. Liver function and image were followed during 1 year. *Results:* Eight patients received $2.0–15.0 \times 10^8$ cells. Three and 24-h WBS showed mean hepatic radiotracer retentions of 41 and 32% respectively. One case of dissection of the hepatic artery and one case of Tako-tsubo syndrome occurred as early complications. A patient developed a cutaneous immune-mediated disorder and another patient developed hepatocellular carcinoma (HCC) 12 months after infusion. A reduction in bilirubin was shown at 1 week while serum albumin increased above baseline up to 1 month after infusion ($P < 0.05$). *Conclusions:* BMMCs infusion is feasible and practical in a clinical setting. *In vivo* tracking of labelled cells demonstrated that the hepatic artery route successfully delivered BMMCs to the liver. The early improvement of laboratory indices of liver function should be interpreted with caution, because this study was not designed to evaluate efficacy. The median Model for End-Stage Liver Disease score had not deteriorated 1 year later. The occurrence of a graft-versus-host disease-like phenomenon highlights the importance of sustained vigilance even when giving autologous cells. Controlled studies are needed to determine whether BMMCs infusion affects HCC development in cirrhosis.

Liver transplantation is the only definitive therapy for end-stage liver disease. Although survival after transplantation can reach 90%, organ shortage has limited its impact, leading to transplant rates among patients listed and awaiting liver transplant of < 50% (1). In developing countries, the picture is alarming, and yearly mortality rates have recently reached 49% (2).

Experimental studies of stem cell therapy in rodent models of cirrhosis have shown encouraging results (3–6). Phase 1 clinical trials have been conducted in order to evaluate the safety and feasibility of autologous bone marrow stem cells (BMSCs) infusion in patients with cirrhosis (7–9). Despite suggestion that the procedures were safe and may improve liver function, mean follow-up periods were relatively short, ranging from 60 days to 52 weeks.

Furthermore, a study evaluating BMSCs therapy for cardiovascular diseases revealed that the improvement in myocardial function after a single injection may not be sustained, lasting just months (10). Short-term follow-up

after stem cell therapy may not detect whether a benefit is sustained or transient. In addition, the site of injection, amount and specific subpopulation of cells injected have varied widely among studies, making interpretation difficult. Another relevant aspect, the cell kinetics after infusion, has not been adequately evaluated previously as most of the clinical trials have not used imaging techniques to track bone marrow cells to establish their *in vivo* distribution. Given that the improvement in organ function is likely to be induced by paracrine mechanisms, rather than differentiation into liver parenchyma (11), the cellular distribution and homing are key to understanding and predicting the magnitude of therapeutic effect.

Overall, recent preclinical and clinical research suggest the potential benefit of stem cell therapy in liver disease, but major aspects guiding future randomized studies require clarification. In order to evaluate the feasibility, safety and cell kinetics of autologous bone marrow-derived mononuclear cells (BMMCs) infusion via the hepatic artery in patients with cirrhosis, we conducted a 1-year follow-up clinical study.

Patients and methods

This is a Phase 1 single-arm open interventional study designed to evaluate the safety of a single injection of autologous BMMC via the hepatic artery for treating patients with cirrhosis as well as to evaluate the pharmacokinetics of the injected cells. This study (ClinicalTrials.gov NCT00382278) was approved by the institutional and national review committees and informed consent in writing was obtained from each patient.

Because of invasive aspects of the protocol, such as bone marrow aspiration and hepatic artery catheterization, the inclusion of a blinded control group in this early study was considered ethically unjustifiable. Patients were eligible for inclusion if they were between 18 and 75 years of age, and presented with cirrhosis with moderate liver dysfunction, defined as a Child–Pugh score (12) of B7 to C10 and a Model for End-Stage Liver Disease (MELD) score (13) below 20. The main goal of this protocol was to evaluate its safety and feasibility in cirrhotic patients, regardless of disease aetiology; therefore, different causes of cirrhosis were accepted. Although this strategy reduces group homogeneity, it provides broader information to allow the subsequent development of efficacy trials for cirrhosis because of distinct aetiologies. In order to reduce the effect of spontaneous liver function recovery, a patient with cirrhosis of alcoholic aetiology could be included in this study only after a year of abstinence, when liver function is usually stable.

Exclusion criteria were hepatocellular carcinoma (HCC) or history of other cancer; hepatic artery, portal vein or hepatic vein thrombosis; high risk for bleeding with international normalized ratio (INR) >2.0 or platelet count $<40 \times 10^9$/L; serum creatinine >2.0 mg/dl; decompensated ascites; decompensated hepatic encephalopathy; and significant comorbidities.

Prospective patients were evaluated clinically and with three-phase computerized tomography (CT), Doppler ultrasonography (DUS), liver and renal function tests, full blood count, coagulation profile, α-fetoprotein levels and 12-lead electrocardiography.

Study design

Bone marrow aspiration and cell isolation

Included patients were admitted to hospital the night preceding the procedure, for an up-to-date clinical and biochemical assessment to be made. The day of cell infusion was termed 'day 1'. Under general anaesthesia, 100 ml of bone marrow was aspirated from the posterior iliac crest. BMMCs were isolated by density gradient centrifugation on Ficoll-Paque Plus (Amersham Biosciences, São Paulo, Brazil) and manipulated under GMP-certified aseptic conditions. Mononuclear cells were thoroughly washed with saline containing 5% human serum albumin. The cells were finally resuspended in 40 ml of saline with 5% human serum albumin and filtered through 100 μm nylon mesh to remove cell aggregates. A small fraction of the cell suspension was used for cell counting and viability tested by trypan blue exclusion. *Post hoc* characterization of leucocyte differentiation markers by flow cytometry and functional assays was performed. When possible, fibroblast colony-forming assay (CFU-F) was performed ($n = 4$) to determine the presence of putative progenitor cells of mesenchymal lineages, as described previously (14).

Antibodies and staining procedure for flow cytometry analysis

The following antibodies conjugated with either fluorescein isothiocyanate, phycoerythrin, peridinin chlorophyll protein, allophycocyanin or phycoerythrin-Cy7 were used: anti-CD45 (clone 2D1), anti-CD34 (clone 8Y12), anti-CD3 (clone SK7), anti-CD4 (clone SK3), anti-CD8 (clone SK1), anti-CD14 (clone MΦP9) and anti-CD64 (clone 10.1), anti-CD19 (clone 4G7), anti-HLA-DR (MHC-II, clone TU36), anti-CD56 (clone MY31), anti-CD73 (clone AD2) and anti-CD90 (clone 5E10) were obtained from BD Biosciences (San Jose, CA, USA) and anti-CD105 (clone 166707), from Immunostep (Salamanca, Spain). After staining, erythrocytes were lysed with the BD Bioscience cell lysis buffer solution, and CD45 antibody was used to assess the percentages of leucocytes. Data acquisitions were performed on a BD FACSCanto™ Flow Cytometer and the indicated percentages of immune cell subtypes have been calculated with the BD Paint-a-Gate PROTM software, which displays a combination of the morphological characteristics (side scatter and forward scatter) and specific CD marker expression, thus allowing multiparametric analysis and identification of the cell subtype percentages.

Cell labelling

Cell suspension was radiolabelled with ^{99m}Tc (radioactivity 111 MBq, physical half-life 6 h), as described previously (15, 16). Briefly, a saline solution of 500 μl of $SnCl_2$ was added to the cell suspension in 0.9% NaCl and the mixture was incubated at room temperature for 10 min. Then, 45 mCi ^{99m}Tc was added and the incubation continued for another 10 min. After centrifugation (500*g* for 5 min), the supernatant was removed and the cells were washed again in saline solution. The pellet was suspended in saline solution and then added back to the unlabelled cell suspension. Viability of the labelled cells was assessed by the trypan blue exclusion test. Labelling efficiency (%) was calculated by dividing the activity in the pellet by the sum of radioactivity in the pellet plus supernatant and found to be 83%. Labelled cell viability was >93% in all cases.

