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SAMUEL GREGGIO

TERAPIAS INOVADORAS EM MODELO EXPERIMENTAL DE HIPÓXIA-ISQUEMIA NEONATAL: NEUROPEPTÍDEO NAP E CÉLULAS MONONUCLEARES DE SANGUE DE CORDÃO UMBILICAL HUMANO

PORTO ALEGRE
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Tese de Doutorado apresentada como requisito para obtenção do grau de Doutor pelo Programa de Pós-Graduação em Pediatria e Saúde da Criança, da Faculdade de Medicina da Pontifícia Universidade Católica do Rio Grande do Sul.

Orientador: Prof. Dr. Jaderson Costa da Costa

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Aprovado em 01 de Março de 2013

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PORTO ALEGRE

2013

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RESUMO

Objetivos: investigar, separadamente, o potencial terapêutico do neuropeptídeo NAP e do transplante intra-arterial de células mononucleares de sangue de cordão umbilical humano (CMSCUH) em um modelo experimental de hipóxia-isquemia (HI) neonatal.

Métodos: ratos Wistar machos com 7 dias de vida foram submetidos ao modelo de HI neonatal através da oclusão permanente da artéria carótida comum direita e hipóxia sistêmica (8% O_2 por 2 h). A administração do neuropeptídeo NAP (3 µg/g, i.p.) ocorreu imediatamente (0 h) e 24 h após a aplicação do modelo. Já o transplante de CMSCUH (1 x 10^6 ou 1 x 10^7 células/50 µL) foi realizado 24 h pós-HI através de técnica de microagulha na artéria carótida comum esquerda. Somente no estudo do NAP, ensaio cometa alcalino, peroxidação lipídica e níveis de glutationa reduzida foram determinados no hipocampo e córtex cerebral 48 h pós-HI. Para ambas as terapias empregaram-se a versão espacial do labirinto aquático de Morris (LAM) e determinação estereológica da volumetria hemisférica cerebral na fase adulta destes animais. Utilizou-se o teste do rotarod acelerado e pesagem corporal e cerebral dos animais adultos apenas no estudo do transplante intra-arterial de CMSCUH. A migração das CMSCUH foi monitorada através da técnica de *nested-PCR* no cérebro e órgãos sistêmicos.

Resultados: o tratamento com NAP restabeleceu a integridade hipocampal e cortical do DNA e membranas lipídicas, junto ao incremento do sistema glutationa. Além disso, o NAP impediu o desenvolvimento de déficits do aprendizado e da memória espacial juntamente à redução da atrofia cerebral em ratos adultos. O transplante intracarotídico de CMSCHU em ratos neonatos HI demonstrou-se factível e seguro. Este tratamento preveniu, de maneira dose-dependente, o desenvolvimento de prejuízo cognitivo de ratos adultos previamente expostos à HI neonatal, sem influência sobre a atrofia cerebral. A função motora e peso corporal de ratos adultos não sofreram influência da HI neonatal ou da administração intra-arterial de CMSCHU. As CMSCHU puderam ser detectadas tanto logo após o transplante como tardiamente (30 dias) no cérebro e órgãos sistêmicos dos animais transplantados.

Conclusões: os efeitos benéficos e duradouros do neuropeptídeo NAP e do transplante intra-arterial de CMSCHU, assim como sua viabilidade, fornecem evidências de uma possível translacionalidade destas duas terapias inovadoras para o tratamento da HI neonatal. Desta forma, o neuropeptídeo NAP e a via intracarotídica para o transplante de CMSCHU tornam-se candidatos potenciais para a prevenção do dano HI cerebral e decorrentes sequelas.

Descritores: hipóxia-isquemia neonatal, modelo animal, neuropeptídeo NAP, terapia celular, neuroproteção, estresse oxidativo, memória, lesão cerebral e função motora.

ABSTRACT

Objectives: to investigate separately the therapeutic potential of neuropeptide NAP and the intra-arterial (IA) transplantation of human umbilical cord blood (HUCB) mononuclear cells in an animal model of neonatal hypoxia-ischemia (HI).

Methods: male Wistar rats at postnatal day 7 were subjected to an HI model by permanent occlusion of right common carotid artery and systemic hypoxia (8% O_2 for 2 h). The animals were randomly assigned to groups receiving an intraperitoneal injection of NAP (3 μg/g) immediately (0 h) and 24 h after HI. Other animals received HUCB mononuclear cells (1 x 10^6 or 1 x 10^7 cells/50 μL) into the left common carotid artery 24 h after HI insult by using the microneedle technique. Only for NAP study, brain DNA damage, lipid peroxidation and reduced glutathione (GSH) content were determined 48 h post-HI insult. The spatial version of the Morris water maze learning task and the stereological volume assessment were performed in adult animals that received both therapies. The accelerated rotarod test and cerebral and body weight determination were applied only for cellular therapy study. The HUCB mononuclear cells migration was monitored through nested-PCR analysis in the brain and systemic organs of transplanted-HI rats.

Results: we observed that NAP prevented the acute HI-induced DNA and lipid membrane damage and also recovered the GSH levels in the injured hemisphere of the HI rat pups. Further, NAP was able to hinder impairments in learning and long-term spatial memory and to significantly reduce brain damage in adult animals previously subjected to neonatal HI. The IA transplantation in neonatal HI rat seemed to be a feasible and safe delivery route for HUCB mononuclear cells. The intra-arterially delivered cells hindered dose-dependently the learning and spatial memory impairments without brain damage recovery in adult rats. Additionally we further showed that HI insult or IA cell transplantation had no long-term impact in the body weight and motor function in rodents. The HUCB mononuclear cells could be promptly identified in the ischemic brain after IA transplantation and also in some peripheral organs until at least 30 days later.

Conclusions: the viability and long-lasting beneficial effects of neuropeptide NAP and IA transplantation of HUCB mononuclear cells support its translational characteristic for neonatal HI management. Therefore, NAP and intracarotid delivery of cells became promising candidates for the treatment of HI-induced brain damage and life-long disabilities.

Keywords: neonatal hypoxia-ischemia, rodent model, neuropeptide NAP, cellular therapy, neuroprotection, oxidative stress, memory, brain damage and motor function.