Delivery of autologous bone marrow mononuclear cells

About 4 h after bone marrow aspiration, celiac trunk catheterization was performed by femoral artery puncture under local anaesthesia and the cell suspension was delivered via the common hepatic artery. A sample of the final BMMC suspension was cultured to exclude fungal and bacterial contamination.

Follow-up

Whole body scintigraphy (WBS) was performed 3 h after infusion, to determine qualitative and quantitative distribution of the labelled cells. A second WBS was scheduled for the 24th hours after infusion, in order to estimate time-related reduction of labelled cells retained in the liver. Blood samples were collected daily until day 2, when a DUS was performed and patients were discharged from hospital.

Patients returned to the outpatient clinic on days 5, 14, 30, 45, 60, 90, 120, 180, 270 and 360 for clinical and laboratory evaluation, including full blood count, liver function tests, coagulation profile, α-fetoprotein assay, Child–Pugh and MELD scores. In addition, DUS was performed on days 14, 90, 180 and 360 to detect the appearance of nodules that could suggest HCC, or thrombus formation in portal vein or hepatic artery. A three-phase-CT scan was performed at the end of the follow-up, on day 360.

Endpoints

The following serious adverse events were considered primary safety endpoints: hepatic artery or portal vein thrombosis with permanent vascular damage secondary to catheter manipulation; acute renal failure; shock; acute liver failure demanding urgent transplantation; evidence of new HCC by α-fetoprotein elevation or by imaging methods; or death. Albumin and total bilirubin serum levels, INR, Child–Pugh and MELD scores were periodically evaluated as liver function parameters to exclude deterioration by the procedure itself.

Autologous BMMCs kinetics were assessed in terms of the percentile distribution to the liver and other organs both at the first hours and 24 h after infusion.

In addition to determining the safety profile of autologous cell therapy over the course of a year, we sought to investigate whether there were improvements in blood test results at the early timepoints indicated by preclinical investigations (3–6). Differences were analysed using repeated measures ANOVA.

Results

Patients

All the eight patients recruited in this study underwent bone marrow aspiration and injection of the autologous BMMCs but patient 7 did not complete the follow-up period. A liver nodule suggestive of HCC was detected in her DUS of day 14. Subsequent review of the screening three-phase-CT confirmed that the nodule was present before cell infusion. The patient underwent liver transplantation by month 2 but died shortly afterwards because of surgical complications. This patient's data were used only for in hospital follow-up and cell kinetics evaluation, while liver function and late follow-up were evaluated in the remaining seven patients.

Characteristics of the eight patients enrolled are presented in Table 1. The age ranged from 49 to 68 years, and chronic C hepatitis was the most frequent aetiology of cirrhosis. Although patients had cirrhosis because of several causes, the stages of liver dysfunction was quite similar as determined by the band of Child–Pugh and MELD scores classifying them as of intermediate severity. The mean MELD score was relatively low because creatinine elevation excluded patients from this study. A history of ascites or hepatic encephalopathy did not exclude patients from this study, as long as they were free from these complications at baseline.

Bone marrow aspirate and autologous bone marrow-derived mononuclear cell infusion

Patients received a total of 2.0–15.0 × 10^8 BMMCs. Cell viability was over 94% and CFU-F showed 35.00 ± 7.37 colonies × 10^6 BMMCs seeded. Cell phenotypes and final

Table 1. Individual patient characteristics at baseline

Patient	Sex	Age	Etiology	CP	MELD	Ascites	HE
1	F	67	NAFLD	8	11	Y	N
2	M	55	OH	8	13	N	N
3	F	51	HCV	8	12	Y	Y
4	M	55	HCV/OH	10	16	Y	N
5	M	68	HCV	9	14	Y	Y
6	M	51	HCV	7	12	N	N
7	F	49	HCV/OH	7	13	Y	Y
8	M	65	OH	8	11	N	N

CP, Child–Pugh; HCV, hepatitis C virus; HE, hepatic encephalopathy; MELD, model for end-stage liver disease; NAFLD, non-alcoholic fatty liver disease; OH, alcohol.

Table 2. Cells immunophenotype and composition of the injected cell suspension

Phenotype	CD markers	%	Number of cells ($\times 10^5$)
Haematopoietic stem cells	$CD45^{low}CD34^{high}$	1.257 ± 0.202	5.89 ± 1.831
Mesenchymal stem cells	$CD45^-CD34^-CD105^+CD90^{high}CD73^+$HLA-DR −	0.0138 ± 0.0042	0.07 ± 0.0267
Endothelial progenitor cells	$CD45^-CD34^+CD105^+CD90^{high}CD73^+$	0.0086 ± 0.0040	0.05 ± 0.0265
Promonocytes	$CD45^+CD34^+CD64^+CD14^-$	1.288 ± 0.4027	4.10 ± 1.345
Monocytes	$CD45^+CD34^-CD64^+CD14^+$HLA-DR+	5.191 ± 1.194	34.72 ± 18.281
T Lymphocytes	$CD45^+CD34^-CD3^+$	5.434 ± 1.809	46.98 ± 30.666
Helper T cells	$CD45^+CD34^-CD3^+CD4^+$	3.119 ± 1.312	30.15 ± 21.23
Cytotoxic T cells	$CD45^+CD34^-CD3^+CD8^+$	2.123 ± 0.5998	15.78 ± 9.645
B cells	$CD45^+CD34^-CD3^-CD19^+$	1.918 ± 0.9433	10.33 ± 2.574
Natural killer cells	$CD45^+CD34^-CD56^+$	5.734 ± 1.856	21.77 ± 9.656

CD, cluster of designation for cell surface molecules. Percentages and absolute numbers of cell subpopulations within the cell suspensions are presented as mean ± standard deviation.

composition of the cell suspension obtained from bone marrow aspirates are shown in Table 2.

Follow-up

From November 2005 to January 2008, patients were regularly evaluated according to the study protocol.

In hospital follow-up

Six patients were successfully submitted to the invasive procedure without complications and were discharged from the hospital on day 3. Patient 4 developed post-extubation laryngospasm and was managed with non-invasive ventilation and corticosteroids. Later on the same day, because of asymptomatic hypotension, he was diagnosed with a transient cardiomyopathy compatible with a mild presentation of Tako-tsubo syndrome. He was discharged from hospital on day 6 after complete recovery.

During arteriography, patient 3 suffered a hepatic artery dissection before cell infusion. There was not complete occlusion of the vessel and normal blood flow was promptly restored. The catheter was repositioned in the celiac trunk artery and cells were delivered towards both liver and spleen.

Bone marrow-derived mononuclear cell distribution by scintigraphy

Three- and 24-h WBS were performed in eight and seven patients respectively (Table 3); the absent examination was not conducted because of logistical problems. Six patients received BMMCs in the common hepatic artery but in two patients the infusion site differed because of anatomic variations. Patient 2 received the cells in a left segmental hepatic artery and patient 3 in the celiac trunk. WBS performed 3 h after infusion showed a strong correlation between the site of injection and radioactivity distribution (Fig. 1a–c), with a mean hepatic retention of 41% of the radiotracer. Cell retention in the liver was inversely related to the time from infusion; 24-h WBS performed in seven patients showed a mean hepatic retention of 32% (Fig. 1d).