SUMÁRIO

Capítulo I

1 APRESENTAÇÃO

Na presente tese de doutorado investiguei, separadamente, duas possíveis estratégias terapêuticas para o tratamento da hipóxia-isquemia (HI) neonatal em um modelo animal: neuropeptídeo NAP e transplante intra-arterial de células mononucleares de sangue de cordão umbilical humano (CMSCUH). Sendo assim, a composição desta tese foi elaborada na forma de quatro capítulos. O capítulo I destina-se a explanar a logística de organização da tese, justificar o assunto de pesquisa e apresentar os objetivos que guiaram este trabalho. O capítulo II contempla a introdução e aborda os pontos mais relevantes para a elaboração do *rationale* do estudo de duas terapias inovadoras para o tratamento da HI neonatal em um contexto experimental. Inicialmente, uma abordagem clínico-epidemiológica da HI neonatal embasa a problemática e enfatiza a necessidade de terapias efetivas. No segundo tópico, abordam-se as propriedades neuroprotetoras do peptídeo NAP em estudos *in vitro* e *in vivo*, os mecanismos de ação envolvidos e sua aplicabilidade nesta patologia. A terceira parte, apresentada no formato de artigo de revisão, publicado no *Jornal de Pediatria* em 2010, abrange os estudos pré-clínicos e ensaios clínicos de terapia celular em HI neonatal, e discute os aspectos translacionais associados a esta terapêutica, a fim de fundamentar o transplante intra-arterial de CMSCUH na HI experimental. O capítulo III constitui-se de dois artigos originais, cada qual específico para cada uma das abordagens terapêuticas avaliadas nesta tese. O primeiro deles refere-se à ação neuroprotetora do neuropeptídeo NAP em animais hipóxico-isquêmicos, publicado na *Neurobiology of Disease* em 2011. O segundo item deste capítulo apresenta os resultados acerca do transplante intra-arterial de CMSCUH em modelo animal de HI neonatal, como artigo submetido para publicação nesta mesma revista científica. Como fechamento, no capítulo IV são listadas as principais conclusões oriundas desta tese.

2 JUSTIFICATIVA

Os trabalhos aqui apresentados são oriundos de duas linhas de pesquisa inicialmente desenvolvidas no Laboratório de Neurociências do Instituto de Pesquisas Biomédicas da PUCRS, utilizando terapia com neuropeptídeos, iniciada com o desenvolvimento do projeto de terapia com NAP para o tratamento de crises convulsivas neonatais[1] (Greggio et al., 2008, Greggio et al., 2009), e terapia celular em modelo animal de HI neonatal[2] (de Paula et al., 2009, de Paula et al., 2012). Além deste enfoque terapêutico, o laboratório de Neurociências também se dedica na investigação do potencial terapêutico de células-tronco em outras neuropatologias, tais como hemorragia da matriz germinativa/intraventricular, epilepsia, esclerose lateral amiotrófica, doença de Parkinson e trauma raquimedular. Tais esforços na pesquisa básica (Costa-Ferro et al., 2010, Venturin et al., 2011, Costa-Ferro et al., 2012) contribuíram para a elaboração pioneira de um ensaio clínico[3] (fase I) acerca do transplante autólogo de células-tronco da medula óssea em pacientes com epilepsia do lobo temporal.

Na presente tese, objetivou-se avaliar a utilização do neuropeptídeo NAP e, separadamente, a efetividade da via intra-arterial para transplante de CMSCUH no tratamento da HI neonatal, inferindo as repercussões em termos funcionais e de dano cerebral. Para este fim, foram utilizados ratos neonatos de 7 dias de vida e estes foram submetidos à oclusão permanente da artéria carótida comum direita e, subsequentemente, submetidos à hipóxia global. Tal modelo é clássico nos estudos de HI neonatal (Vanucci & Vanucci, 2005), assemelhando-se em vários aspectos com a condição humana. No sentido de propor novas alternativas terapêuticas para o manejo desta patologia, que atualmente não dispõe de nenhum tratamento efetivo, foi analisada uma série de variáveis que, em conjunto, forneceram evidências da plausibilidade das terapias aqui propostas. Especialmente no projeto do neuropeptídeo NAP, foi realizada uma análise bioquímica de importantes estruturas moleculares cerebrais que são severamente comprometidas nesta condição. Para tal, parâmetros de estresse oxidativo no DNA nuclear e membranas lipídicas foram investigados, assim como sistema de defesa antioxidante. Paralelamente, em ambos os projetos, buscou-se também avaliar a memória espacial e volume hemisférico cerebral destes animais, variáveis comprovadamente acometidas pela lesão HI as quais se baseiam o modelo. No projeto com CMSCHU, optou-se por avaliar também o desempenho motor, peso corporal e cerebral, assim

[1] Apêndice C.
[2] Apêndices D e E, respectivamente.
[3] http://clinicaltrials.gov/ct2/show/NCT00916266

como a monitorização da migração das células transplantadas. Assim, os dados experimentais obtidos nos dois projetos permitem consolidar e avançar o conhecimento científico sobre o tratamento com neuropeptídeos e CMSCUH na HI neonatal. Por fim, estes trabalhos servirão de alicerce para que estas terapias inovadoras possam, em um futuro próximo, translacionar para a elaboração de ensaios clínicos na área pediátrica.

É importante destacar que os protocolos experimentais usados nesta tese seguiram as normas internacionais de experimentação com animais de laboratório. Todos os procedimentos foram realizados tomando os cuidados necessários para reduzir ao máximo o número de animais empregados. Ressalta-se também que os animais receberam cuidados adequados e foram submetidos ao mínimo possível de desconforto e estresse. Durante os experimentos, foi instituída sedação de acordo com a prática veterinária quando necessário. Sendo assim, os projetos estavam de acordo com as normativas de experimentação animal do Comitê de Ética para Uso de Animais da Pontifícia Universidade Católica do Rio Grande do Sul, sob aprovação dos registros CEUA 10/00172[4] e CEUA 09/00105[5]. Na presente pesquisa, a coleta de CMSCUH foi realizada somente após assinatura do Termo de Consentimento Livre e Esclarecido[6] e não acarretou riscos para a mãe doadora e nem para o recém-nascido.

[4] Anexo A.
[5] Anexo B.
[6] Apêndice A.

3 OBJETIVOS

3.1 Objetivo Geral

Os objetivos desta tese são verificar, separadamente, o potencial terapêutico do tratamento com neuropeptídeo NAP e transplante intra-arterial de CMSCUH em um modelo experimental de HI neonatal.

3.2 Objetivos Específicos: Neuropeptídeo NAP e HI neonatal

- Avaliar o possível impacto do tratamento com neuropeptídeo NAP sobre o DNA hipocampal e do córtex cerebral 48 horas após aplicação do modelo de HI experimental, através do ensaio cometa alcalino;
- Verificar o possível efeito protetor do neuropeptídeo NAP sobre a peroxidação lipídica no hipocampo e córtex cerebral 48 horas após aplicação do modelo de HI experimental, através da determinação de substâncias reativas ao ácido tiobarbitúrico;
- Quantificar se o possível efeito protetor do neuropeptídeo NAP está associado à modulação do conteúdo de glutationa reduzida no hipocampo e córtex cerebral 48 horas após aplicação do modelo de HI experimental, através de dosagem dos níveis de glutationa reduzida;
- Verificar o impacto do tratamento com neuropeptídeo NAP sobre a curva de aprendizagem, latência de escape e no percentual de tempo gasto no quadrante alvo na versão espacial do labirinto aquático de Morris em animais hipóxico-isquêmicos adultos;
- Determinar se o peptídeo NAP promove neuroproteção a longo prazo do hemisfério cerebral lesionado, empregando técnica estereológica de contagem de pontos associado ao princípio de Cavalieri para determinação de volumetria hemisférica.

3.3 Objetivos Específicos: Transplante intra-arterial de CMSCUH e HI neonatal

- Desenvolver o procedimento de administração intra-arterial de CMSCUH na artéria carótida de ratos neonatos com 8 dias de vida, baseado na técnica de microagulha;
- Determinar se o transplante intra-arterial de CMSCUH, em diferentes dosagens, tem influência sobre a curva de aprendizagem, latência de escape, percentual de tempo gasto no quadrante alvo, número de cruzamentos sobre o aro alvo e velocidade de natação na versão espacial do labirinto aquático de Morris em animais hipóxico-isquêmicos adultos;
- Avaliar o equilíbrio e coordenação motora, através do teste do rotarod acelerado, em ratos adultos previamente submetidos ao modelo de HI neonatal e transplantados intra-arterialmente com CMSCUH;
- Verificar os possíveis efeitos do modelo experimental de HI neonatal e transplante intracarotídico de CMSCHU sobre o peso corporal e cerebral de animais na fase adulta;
- Determinar se o transplante intra-arterial de CMSCUH, em diferentes dosagens, promove neuroproteção a longo prazo do hemisfério cerebral lesionado, empregando a técnica estereológica de contagem de pontos associado ao princípio de Cavalieri para determinação de volumetria hemisférica cerebral;
- Investigar se as CMSCUH transplantadas intra-arterialmente em ratos neonatos submetidos ao modelo de HI podem ser identificadas, em diferentes órgãos (hemisférios cerebrais direito e esquerdo, fígado, baço e pulmões) e em diferentes tempos de análise (1, 3, 6 e 24 h; 7 e 30 dias) através da técnica da reação em cadeia da polimerase (*nested-PCR*), utilizando como marcador o gene da β-actina humana.

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Capítulo II

1 Hipóxia-isquemia Neonatal

A lesão cerebral hipóxico-isquêmica (HI), que ocorre no período neonatal, é uma das maiores causas de mortalidade e morbidade neurológica em crianças. Estatísticas sugerem uma incidência de asfixia em 2,5 (1,2-7,7) por 1000 nascimentos a termo em países desenvolvidos (Liao et al., 2012). No Brasil, estima-se que a prevalência de asfixia neonatal seja de, aproximadamente, 2% dos nascidos vivos (Souza 2003). Neonatos hipóxico-isquêmicos apresentam um baixo escore Apgar nos primeiros 5 minutos, exibem anormalidades na avaliação neurológica, acidose metabólica e, geralmente, crises convulsivas, podendo haver a necessidade de suporte respiratório nas primeiras horas de vida pós-natal (Liao et al., 2012). No passado, a taxa de mortalidade para recém-nascidos asfixiados variava de 50-75%. Devido aos avanços no manejo obstétrico e nos cuidados dos neonatos de risco, houve uma redução da prevalência para 27-50%. No entanto, em decorrência de uma maior taxa de sobrevivência dessas crianças, houve um aumento na prevalência de sequelas neurológicas a longo-prazo resultantes da lesão HI. Aproximadamente 90% das crianças com diagnóstico de encefalopatia severa (Sarnat III) no período perinatal apresentam incapacidades neuropsicológicas permanentes, incluindo retardo mental, paralisia cerebral, epilepsia, dificuldades de aprendizado e déficits visual e/ou auditivo. Nos casos de encefalopatia moderada (Sarnat II), 30-50% desenvolvem sérios déficits neurológicos. Já neonatos com encefalopatia HI leve geralmente não apresentam desfechos desfavoráveis (Dilenge et al., 2001).

Dados indicam que a lesão HI neonatal ocorre em aproximadamente 10 a 20% dos casos de paralisia cerebral. Crianças nos estágios I e II da encefalopatia HI frequentemente exibem déficits motores específicos dependendo da lesão, tais como a diplegia ou a hemiplegia espástica, geralmente sem transtornos cognitivos. Já as crianças na categoria III, usualmente, irão apresentar dificuldades motoras precoces, tais como atrasos no desenvolvimento neuropsicomotor e podem apresentar déficits cognitivos e epilepsia (Perlman 2006). A presença, a severidade e a distribuição das lesões neuropatológicas originárias da HI dependem de diversos fatores, incluindo a natureza e a duração do insulto, a idade gestacional do feto ou recém-nascido e a presença ou ausência de estresse sistêmico. A maioria dos episódios de lesão HI severa causa danos variados à estruturas cerebrais, incluindo porções específicas do córtex cerebral, hipocampo, gânglios da base, tálamo, tronco encefálico e a substância branca periventricular e subcortical (Berger and Garnier 1999).