Table 3. Proportion of liver radioactivity in relation to whole body radioactivity, 3 and 24 h after infusion of bone marrow-derived mononuclear cell radiolabelled with ^{99m}Tc

Patient	3-h WBS (%)	24-h WBS (%)
1	50.6	38.8
2	45.3	32.2
3	32.9	–
4	46.7	34.4
5	35.9	32.8
6	39.2	37.2
7	43.5	27.7
8	33.2	20.6
Mean ± SD	40.9 ± 6.6	31.9 ± 5.3

SD, standard deviation; WBS, whole body scintigraphy.

Liver function

We sought to determine the safety of BMMCs therapy in cirrhotic patients over 12 months of follow-up. The biochemical measures of liver function did not deteriorate during this period (Fig. 2). Paired analysis of all consecutive biochemical tests during the first month after treatment revealed improvements in serum bilirubin ($P = 0.02$) and albumin ($P = 0.03$) from baseline. By day 30, bilirubin levels had decreased in six out of the seven patients that completed follow-up. These six patients also experienced a rise in albumin levels that was maintained for 2 months, the exception being patient 6. There were corresponding trends towards improvement in INR and Child–Pugh and MELD scores during the first month. The early improvements in markers of liver function were not sustained over 12 months. By the end of follow-up, the mean albumin and bilirubin levels were superior to baseline but no longer statistically significant. The median Child–Pugh and MELD scores were unchanged from baseline by the end of the 12-month period. Of note, the measured biochemical parameters

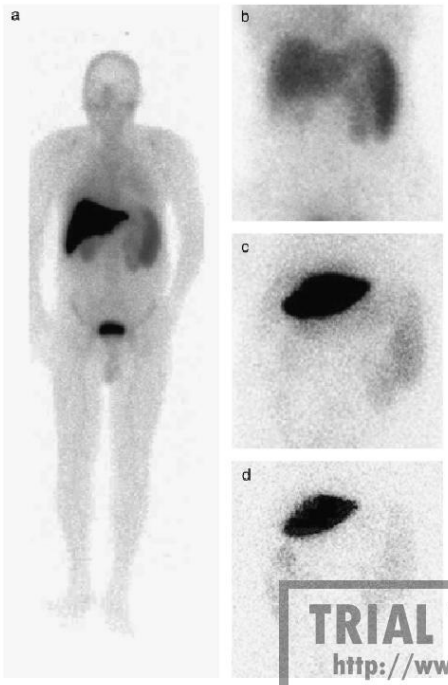

Fig. 1. Anterior view of whole body scintigraphy (WBS) showing ^{99m}Tc-$SnCl_2$ biodistribution after infusion of labelled bone marrow-derived mononuclear cell. Three-hour WBS of patient 4 (a, left), showing 47% of the total radioactivity uniformly distributed in the liver. The remaining uptake was distributed predominantly in spleen, bone marrow and kidneys. The high bladder uptake is attributed to renal excretion of the radiotracer. The 3-h WBS in patient 3 (b, top right) showed high spleen uptake because of cell infusion in the celiac trunk. Patient 2 received the BMMC in a left segmental hepatic artery. Radiotracer uptake was restricted to the site of injection in both 3-h (c, middle right) and 24-h WBS (d, bottom right).

worsened in patient 8 during two episodes of gastric variceal bleeding.

Adverse events

During long-term follow-up, Patient 2 developed type 2 diabetes mellitus (DM), episodes of erysipelas and an immune-mediated condition, eosinophilic fasciitis (EF). By month 6, patient 2 developed bullous erysipelas, which responded promptly to antibiotics. However, by month 10, he noticed subcutaneous nodules with inflammation in both forearms and at venepuncture sites, associated with swelling of the back of hands, fatigue and weight loss. He was diagnosed with new-onset diabetes in a non-ketotic hyperosmolar state. The skin changes regressed during inpatient care and DM treatment. After 2 months, the cutaneous lesions recurred in the same sites, in a milder form. Biopsies revealed inflammatory lymphocytic and eosinophilic infiltration with fibrosis of subcutaneous septa and fascia, compatible with EF (Fig. 3).

The following antibodies were negative: anti-Scl 70, anti-centromere, anti-Ro (SS-A), anti-La (SS-B), anti-U1 ribonucleoprotein, antinuclear antibody, anti-single strand DNA, anti-islet and anti-GAD. Complement levels (C3, C4 and CH100) were decreased. Peripheral lymphocytes and eosinophil counts were normal. By month 15, the cutaneous inflammation worsened with development of underlying fibrotic plaques. Prednisone was initiated in the lowest effective dose (40 mg daily). Shortly after, the addition of azathioprine (100 mg daily) allowed weaning of prednisone, inducing complete remission of inflammatory and fibrotic changes. After 6 months of treatment, azathioprine withdrawal was attempted with success, and the patient has remained asymptomatic and free of immunosuppression for 2 years.

Liver function was temporarily impaired in patient 6 because of severe lower limb cellulitis by day 60. He was admitted for intravenous antibiotic therapy and recovered gradually from the cutaneous infection. This patient did not present with improvement of either bilirubin or albumin levels. Furthermore, a 3 cm HCC was diagnosed on the day 360 CT scan and DUS. Ten months after the end of follow-up another patient (patient 3) developed HCC.

In patient 8, the first episode of gastric variceal bleeding on day 80 was complicated with spontaneous bacterial peritonitis. A recurrence of haemorrhage on day 100 contributed to the transient worsening of liver function. Nevertheless, laboratory parameters by the end of followup were superior to baseline.

Self-limited ascites occurred in association with clinical events such as Tako-tsubo syndrome (patient 4), cutaneous infection (patient 6) and gastrointestinal bleeding, that also resulted in acute encephalopathy (patient 8). Two out of five patients that had compensated ascites at baseline developed clinically detectable ascites towards the end of follow-up (patients 4 and 5). Table 4 summarizes the adverse events and complications diagnosed during follow-up.

Discussion

We demonstrated that the infusion of BMMCs through the hepatic artery in patients with cirrhosis was feasible but needs close and long-term follow-up. A transient improvement of laboratory tests of liver function occurred after the intervention in some patients, but it needs to be interpreted critically, because this is a single-arm study not designed to evaluate efficacy. These data will inform the design of Phase 2 clinical trials regarding the timing of such endpoints and the potential requirement for repeated cell infusion.

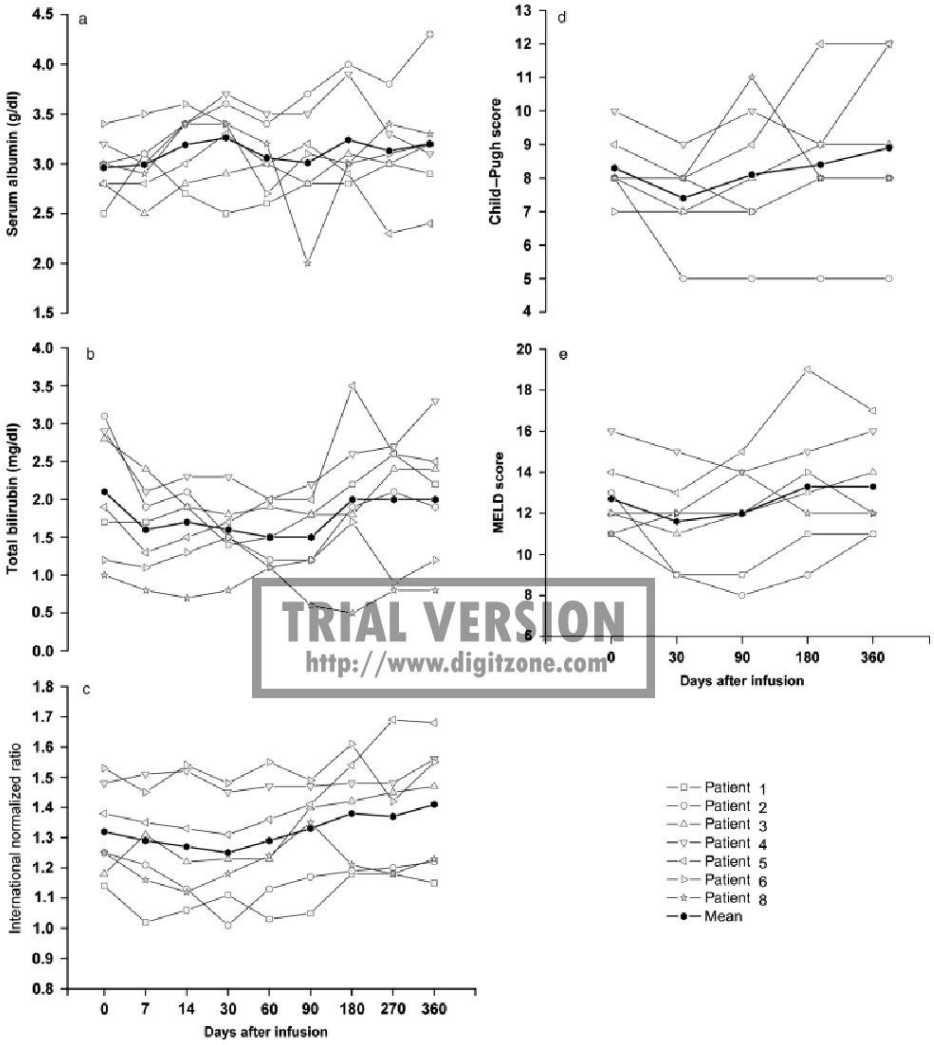

Fig. 2. Liver function after BMMC infusion. Serum albumin (a), total bilirubin (b), INR (c), Child–Pugh score (d), MELD score (e). D, day; INR, international normalized ratio; MELD, model for end-stage liver disease; Pt, patient.

Adult stem cells are responsible for maintaining the integrity within an organ by replacing aged or damaged cells (17). There are two major types of adult stem cells in the bone marrow: haematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). The mechanisms involved in stem cell-mediated regeneration in the liver are not yet clear, although differentiation or fusion with local cells and immunomodulatory, antifibrotic and regenerative stimuli have been demonstrated (18). There is some evidence to support the use of either HSCs (19–22) or MSCs (3, 4, 6, 23, 24) in liver disease. The potential differentiation into hepatocyte-like cells and the

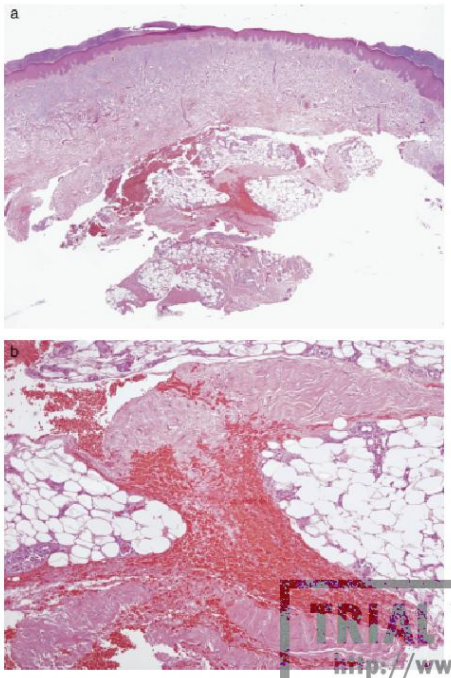

Fig. 3. Skin biopsy, patient 2. Severe thickening of interlobular septa is present, extending deep into the hypodermis. There is scarce inflammatory infiltrate and adipose lobules are spared, apart from small foci of steatonecrosis adjacent to affected septa. Epidermis and dermis present usual aspect. Haematoxylin–eosin, original magnification × 20 (a, top) and × 100 (b, bottom).

Table 4. Adverse events and complications

Patient	Event (moment of presentation)
1	Portal vein thrombosis, variceal bleeding and ascites (sixth month)
2	Bullous erysipela (fifth and sixth months) Eosinophilic fasciitis and type 2 DM (10th month)
3	Hepatic artery dissection (day 1) Type 2 DM (sixth month) HCC (22nd month)
4	Post-extubation laryngospasm, Tako-tsubo syndrome (day 1) Mild ascites (11th month)
5	Mild ascites (12th month)
6	Lower limb cellulitis, mild ascites (second month) HCC (12th month)
7	Post-liver transplant death (third month)
8	Variceal bleeding, moderate ascites, SBP, encephalopathy (second month) Variceal rebleeding (fourth month)

DM, diabetes mellitus; HCC, hepatocellular carcinoma; SBP, spontaneous bacterial peritonitis.

immunomodulatory properties of MSCs make them attractive for clinical use, although caution is advised because of a possible fibrogenic effect of purified cell fractions (25).

Because the only means to directly obtain MSCs in relevant numbers is by aspiration of bone marrow, this was our method of choice. Compared with the peripheral harvest of BMSCs, it requires general anaesthesia and patients may experience local complications. Although there is evidence that patients with compensated cirrhosis can undergo elective surgery with an acceptable rate of morbidity and mortality (26), we observed an episode of laryngospasm that resulted in a transient cardiomyopathy. Tako-tsubo syndrome is a unique reversible cardiomyopathy that is frequently precipitated by a stressful event and has a clinical presentation that is indistinguishable from a myocardial infarction. The pathophysiology remains unknown, but catecholamine-mediated myocardial stunning is the most favoured explanation. Recovery from general anaesthesia has been described as a cause (27). The peripheral acquisition of significant amounts of MSCs would be preferable. High doses of granulocyte-colony-stimulating factor (G-CSF) may be required for this. The associated hypothetical risk of splenic rupture in cirrhotic patients (28) has not been observed in clinical trials using this drug (8, 22, 29, 30, 31).

Given the expectation of MSCs in cell therapy, we considered relevant to confirm their presence in the injected cell suspension, performing CFU-F and immunophenotyping. However, this study was not designed to study the mechanisms involved in cell therapy and it is impossible to determine the positive or negative role played specifically by MSCs in this protocol, if there ever was one.

Our choice of using a simple, non-automated method for cell separation without the need for cell culture enhanced feasibility and has been used in other clinical trials (7, 9). The *ex vivo* expansion of bone marrow subpopulations increases the cost and risk of contamination. However, the BMMCs fraction includes diverse cell populations, making it difficult to identify which subpopulation or cell interactions may be responsible for the changes in liver function.

This study found some complications of the procedure that were not shown in similar cell-based protocols (8, 9, 22–24, 29–31), and the safety of the procedure remains to be proved. Studies evaluating the effects of BMMCs infusion in cirrhosis reported that the patients exhibited fever at one day after infusion (9) and an episode of hepatic encephalopathy in a patient with a history of previous episodes (7). Others have studied the injection of $CD34^+$ cells into the hepatic artery, collecting the cells directly from bone marrow (30, 32) or by leukapheresis procedure (8, 22, 29), most of the protocols using prior mobilization with G-CSF (8, 22, 29, 30). Transient thrombocytopenia consequent to leukapheresis was reported (8, 22) and a trial was prematurely stopped because of the development of progressive renal failure

in two out of four patients, although the complication was not assigned to a direct effect of $CD34^+$ cells but to radio-contrast nephropathy (32). The injection of cultured autologous MSCs into peripheral or the portal vein has also been evaluated in Phase 1 clinical trials (23, 24). Although the reasons for patients loss during follow-up in one of these studies are not evident (24), they agree on the safety of the procedure. A randomized controlled trial was conducted to investigate the therapeutic effect of peripheral blood monocyte cell transplantation in decompensated cirrhosis (31), both groups of treatment receiving G-CSF. No major adverse effects were noted over the 6-month follow-up. Except for mild pain at the bone marrow needle puncture site – when cells were harvested from bone marrow – and some discomfort at the site of cell infusion, no other complication, specific side effects related to the infusion procedure or development of focal liver lesion were reported in all these studies (8, 9, 22–24, 29–31).