A causa mais frequente da encefalopatia HI é a asfixia intra-útero e o principal mecanismo patogênico atribuído à sua neuropatologia é a redução do fluxo sanguíneo cerebral (Berger and Garnier 1999, Perlman 2006). Eventos neurotóxicos interligados, tais como, falência energética, despolarização da membrana, liberação de aminoácidos excitatórios, acúmulo de radicais livres e apoptose ocorrem simultaneamente e contribuem para a disfunção celular e morte neuronal após insultos (Vexler and Ferriero 2001). Em relação às respostas celulares, a redução do fluxo sanguíneo cerebral inicia uma cascata de eventos bioquímicos deletérios que duram horas ou dias. A depleção do oxigênio impossibilita a fosforilação oxidativa e ocorre uma mudança para o metabolismo anaeróbico. Este é um estado de energia ineficiente que resulta na rápida depleção de reservas de fosfato de alta energia, incluindo a molécula de adenosina trifosfato (ATP). A redução da síntese de ATP resultante da hipóxia acentuada altera o equilíbrio iônico através da membrana celular (Golan and Huleihel 2006, Perlman 2006). Em termos histológicos, a lesão HI produz cistos necróticos dentro do parênquima cerebral. A necrose decorre de uma agressão intensa às células, com inibição total das mitocôndrias, falência completa das bombas iônicas, edema e lise celular, ruptura das organelas, extravasamento do citoplasma no meio extracelular e presença de resposta inflamatória intensa com fagocitose. Trata-se de um processo passivo de morte celular. Após este processo, dá-se início a uma fase tardia de dano tecidual caracterizada pela morte celular apoptótica. A apoptose decorre da agressão lenta à célula, com inibição parcial da fosforilação oxidativa, redução do tamanho da célula e ruptura do DNA (Liao et al., 2012, Shankaran 2012).

Atualmente, nosso grupo de pesquisa investiga o uso de estratégias neuroprotetoras no modelo experimental mais utilizado para o estudo da lesão HI no período neonatal (Levine 1960, Rice et al., 1981, Vannucci and Vannucci 1997). Neste modelo, o dano cerebral é obtido pela associação de oclusão unilateral da artéria carótida comum, com subsequente exposição a ambiente hipóxico em ratos de 7 dias de vida. As lesões são encontradas no hemisfério ipsilateral à oclusão da artéria carótida nas regiões do córtex cerebral, substância branca periventricular e subcortical, estriado (gânglios da base) e hipocampo. A região hipocampal de CA3 (corno de Amon) é considerada a região mais suscetível, seguida da camada de células granulares, CA1 e hilo (Hossain 2005, Vannucci and Vannucci 1997). Aproximadamente 79% dos filhotes de ratos sobrevivem ao protocolo de HI e cerca de 90% dos sobreviventes apresentam danos cerebrais. Infarto do córtex cerebral ipsilateral e do hipocampo estão presentes em 56% e, dependendo da duração da sobrevivência pós-asfixia,

achados adicionais também são vistos, incluindo alterações na função neurológica e comportamental (Raju 1992).

Apesar dos avanços tecnológicos e científicos nos cuidados perinatais dos recém-nascidos de risco, o manejo clínico de crianças asfixiadas tem sido limitado à manutenção da oxigenação, ao controle da pressão sanguínea e da homeostase, ao tratamento das convulsões e ao controle da hipertensão intracraniana. No entanto, essas medidas não são dirigidas à prevenção ou à interrupção dos mecanismos de lesão cerebral (Vannucci 2000). Em decorrência da significância clínica e socioeconômica do dano cerebral neonatal, estratégias neuroprotetoras vêm sendo estudadas em ensaios experimentais e clínicos na tentativa de reduzir a lesão cerebral e, assim, melhorar os desfechos comportamentais. Bloqueadores de cálcio, inibidores de aminoácidos excitatórios, de radicais livres, de óxido nítrico, fatores de crescimento, hipotermia e terapias celulares são algumas das abordagens terapêuticas atuais que tentam interromper a cascata de eventos prejudiciais iniciada pelo evento hipóxico-isquêmico (Johnston et al., 2011, Shankaran 2012). Atualmente, a hipotermia é considerada uma alternativa no tratamento da síndrome hipóxico-isquêmica. O resfriamento da cabeça tem a capacidade de reduzir a extensão da lesão em modelos experimentais, assim como em estudos clínicos relacionados à isquemia e ao trauma. Os mecanismos potenciais de neuroproteção da hipotermia incluem inibição da liberação de glutamato, redução do metabolismo cerebral e inibição da apoptose. No entanto, aspectos como a duração da terapia, o grau de hipotermia, o momento e o método da intervenção e, principalmente, os efeitos adversos, ainda devem ser investigados (Shankaran 2012, Shankaran et al., 2012).

A terapia celular também vem sendo explorada como uma atual e plausível abordagem de tratamento para doenças neurológicas severas, incluindo a HI neonatal. Inicialmente usado no tratamento de doenças hematológicas malignas e distúrbios autoimunes, o transplante de células imaturas e indiferenciadas do sangue de cordão umbilical humano tem sido proposto atualmente como um candidato terapêutico para minimizar o dano de lesões no sistema nervoso central imaturo (Bennet et al., 2012, Dalous et al., 2012, de Paula et al., 2010, Liao et al., 2012, Pimentel-Coelho et al., 2012, Rosenkranz and Meier 2011, Wang et al., 2012). Apesar do número crescente de estudos sobre o uso de terapias neuroprotetoras inovadoras no tratamento de lesões HI em modelos animais, incluindo recentes contribuições do nosso laboratório (de Paula et al., 2012, de Paula et al., 2009, Greggio et al., 2009), infelizmente ainda não há terapia eficaz na prática clínica para o tratamento de HI neonatal e decorrentes sequelas.