We chose to follow patients for a longer period than most stem cell trials in liver disease in order to detect late complications. Moreover, if any adverse events were noticed, the patient was followed indefinitely. This proved useful in patients 2 and 6, who developed complications towards the end of the follow-up.

Patient 6 had an HCC nodule diagnosed by day 360. To the best of our knowledge, there are no reports of malignancy induced by adult stem cell therapy. HCC is a major complication of cirrhosis and its incidence may reach 7.1% a year (33). With regard to our sample size and the follow-up period, the probability of one of the patients developing HCC was 30%. Of note, patient 6 had a multifocal presentation unresponsive to three sessions of chemoembolization and died 18 months after diagnosis. Patient 3 also developed a multifocal HCC, but just 10 months after the end of follow-up, dying 8 months later. Although the development of HCC cannot be directly attributed to the BMMCs infusion, the disease behaviour in these two patients was similar. Even in a small fraction, MSCs were part of the cells infused in this protocol and their immunomodulatory effects have already been demonstrated, which may contribute to neoplastic transformation (18, 25). The investigation of the cellular mechanisms involved in cell-based research protocols is out of the scope of this study and a possible relationship with the cell infusion remains to be clarified.

Ten months after receiving BMMCs, patient 2 developed EF, a rare disorder characterized by chronic inflammation and fibrosis of the subcutaneous septa and muscular fascia. It has been considered a variant of scleroderma but has also been described as a manifestation of graft-versus-host disease (GVHD) following allogeneic HSCs transplantation (34). Currently, the aetiology of EF is still uncertain with a number of suggested associated conditions (35–37). None of these conditions were present in this patient.

A theoretical risk of GVHD-like manifestations should be considered in autologous stem cell therapy for chronic or degenerative diseases, even though this complication has been reported only in the context of bone marrow transplantation. Nakamura *et al.* (38) described cutaneous GVHD after autologous peripheral blood stem cell transplantation that was clinically and histologically similar to EF. The development of GVHD following autologous transplantation depends upon the derangement of self-tolerance, typically following preparative chemoradiotherapy or cyclosporine A administration (39), both absent from our protocol. However, advanced cirrhosis may account for a degree of immunodepression and thymic dysfunction, creating a favourable environment for immune system derangements. Autoreactive T cells that escape negative selection because of dysfunction of the thymus take part in this process (34). Furthermore, it has been postulated that HSC transplantation may generate a distorted immune response with secondary autoimmune-like phenomena (40). Introduction of immature T cells from bone marrow in peripheral blood, even at small amounts, might deflagrate an autoimmune-like event or GVHD.

As far as we are aware, this is the first clinical trial to investigate the cell kinetics of stem or progenitor cell therapy in liver disease. *In vivo* cell tracking techniques have been researched in cardiac stem cell-based therapy, improving knowledge of biodistribution and guiding the best route for cell delivery (41). We developed a novel technique using $^{99m}Tc\text{-}SnCl_2$ specific for mononuclear cell labelling (15). Both labelling efficiency and cell viability in our study were adequate and allowed us to show that approximately 41% and then 32% of the radioactivity was retained in the liver 3 and 24 h after injection respectively. Our preclinical studies have shown that liver and spleen retain a large proportion of cells injected peripherally (15, 16). Considering this experimental evidence and encouraging clinical results showing improvement of the liver function of cirrhotic patients after injection of BMMCs in a peripheral vein (9), the adoption of the peripheral route for stem cell injection in future protocols seems attractive.

Liver function evaluation suggested a transient effect of the cell infusion. Other studies using BMMCs injection have observed a continuous improvement of mean albumin levels (7, 9), but the maximum follow-up was 6 months. Although most of the clinical trials evaluating stem cells in liver diseases have restricted follow-up to < 6 months (7, 9, 29, 31, 32, 42), others groups extended this period (22, 23). Levicar *et al.* (22) harvested $CD34^+$ cells from peripheral blood in five patients with cirrhosis and injected into portal vein or hepatic artery, following one of them for 6 months and the others for 12–18 months. Similar to our results, they found an improvement in albumin and total bilirubin levels that was more pronounced between 2 and 6 months after cell infusion. Mohamadnejad *et al.* (23) were the first to infuse cultured autologous mesenchymal bone marrow cells in patients with cirrhosis. Four patients received cells through a peripheral vein and were followed for 12

months. Three of them had an improvement in liver function tests and MELD score, most significant in month 6. Yannaki *et al.* (42) reported two patients with alcoholic cirrhosis who were submitted to courses of peripheral harvest and injection of $CD34^+$ cells and followed for 30 and 34 months. MELD and Child–Pugh scores improved in both patients, showing a nadir at month 12. The authors also observed that both patients had repeated hospitalizations for major complications before the enrollment and none during follow-up.

In accordance with our observation, longer follow-up studies tend to indicate that, if a single injection of BMMCs is beneficial, its effect seems to be transitory.

In conclusion, although clinical trials have pointed towards the safety of stem cell-based protocols in cirrhosis, the complications shown in this study reinforce the need for close and long-term follow-up of the patients. Randomized studies with larger number of patients are essential to distinguish between natural complications of the disease, such as HCC, and adverse events of the therapy. Such controlled trials are now required to determine whether BMMCs therapy causes a definitive positive effect on liver function. Novel studies may also elucidate if stem or progenitor cells induce a sufficient derangement of the immune system capable of resulting in clinical manifestations. Cell tracking techniques may prove helpful in defining the optimal route of injection.

While studies comparing the mechanisms of action of bone marrow subpopulations in the diseased liver are not available, the use of BMMCs in stem cell-based protocols seems reasonable, considering its low cost and simplicity. Although designed to evaluate safety, this and other studies have shown a transient effect of bone marrow cell infusions. This raises the possibility that repeated doses may be required to result in longer term clinical benefit.

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APÊNDICE D - Artigo 5 – Safety of autologous bone marrow mononuclear cell transplantation in patients with nonacute ischemic stroke

RESEARCH ARTICLE

Safety of autologous bone marrow mononuclear cell transplantation in patients with nonacute ischemic stroke

Aims: To assess the safety and feasibility of intra-arterial transplantation of autologous bone marrow mononuclear cells in patients with middle cerebral artery ischemic stroke within 90 days of symptom onset. **Patients & methods**: Six patients were included in the study, and they received 1–5 × 10^8 bone marrow mononuclear cell and were evaluated using blood tests, neurological and imaging examination before treatment, and 1, 3, 7, 30, 60, 90, 120 and 180 days after transplantation. Scintigraphies were carried out 2 and 24 h after the procedure to analyze the biodistribution of labeled cells. Electroencephalogram was conducted within 7 days after transplantation. **Results:** No patients exhibited any complication or adverse events during the procedure. There was no worsening in the neurological scales until the end of the follow-up. **Conclusion:** Intra-arterial bone marrow mononuclear cell transplantation is feasible and safe in patients with nonacute ischemic strokes of the middle cerebral artery. Further studies are required to evaluate the efficacy of this therapy.

KEYWORDS: bone marrow stem cells · cell therapy · ischemic stroke · middle cerebral artery · stem cells

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Stroke is the third leading cause of death in developed countries [1] and the leading cause of death in some developing countries [2] including Brazil [3]. Furthermore, stroke remains the leading cause of disability in the world; at least 30% of stroke survivors do not make a complete recovery, and a further 20% require assistance for activities of daily living [4]. Although acute-stroke care facilities such as stroke units and the use of thrombolytics may improve prognosis, there are few clinical trials evaluating effective ways to enhance recovery in patients with residual disability [5]. In recent years, several studies have investigated the potential neuroprotective and restorative role of stem cells from different sources in animal models of brain ischemia [6]. Although the mechanisms of action are still unclear, these studies demonstrated that stem cell administration ameliorates the functional loss observed after ischemia, making stem cell transplantation an attractive approach to restore brain function after stroke in humans.

To date, only a few clinical studies evaluated stem cell transplantation in stroke patients [6]. Some of these studies employed cells derived from a human teratocarcinoma [7,8], in one study porcine cells were used [9] and in all of the studies the patients included were in the chronic phase of the stroke. Cell therapies using bone marrow-derived stem cells have also been reported in stroke patients. For example, Bang and colleagues examined the safety and efficacy of cultured expanded autologous mesenchymal bone marrow-derived cells administered intravenously 4–5 and 7–9 weeks after symptom onset in five stroke patients [10]. More recently, bone marrow mononuclear cells (BMMCs) harvested from the patients were stereotactically implanted into the perilesional area in five patients bearing chronic sequels of stroke [11]. Both studies concluded that bone marrow-derived stem cells are safe in patients with stroke in the subacute or chronic phase.

In our study we have used autologous BMMCs, as they are easily obtained and can be isolated in a short period of time just prior to transplantation, minimizing the chances of contamination and allowing treatment of the patients in the acute or subacute phase of stroke. Furthermore, the mononuclear fraction contains several types of bone marrow cells including stem cells and precursors, which can produce large amounts of cytokines and trophic factors that promote angiogenesis, neuroprotection and neuroregeneration after CNS injury in animal models of neurological diseases [12–18].

An important question to be addressed in stroke studies is related to the time window for treatment. Transplantation of stem cells during the first few days of ischemia presents several obstacles since the patients are very unstable in the acute phase. On the other hand, in the nonacute phase, the formation of scar tissue and the restoration of the blood–brain barrier might

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adversely affect the migration and homing of the transplanted cells. In order to address these issues, in our study a small percentage of the transplanted cells were labeled with ^{99m}Technetium (^{99m}Tc). We were able to demonstrate that the intra-arterially injected BMMCs were able to migrate and locate to the lesion at least in the first 2 h after transplantation [19,20]. However, it remained to be determined whether this method of transplantation of BMMCs was safe and whether these cells were also well tolerated for longer periods of time. The results reported here showed no adverse effects related to the transplanted cells during the procedure and during the follow-up period of 180 days in these patients. We conclude that intra-arterial transplantation of autologous BMMCs seems to be feasible and safe in nonacute stroke patients.

Patients & methods

Subjects

This is an unblinded, uncontrolled Phase I study. The protocol and the consent form were approved by the Institutional Research Ethics Committee and the National Committee of Ethic and Scientific Research of Brazil. Written informed consent was obtained from all subjects. This study is registered at ClinicalTrials.gov (NCT00473057) [21].

Individuals aged 18–75 years of both sexes were eligible for the study if they had the following characteristics:

- Ischemic stroke in the middle cerebral artery (MCA) territory evidenced by computed tomography or MRI occurring within 90 days;
- Recanalization of the involved MCA as assessed by transcranial Doppler studies or by magnetic resonance angiography;
- A score between four and 17 according to the National Institutes of Health Stroke Scale (NIHSS).

We excluded patients who met any of the following criteria:

- Difficulty in obtaining vascular access for percutaneous procedure;
- Ipsilateral carotid stenosis (>50%, by Doppler studies);
- Neurological worsening (>four points in the NIHSS) before injection due to either edema or intracerebral hemorrhage;
- Thrombophilias or primary hematological diseases;
- Neurodegenerative disorders;
- Previous stroke with modified Rankin Scale (mRS) of more than 2;
- Intracardiac thrombus;
- Autoimmune disorders;
- Sepsis (according to the Society of Critical Care Medicine and the American College of Chest Physicians 1992 criteria);
- History of neoplasia or other comorbidity that could impact a patient's short-term survival;
- Any condition that in the judgment of the investigator would place the patient under undue risk;
- Bone disorders that could increase the risk of the bone-marrow harvesting procedure;
- Liver failure renal failure (serum creatinine >2 mg/ml);
- Hemodynamic or respiratory instability;
- Lacunar stroke;
- Pregnancy;
- Previous participation in other clinical trials.

Bone marrow aspiration, cell separation, injection & labeling

Cells were obtained by marrow aspiration of the iliac crest (~80 ml) and processed by Ficoll density centrifugation. After washing, counting and viability testing, cells were resuspended in 10 ml of saline solution with 5% autologous serum. Cell isolation and manipulation was performed in a laminar flow cabinet with sterile equipment. Bacteriology and culture were also performed to rule out contamination of the material. Viability of the labeled cells was evaluated by the trypan blue exclusion test, and was estimated to be greater than 93% in all cases. Labeling efficiency (%) was calculated by the activity in the pellet divided by the sum of the radioactivity in the pellet plus supernatant, and was estimated to be greater than 90% in all cases. Approximately 1 ml of this solution was labeled with ^{99m}Tc [19,20]. Labeled cells were added back to the total mononuclear cell suspension (final volume 10 ml) and slowly injected into the territory of the MCA, via the femoral artery, after navigation (Seldinger technique) and under local anaesthesia and conscious sedation. A routine coaxial technique with femoral arterial puncture was used. A 6 Fr guiding catheter (Envoy-Cordis, Miami, FL, USA or Guider Soft tip, Boston Scientific,

Target Therapeutics, Fremont, CA, USA) was positioned at the cervical level with continuous flushing with normal saline. Intravenous heparin was used (bolus of 80 units/kg of body weight) as needed to maintain the activated clotting time between two- and three-times the baseline values. A large-inner-diameter microcatheter (SL 1018 Boston Scientific, Target Therapeutics) was navigated to the M1 portion of the MCA and the infusion was performed in approximately 10 min.

Imaging

Whole-body, planar scintigraphies and single-photon-emission computed tomography were performed using a Millennium GE camera (General Electric Medical Systems, Milwaukee, WI, USA), as previously described [20]. Acquisition protocols were performed 2 and 24 h after cell transplantation. Computed tomography images were acquired before cell therapy with a 40-detector row scanner (Brilliance-40, Philips Medical Systems, Surrey, UK). Computed tomography or MRI were performed before and after the procedure during the follow-up.

Neurologic evaluation

All individuals were evaluated at admission, on the day of transplantation, and 1, 3, 7, 30, 60, 90, 120 and 180 days after cell infusion using the NIHSS, the Barthel Index (BI) and the mRS by a single board-certified neurologist (VB). Routine laboratory tests (complete blood count and biochemical examinations: urea, creatinine and electrolytes) were carried out at these points. An electroencephalogram was obtained within 7 days after transplantation.

Results

The main goal of this study was to examine the safety and feasibility of BMMC transplantation in patients with MCA ischemic stroke between 2 and 3 months after stroke onset. The patients included in the study were evaluated using blood tests, NIHSS, mRS and BI before treatment, and 1, 3, 7, 30, 60, 90, 120 and 180 days after the infusion of the BMMCs. A minimum of 1×10^8 and maximum of 5×10^8 BMMCs were injected 2–4 h after bone marrow aspiration. The pharmacological treatment and rehabilitation therapy of each patient was maintained unaltered during the follow-up period.