2 Neuropeptídeo NAP em Hipóxia-Isquemia Neonatal

A proliferação neuronal, sobrevivência e diferenciação celular são atividades dependentes de fatores de crescimento proteicos durante o desenvolvimento. Sendo assim, a atividade de proteínas neurotróficas é parte fundamental da resposta do SNC a uma grande variedade de agentes que modulam a sua integridade. Geralmente, neurônios, quando estimulados por processos danosos, enviam sinais extracelulares a astrócitos e microglias que os intermedeiam, induzindo a liberação de neurotrofinas específicas (Zemlyak et al., 2007). O número crescente e diverso destas moléculas tornou-se aparente a partir da identificação de um primeiro fator, o peptídeo vasoativo intestinal (VIP; *vasoactive intestinal peptide*). Primeiramente identificado no intestino como uma molécula indutora de vasodilatação, descobriu-se que o VIP é o peptídeo cerebral mais abundante e apresenta capacidade neuroprotetora *in vivo*. Para que este composto garanta uma defesa neuronal adequada, torna-se necessário que astrócitos portadores de receptores específicos para o VIP sejam induzidos a sintetizar e secretar proteínas neuroprotetoras secundárias. Assim, o VIP promove a secreção, pelas células gliais, de determinados fatores tróficos tais como interleucina-1 (IL-1), interleucina-6 (IL-6), neurotrofina-3 (NT-3), protease nexin-1 (PN-1), as quimiocinas RANTES e MIP, fator neurotrófico atividade dependente (ADNF; *activity-dependent neurotrophic factor*) e proteína neuroprotetora atividade dependente (ADNP; *activity-dependent neuroprotective protein*) (Busciglio et al., 2007, Dejda et al., 2005), como apresentado na figura 1.

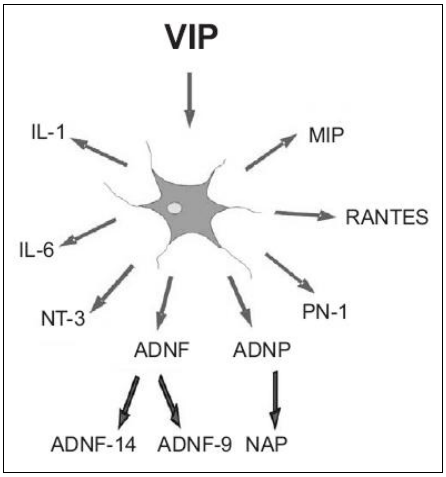

Figura 1 - Secreção do neuropeptídeo NAP. VIP promove a liberação de fatores gliais, tais como IL-1, IL-6, NT-3, PN-1, as quimiocinas RANTES e MIP, e os fatores neuroprotetores ADNF e ADNP. Os fatores ADNF-9, ADNF-14 e NAP são os fragmentos ativos das proteínas originais ADNF e ADNP, respectivamente. Fonte: Dejda *et al.* (2005); pg. 311.

Especificamente acerca do ADNP, este fator é fundamental nos processos de desenvolvimento cerebral e embriogênese (Mandel et al., 2007, Pinhasov et al., 2003). Paralelamente a estas funções, verificou-se um aumento significativo da expressão cerebral de ADNP um mês após lesão cerebral traumática experimental (Gozes et al., 2005b, Zaltzman et al., 2004). Esta evidência levantou a hipótese de que o ADNP estivesse envolvido em um mecanismo compensatório endógeno na neuroproteção. Corroborando tais achados, demonstrou-se uma expressão transitória aumentada de ADNP correlacionada à progressão inicial em um modelo animal de demência (Fernandez-Montesinos et al., 2010).

Dentre os diversos fatores identificados, Bassan e colaboradores (1999) isolaram o menor elemento ativo da molécula da proteína ADNP, denominado NAP (também conhecido por AL-108 e *davunetide*) (Gozes 2011a, c). Este peptídeo, de apenas oito aminoácidos (NAPVSIPQ) e peso molecular de 824,93 Da, confere proteção a culturas neuronais contra toxicidade associada ao peptídeo β-amilóide (Bassan et al., 1999, Zemlyak et al., 2000), NMDA (Bassan et al., 1999), bloqueio elétrico (tetrodotoxina) (Bassan et al., 1999), glicoproteína gp120 (Bassan et al., 1999), fator de necrose tumoral alfa (Beni-Adani et al., 2001), álcool (Wilkemeyer et al., 2003), dopamina (Offen et al., 2000), peróxido de oxigênio (Steingart et al., 2000), privação de nutrientes (Lagreze et al., 2005) e sobrecarga de zinco (Divinski et al., 2006). Na maioria destes casos, a neuroproteção *in vitro* se torna significativa em concentração de NAP na ordem de fentomolar e picomolar. Já em estudos *in vivo*, NAP tem ação protetora contra colinotoxicidade (Gozes et al., 2000), lesão retiniana (Belokopytov et al., 2011), crises convulsivas induzidas por hipóxia neonatal (Greggio et al., 2009) e por ácido caínico (Zemlyak et al., 2009), deficiência de apolipoproteína E (Bassan et al., 1999, Rotstein et al., 2006), traumatismo craniano (Beni-Adani et al., 2001, Zaltzman et al., 2004), oclusão da artéria cerebral média (Leker et al., 2002), síndrome alcoólica fetal (Spong et al., 2001), hipóxia-isquemia neonatal (Kumral et al., 2006), disfunções cognitivas e de ansiedade associadas à meia idade (Alcalay et al., 2004, Gozes et al., 2002, Gozes et al., 2005a), esquizofrenia (Gozes 2011b, Merenlender-Wagner et al., 2010), doença de Alzheimer/taupatias (Matsuoka et al., 2008, Shiryaev et al., 2009, Shiryaev et al., 2011, Vulih-Shultzman et al., 2007) e doença de Parkinson (Fleming et al., 2011).