Baseline characteristics

The main characteristics of the patients included are shown in TABLE 1. In summary, six male patients (aged 24–65 years) received intra-arterial BMMCs 59–82 days after mild-to-moderate MCA infarcts (NIHSS between four and 13). Three of the patients had received thrombolytic therapy and one of them was submitted to a craniectomy during the acute stage of stroke. Two patients were taking an oral anticoagulant drug to prevent recurrent strokes, and were switched to low-molecular-weight heparin (enoxaparine 1 mg/kg twice daily) 1 week before the procedure.

Imaging

The patients included in this study were in the nonacute phase of stroke and the blood–brain barrier was likely closed by this time. To assess whether the injected cells were still able to reach the lesioned region we investigated the distribution of BMMCs labeled with ^{99m}Tc 2 and 24 h after transplantation. Whole-body scans obtained 2 h after cell transplantation showed the presence of ^{99m}Tc-labeled cells in the brains of all patients. The activity of the radioisotope in the brain was 0.6–5.1% of the activity in the whole body as described previously [20]. A representative image illustrating the whole-body biodistribution of BMMCs is shown in FIGURE 1.

At 24 h, cell homing could only be visualized in the brains of two patients, while in all patients uptake was seen in the liver, lungs, spleen, kidneys and bladder [20]. The absence of labeled cells in the brain of the remaining patients 24 h after the transplant could be due to the decay of the radioactivity compound below the levels of detection and/or to the decrease in the number of cells at the lesion site.

Safety of the procedure

Serial clinical, laboratory and radiographic evaluations showed no deaths, stroke recurrence or cell-related adverse events during the procedure or during the follow-up period of 180 days. These results confirm the excellent tolerance to the procedure.

The electroencephalogram performed after the transplantation showed polymorphic slow activity in the ischemic area and only one patient exhibited spike-wave activity without clinical manifestation. Two patients suffered generalized seizures after the end of follow-up (around 200 days after the BMMC infusion); one was successfully treated with phenytoin and the other was treated with a combination of oxcarbazepine and lamotrigine.

Neurological evaluation

TABLE 1 shows a summary of the values of the NIHSS, BI and mRS before and after transplantation of BMMCs.

Table 1. Baseline characteristics and clinical follow-up.

Characteristics	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Sex/age	M/24	M/65	M/47	M/65	M/57	M/47
Risk factor	PFO	Hypertension, DM, dislipidemia	Hypertension	AF, DM	Hypertension	DM, hypertension
Symptoms	Aphasia, dysarthria hemiplegia	Dysarthria, hemiplegia	Aphasia, hemiparesis	Aphasia, dysarthria hemiplegia	Dysarthria, hemiplegia	Dysarthria, hemiplegia
Infarct side	Left	Right	Left	Left	Right	Right
Stroke mechanism	Cardioembolic	Atherosclerotic	During aneurism clipping	Cardioembolic	Atherosclerotic	Atherosclerotic
Acute treatment	Conservative	Conservative	Conservative	iv. thrombolysis	iv. thrombolysis	iv. plus ia. thrombolysis plus craniectomy
Hemorrhagic transformation	No	Yes	No	Yes	No	Yes
Infusion day: NIHSS/BI/mRS (days since stroke)	7/100/2 (67)	9/35/4 (82)	4/95/1 (62)	13/25/5 (72)	9/30/4 (59)	13/10/5 (73)
NIHSS/BI/mRS at day 3	6/100/2	9/35/4	3/95/1	12/25/5	6/30/4	13/10/5
NIHSS/BI/mRS at day 7	5/100/2	8/35/4	3/95/1	12/25/5	5/30/4	12/15/5
NIHSS/BI/mRS at day 30	5/100/1	6/50/4	3/100/1	11/25/4	3/50/3	12/25/5
NIHSS/BI/mRS at day 60	4/100/1	6/55/4	3/100/1	9/30/4	4/90/1	12/30/5
NIHSS/BI/mRS at day 90	4/100/1	6/65/3	3/100/1	10/35/4	4/90/1	12/30/5
NIHSS/BI/mRS at day 120	4/100/1	6/75/3	3/100/1	10/35/4	1/100/0	11/50/3
NIHSS/BI/mRS at day 180	4/100/1	6/80/3	3/100/1	10/35/4	1/100/0	11/50/3
n of injected cells	5×10^8	1.25×10^8	3.9×10^8	4×10^8	3.2×10^8	1×10^8

AF: Atrial fibrillation; BI: Barthel index; DM: Diabetes mellitus; ia.: Intra-arterial; iv.: Intravenous; M: Male; mRS: Modified Rankin scale; n: Number; NIHSS: National Institutes of Health Stroke Scale; PFO: Patent foramen ovale.

Only one patient (patient three) was independent in daily activities at BMMC infusion day (BI: 95, mRS: 1). No signs of worsening in the neurological condition were observed immediately after the procedure or during the follow-up period. In fact, at the 180-day follow-up evaluation, all patients had improved their scores in comparison with the values before transplantation. For example, the NIHSS scores improved (range -1 to -8 points) during follow-up in all patients (TABLE 1 & FIGURE 2).

Discussion

In the present series of patients, BMMC transplantation proved to be safe and feasible in six patients with ischemic stroke in the MCA territory within 90 days after onset. There were no complications or unexpected outcomes related to the procedure. All patients improved their neurological scores (NIHSS, BI and mRS) at the end of follow-up.

A small percentage of the injected cells were labeled with ^{99m}Tc, which allowed us to demonstrate that BMMCs were capable of migration and homing to the lesion site. ^{99m}Tc has a half-life of 6 h, which is a significant advantage over ^{18}F-fluorodeoxyglycose, which has a half-life of 110 min. ^{111}Indium-oxine (^{111}In-oxine) has a half-life of 2.81 days and allows monitoring for approximately 96 h, whilst ^{99m}Tc permits tracking for 24–48 h; however, ^{111}In-oxine has shortcomings including suboptimal photon energies, low-resolution images and the 18–24-h period between injection and imaging that is generally necessary [21,22]. Moreover, studies have suggested that ^{111}In-oxine may have deleterious effects on different cells, including hematopoietic progenitor cells [23] and mesenchymal stem cells [24]. ^{99m}Tc allows imaging for 24–48 h and results in higher image resolution and a lower radiation burden to the patient [21,22].

Because of this window for cell tracking, it was not possible to assess exactly how long the cells remained in the brain. This has been an unsolved issue in the few clinical trials performed so far and even in animal models, and new methods of cell labeling have to be developed in order to demonstrate the presence of transplanted cells for longer periods of time and/or cell fate. In spite of that, we were able to demonstrate that BMMCs could be used as therapy in the nonacute phase of the stroke since they were able to migrate to the ischemic region even after restoration of the blood–brain barrier.

The injection of large amounts (up to 5×10^8) of bone marrow-derived cells intra-arterially and the observation that some of these cells may be able to cross the blood–brain barrier could raise many safety concerns that have to be addressed in clinical trials. We have used the maximal amount of cells that could be obtained with local anesthesia and mild sedation. The use of general anesthesia, which could allow us to increase the volume of bone marrow aspirate, may also potentially cause several adverse events. Moreover, the amount of cells used was comparable to other clinical trials using BMMCs for myocardial diseases [25–31].

The results of our study showed that no adverse events were observed up to 180 days after transplantation that could be related to the procedure or to the presence of the transplanted BMMCs in the brain. Similar results have also been reported by our group in patients transplanted in the acute phase of the stroke (up to 10 days after ictus) [FRIEDRICH M *ET AL.*, UNPUBLISHED DATA].

In our series, two patients suffered seizures approximately 200 days after the cell infusion that were controlled pharmacologically. These patients are under extended follow-up. Late seizures (at least 2 weeks after the stroke) [32,33] occur in 3–67% of the patients, varying considerably among series [32–35], and although several risk factors for early or late seizures have been identified, there is no clear predictor of poststroke epilepsy [36]. Based on these observations and on the small sample of our study we cannot at present either rule out or conclude that the incidence (two out of six) of seizures is explained by chance or attributed to the cell therapy.

In animal models of ischemia, stem cells from different sources have been used with promising results, and different mechanisms of action have been suggested to explain these functional benefits [37–39]. For example, it was argued that the introduction of neural stem cells into areas of cell loss may result in repair and restoration of circuitry [40] through neuroprotective [41,42] and immunomodulatory effects [43,44], as well as potentially leading to some cell replacement [45,46]. Alternatively, when bone marrow-derived stem cells (mesenchymal or mononuclear cells) are employed, the functional benefits observed are probably due to the release of cytokines and/or trophic factors, which may have an immunomodulatory effect and/or contribute to a reduction in apoptosis in the penumbra area in earlier periods

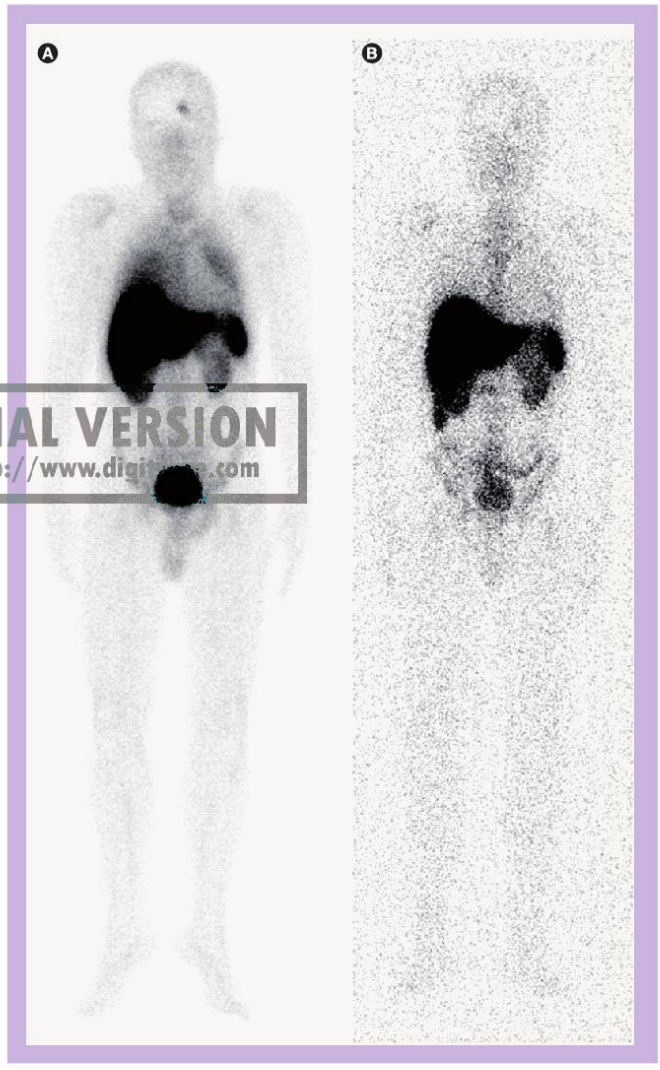

Figure 1. Whole-body scintigraphies. Anterior view of patient 3 at **(A)** 2 h and **(B)** 24 h after cell transplantation shows the distribution of bone marrow mononuclear cells labeled with ^{99m}Tc.

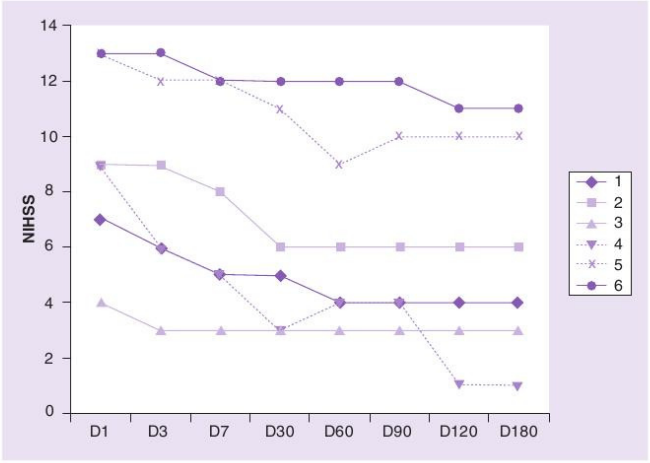

Figure 2. Evolution of the National Institutes of Health Stroke Scale score after the procedure.
D: Days; NIHSS: National Institutes of Health Stroke Scale.

after ischemia [13,14] . In addition, it has been suggested that cell-based therapies amplify the endogenous processes of brain repair and plasticity, including neurogenesis, angiogenesis and synaptogenesis, which could explain the benefits observed in experimental models of stroke, even when cells are administered at later times [8,15–19].

Cell therapies using bone marrow-derived stem cells (mesenchymal cells or the mononuclear cell fraction) have been well documented in several diseases, including myocardial infarction and limb ischemia [25–30,50–52], and may also represent new and important strategies for the treatment of stroke.

At present, only a few clinical studies involving cellular therapies in patients with acute and chronic stroke have been published and some of them involved therapy with autologous bone marrow-derived stem cells [7–10,53–55]. Bang and coworkers investigated intravenous infusion of autologous mesenchymal bone marrow cells, expanded *in vitro*, in patients with acute cerebral infarcts [10]. The procedure was safe and feasible; the group receiving mesenchymal cells (n = 5) had improved BI and mRS scores after the infusion 3 and 6 months after the onset of symptoms. However, a shortcoming of the study is cell preparation; although feasible, it is time consuming, potentially dangerous and expensive. Two other studies carried out during the acute phase of stroke (3–10 days postictus) evaluated the safety of intra-arterial BMMC transplantation in patients with MCA territory infarcts in two different Brazilian institutions [54,55]. Preliminary reports showed that the procedure is safe and feasible [54,55].

Although the transplantation of bone marrow-derived stem cells in stroke patients seems to be safe and feasible, many questions remain unanswered, including the dose of cells, administration route, timing of the infusion (acute vs nonacute vs chronic stage of stroke), the territory of stroke (cortical vs subcortical territories, anterior vs posterior circulation) and the mechanisms of action of bone marrow-derived stem cells in the setting of stroke. In our study, the rationale for selecting the cell dose was based on the results of the preliminary safety studies described previously [53–55] and the reason for selecting nonacute patients was based on results of some experimental models of cell therapy in stroke [56–58]. However, a therapeutic window should be determined as a function of therapeutic dose, and the repeated-dose regimen could optimize recovery benefit [59]. An additional shortcoming of our study is the absence of a control group and this is particularly relevant in studies involving diseases with spontaneous recovery. For this reason, any conclusions regarding the efficacy of the therapy should be postponed until a Phase II clinical trial with a control group included has been conducted.

Conclusion

Transplantation of BMMCs is feasible and appears to be safe in patients with nonacute ischemic stroke. Further randomized clinical trials are necessary to establish the efficacy and long-term safety of this procedure.

Financial & competing interests disclosure

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- Stroke is the third leading cause of death in developed countries and the leading cause of death in some developing countries including Brazil.
- Stroke is the leading cause of disability in the world.
- Stem cell-based therapies can have a potential role in neuroprotection and neuroregeneration after ischemia.
- In animal models, bone marrow-derived stem cells ameliorate the functional loss observed after ischemia.
- Autologous bone marrow mononuclear cells intra-arterially transplanted are safe in patients with nonacute stroke.

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▪ **An important discussion on the current status of cell therapy for stroke with recommendations for future laboratory and clinical investigations.**

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