Sabe-se que o NAP é um peptídeo altamente solúvel e biodisponível, e que sua estrutura secundária é do tipo helicoidal randômica, tanto em soluções aquosas como orgânicas (Gozes et al., 2005a). Assim, sua solubilidade em membranas lipídicas permite que este ultrapasse a barreira hematoencefálica (BHE), podendo atingir prontamente estruturas

cerebrais através de administração intranasal (Gozes et al., 2000), subcutânea (Beni-Adani et al., 2001), intravenosa (Leker et al., 2002) e intraperitonial (Greggio et al., 2009), em estudos pré-clínicos. É válido destacar, também, que o peptídeo NAP não altera o nível proliferativo celular, descartando a hipótese de que o composto possa oferecer algum risco de tumorigenicidade (Alcalay et al., 2004).

Frente ao amplo espectro de proteção do peptídeo NAP, até mesmo em concentrações na ordem de fentomolar em estudos *in vitro*, o seu mecanismo de ação é de grande interesse e foco de intensa investigação. Neste contexto, demonstrou-se que o NAP liga-se especificamente à tubulina, principalmente com a subunidade βIII-tubulina, presente em células neuronais. Esta propriedade, condizendo com função de chaperona, facilitaria a agregação de microtúbulos e aumentaria a sobrevivência e integridade celular (Divinski et al., 2006). Em contrapartida, tal ligação também é possível no citoesqueleto de astrócitos, tipo celular que não possui este tipo de subunidade tubulinar, o que sugere mecanismos de ação subjacentes. A manutenção da funcionalidade neuronal exercida pelo NAP, através da formação e estabilidade microtubular, também pode ser justificada pela regulação da mobilização de cálcio intra- e extracelular. Tais resultados sustentam a hipótese de que este peptídeo lipofílico não requer receptores celulares de superfície e nem ação dependente de quiralidade (Busciglio et al., 2007).

Seu mecanismo de ação também envolve o controle da formação de monofosfato de guanosina cíclico (cGMP) e óxido nítrico (Ashur-Fabian et al., 2001), modulação de mecanismos de inflamação (Beni-Adani et al., 2001), e também interfere em vários níveis do processo de apoptose, tais como sobre a expressão da proteína p53 (Gozes et al., 2004), liberação mitocondrial de citocromo C (Zemlyak et al., 2009), ativação da caspase 3 e fragmentação de DNA nuclear (Leker et al., 2002). Além disso, há evidências que indicam atividade antioxidativa do NAP, tanto em culturas neuronais (Busciglio et al., 2007, Gozes et al., 2004, Offen et al., 2000, Steingart and Gozes 2006, Steingart et al., 2000) como em modelos animais (Greggio et al., 2009, Spong et al., 2001). Alguns dos estudos acima citados demonstram o envolvimento do sistema antioxidante da glutationa endógena no mecanismo de neuroproteção do NAP. Em termos de reparação de biomoléculas, Visochek e colaboradores (2005) demonstraram uma via alternativa para ativação da poli(ADP-ribose)polimerase-1 (PARP-1), por mecanismos de sinalização induzidos por NAP, na ausência de dano ao DNA (Visochek et al., 2005). A ativação de PARP-1 induzida por NAP pode promover efeito neuroprotetor como resultado da poli-ADP ribosilação da histona 1 e relaxamento da cromatina. Assim, esta ação permitiria um acesso facilitado ao DNA pelos

fatores de transição e enzimas de reparo fundamentais para diferenciação e sobrevivência celular (Visochek et al., 2005). Adicionalmente, foi demonstrado que o NAP pode estimular vias bioquímicas envolvidas nos processos de sobrevivência neuronal, diferenciação e plasticidade sináptica, tais como a família das proteínas quinases ativadas por mitógenos (MAPKs) e fosfatidilinositol-3-quinase/Akt (PI3K/Akt) (Pascual and Guerri 2007, Sokolowska et al., 2011), fosforilação da quinase Fyn e proteína substrato associada à Crk (Cas) (Chen and Charness 2008).

Em 2009, nosso grupo de pesquisa demonstrou, de forma pioneira, a capacidade neuroprotetora do peptídeo NAP nas lesões oxidativas hipocampais de ratos neonatos submetidos a crises convulsivas induzidas por hipóxia. Além disto, observou-se que o NAP apresenta um efeito dose-dependente na prevenção de danos ao DNA e em membranas lipídicas paralelamente a um incremento do sistema glutationa (Greggio et al., 2009). Em caráter translacional, os resultados desta pesquisa sugerem a possibilidade desta droga aprimorar a abordagem terapêutica das crises convulsivas neonatais. Levando em consideração que as drogas anticonvulsivantes – que geralmente apresentam baixa eficácia e que podem interferir no neurodesenvolvimento da criança – continuam constituindo o tratamento de primeira linha para crises neonatais, o neuropeptídeo NAP poderia manter a integridade de estruturas cerebrais em um plano molecular, enquanto que a atividade convulsivante não esteja completamente supressa.

Existem apenas dois trabalhos, listados no banco de dados PubMed, verificando o efeito neuroprotetor do peptídeo NAP em animais com lesão HI neonatal. Inicialmente, Rotstein e colaboradores (2006) verificaram que administração de NAP (25 µL/mg; s.c.) promoveu efeitos positivos nos desfechos cognitivos e nas habilidades motoras avaliadas em camundongos deficientes de apolipoproteína E (Rotstein et al., 2006). Apesar de esta linhagem de animais possuir maior suscetibilidade a lesões cerebrais frente à hipóxia, o modelo clássico para estudos pré-clínicos de lesão HI neonatal permanece sendo o de Vanucci & Vanucci (1997) (Vannucci and Vannucci 1997). Além disso, os autores não verificaram se os efeitos terapêuticos do NAP nas avaliações funcionais acompanhavam uma possível recuperação histológica do dano cerebral destes animais. Em outro estudo, Kumral e colaboradores (2006) verificaram que uma única administração de NAP (0,3 µg/g; i.p.) foi capaz de diminuir significativamente a formação de óxido nítrico, preservando a densidade neuronal e reduzindo a quantidade de neurônios em processo de apoptose no hipocampo e no giro denteado de ratos neonatos com lesão HI (Kumral et al., 2006). Neste trabalho, no entanto, mesmo tendo sido usado o modelo animal apropriado de HI, os autores não

investigaram o impacto da terapia com NAP sobre os aspectos motores e cognitivos. Sendo assim, apesar dos indícios favoráveis da utilização do NAP neste tipo de lesão cerebral, ainda são incompletos os argumentos experimentais que sustentam a sua aplicabilidade terapêutica, em virtude das abordagens e desenhos experimentais utilizados.

Desde a sua obtenção sintética, o NAP, atualmente denominado de *Davunetide,* está sob proteção conferida por patente e licenciado exclusivamente para estudos clínicos desenvolvidos pela empresa de biotecnologia Allon Therapeutics Inc.. A partir do composto básico, surgiram variantes moleculares como AL-208, AL-408 e AL-508, sendo todas provenientes do ADNP, com exceção do composto AL-309, oriundo do ADNF. Conforme informações disponíveis no endereço eletrônico da empresa[7], os compostos já está sendo testados em ensaios clínicos, em etapas diferentes de desenvolvimento, para comprometimento cognitivo leve amnéstico[8], taupatias[9] (degeneração lobar frontotemporal e síndrome de degeneração corticobasal), paralisia supranuclear progressiva[10], doença de Alzheimer, déficit cognitivo associado à esquizofrenia[11] e déficit cognitivo leve após cirurgia de revascularização do miocárdio com circulação extracorpórea[12] (figura 2).

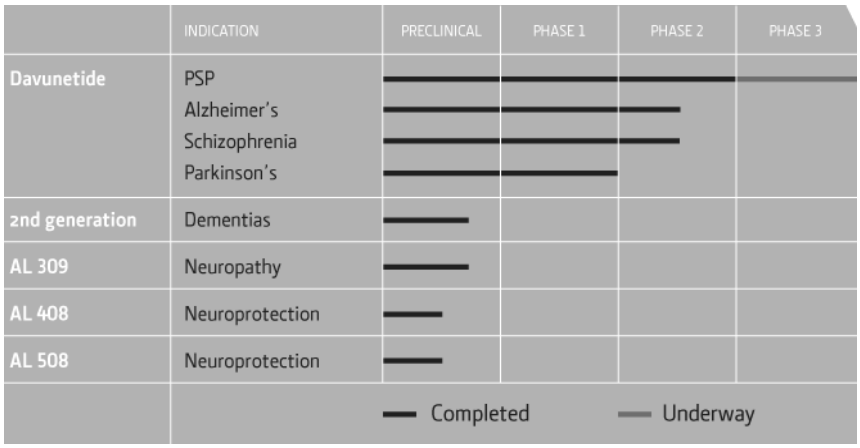

Figura 2 – Estudos pré-clínicos e clínicos da Allon Therapeutics Inc. O esquema apresenta as diversas etapas de investigação para o uso do NAP (Davunetide) e variações em estudos pré-clínicos e ensaios clínicos de fase I, II e III. As barras escuras representam estapas já concluídas, enquanto que as barras claras indicam estudos em andamento. Legenda: *PSP* (paralisia supranuclear progressiva); *Schizophrenia* (esquizofrenia); *Parkinson's* (doença de Parkinson); *Dementias* (demências); *Neuropathy* (neuropatia); *Neuroprotection* (neuroproteção). Fonte: endereço eletrônico da Allon Therapeutics Inc..

[7] http://www.allontherapeutics.com/product-development/development-status
[8] http://clinicaltrials.gov/ct2/show/NCT00422981
[9] http://clinicaltrials.gov/ct2/show/NCT01056965
[10] http://clinicaltrials.gov/ct2/show/NCT01110720
[11] http://clinicaltrials.gov/ct2/show/NCT00505765
[12] http://clinicaltrials.gov/ct2/show/NCT00404014

Apesar dos esforços em viabilizar a terapia com o peptídeo NAP em diversas neuropatologias, ainda são escassas as iniciativas terapêuticas deste composto na área pediátrica. Embora estudos experimentais tenham sido feitos em modelos de dano cerebral no período perinatal – tais como em HI (Kumral et al., 2006, Rotstein et al., 2006), crises convulsiva induzida por hipóxia (Greggio et al., 2009), síndrome alcoólica fetal (Spong et al., 2001) e paralisia cerebral (Sokolowska et al., 2011) – nenhum ensaio clínico com NAP encontra-se em andamento para quaisquer das patologias acima citadas. Assim sendo, frente às evidências experimentais favoráveis do uso do neuropeptídeo NAP, propõe-se aprimorar a abordagem experimental com o intuito de dar força à proposta translacional desta terapia inovadora na lesão HI neonatal.

3 Terapia Celular em Hipóxia-Isquemia Neonatal

ARTIGO DE REVISÃO

Use of stem cells in perinatal asphyxia: from bench to bedside[13]

de Paula S, Greggio S, DaCosta J.C.[14]

Jornal de Pediatria (Rio J). 2010 Nov-Dec;86(6):451-64.

doi:10.2223/JPED.2035

[13] A atualização da Tabela 1, intitulada *Revisão das publicações sobre terapia celular em hipóxia-isquemia experimental no período neonatal*, encontra-se no Apêndice B e está precedida pelas referências de todos os trabalhos tabelados.

[14] Os autores Simone de Paula e Samuel Greggio contribuíram igualmente na elaboração deste artigo de revisão.

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ARTIGO DE REVISÃO

Use of stem cells in perinatal asphyxia: from bench to bedside

O uso de células-tronco na asfixia perinatal: do laboratório à prática clínica

Simone de Paula[1], Samuel Greggio[1], Jaderson Costa DaCosta[2]

Resumo

Objetivos: Apresentar evidências científicas recentes sobre os efeitos do transplante com células-tronco em modelos animais de lesão cerebral hipóxico-isquêmica neonatal e abordar os aspectos translacionais relevantes à aplicação clínica da terapia celular nesse contexto.

Fonte dos dados: Para a seleção dos artigos, utilizou-se a base de dados PubMed e Scopus. O critério de seleção de artigos foi a especificidade em relação ao tema estudado, preferencialmente a partir do ano de 2000. Também foram revisados artigos clássicos de anos anteriores que se aplicavam ao propósito desta revisão.

Síntese dos dados: Células-tronco de diferentes fontes exógenas podem exibir propriedades neuroprotetoras em modelos experimentais de hipóxia-isquemia neonatal. Na maioria dos experimentos animais, os benefícios morfológicos e funcionais observados foram independentes da diferenciação neural, sugerindo mecanismos de ação associados, tais como a liberação de fatores tróficos e a modulação inflamatória.

Conclusões: Baseado nos estudos experimentais analisados, a terapia celular pode tornar-se uma promissora abordagem terapêutica no tratamento de crianças com encefalopatia hipóxico-isquêmica. No entanto, estudos adicionais necessitam ser realizados a fim de elucidar os possíveis mecanismos de ação dessas células e definir estratégias clínicas seguras e efetivas.

J Pediatr (Rio J). 2010;86(6):451-464: Encefalopatia hipóxico-isquêmica, células-tronco, asfixia, terapia celular.

Abstract

Objectives: To present recent scientific evidence on the effects of stem cell transplantation in animal models of neonatal hypoxic-ischemic brain injury and address the translational relevance of cell therapy for clinical application in this context.

Sources: The PubMed and Scopus databases were used to select articles. The selection criterion was the specificity of articles regarding the subject studied, preferably articles published from 2000 onward. We also reviewed classic articles from previous years that were applicable to this review.

Summary of the findings: Stem cells from different exogenous sources may exhibit neuroprotective properties in experimental models of neonatal hypoxia-ischemia. In most animal experiments, the morphological and functional benefits observed were independent of neural differentiation, suggesting associated mechanisms of action, such as the release of trophic factors and inflammatory modulation.

Conclusions: Based on the experimental studies analyzed, cell therapy may become a promising therapeutic approach in the treatment of children with hypoxic-ischemic encephalopathy. However, further studies are warranted to elucidate potential mechanisms of action of these cells and to define safe and effective clinical strategies.

J Pediatr (Rio J). 2010;86(6):451-464: Hypoxic-ischemic encephalopathy, stem cells, asphyxia, cell therapy.

Introdução

A lesão cerebral hipóxico-isquêmica (HI) do recém-nascido é uma das maiores causas de mortalidade e morbidade neurológica em crianças. Estatísticas sugerem uma incidência de asfixia em 2-4 por 1.000 nascimentos a termo. No Brasil, estima-se que a prevalência de asfixia neonatal seja de, aproximadamente, 2% dos nascidos vivos[1]. Além disso, a taxa de mortalidade dos recém-nascidos asfixiados no período neonatal é de 20-50%, sendo que mais de 25% dos sobreviventes podem exibir incapacidades neuropsicológicas permanentes, tais como retardo mental, paralisia cerebral, epilepsia e dificuldades de aprendizagem[2].

A causa mais frequente da encefalopatia HI é a asfixia intraútero severa, e o principal mecanismo patogênico atribuído à sua neuropatologia é a redução do fluxo sanguíneo

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cerebral[3]. Eventos neurotóxicos associados, tais como falência energética, despolarização da membrana, liberação de aminoácidos excitatórios, acúmulo de radicais livres e apoptose ocorrem simultaneamente e contribuem para a disfunção celular e para a morte neuronal após insultos hipóxico-isquêmicos[4].

Apesar dos avanços tecnológicos e científicos nos cuidados perinatais dos recém-nascidos de risco, o manejo clínico de crianças asfixiadas tem sido limitado à manutenção da oxigenação, ao controle da pressão sanguínea e da homeostase, ao tratamento das convulsões e ao controle da hipertensão intracraniana[5].

Novas estratégias neuroprotetoras vêm sendo investigadas em estudos experimentais e em ensaios clínicos em decorrência da significância clínica e do impacto socioeconômico originados pelo dano cerebral neonatal. Bloqueadores de cálcio, inibidores de aminoácidos excitatórios e de radicais livres, o uso de óxido nítrico, de fatores de crescimento, neuropeptídeos e a hipotermia são algumas das abordagens terapêuticas atuais que objetivam interromper a cascata de eventos neuroquímicos iniciada pela HI[4]. Com exceção da hipotermia, que demonstra desfechos satisfatórios apenas em crianças com lesões HI moderadas, essas terapias apresentam resultados limitados[6].

Nesse contexto, a terapia celular vem sendo explorada por ser uma atual e promissora abordagem de tratamento para doenças neurológicas graves. As células-tronco representam uma unidade natural do desenvolvimento embrionário e da reparação tecidual, sendo um subconjunto de células imaturas, indiferenciadas e não especializadas que apresentam a capacidade de autorrenovação e diferenciação em linhagens celulares específicas[7]. Tais células têm sido encontradas em todos os órgãos e tecidos pós-natais, inclusive no sistema nervoso central (SNC), previamente conhecido pela carência de progenitores celulares e de potencial regenerativo[8]. Recentes descobertas revolucionaram a biologia das células-tronco e têm demonstrado o potencial clínico dessas células em uma variedade de doenças humanas. Inicialmente usado no tratamento de doenças hematológicas malignas e distúrbios autoimunes, o transplante de células imaturas e indiferenciadas tem sido proposto atualmente como fonte potencial de novas células e de fatores tróficos para minimizar o dano celular e regenerar tecidos necróticos decorrentes de lesões no SNC[9]. Estudos experimentais demonstram que o transplante de células-tronco tem melhorado a funcionalidade em modelos experimentais de isquemia cerebral, doença de Parkinson, doença de Huntington, epilepsia e traumatismo raquimedular[10-14].

Basicamente, duas categorias de células-tronco têm sido utilizadas em estudos experimentais de HI neonatal: as células-tronco neurais, provenientes de tecido neuronal embrionário ou adulto; e as células-tronco somáticas de origem não neuronal, nas quais se destacam as provenientes do sangue da medula óssea e do cordão umbilical.

O objetivo desta revisão é apresentar o estado da arte dos estudos experimentais de terapia celular em modelo animal de HI neonatal (Tabela 1), abordando os possíveis mecanismos de ação desse recurso terapêutico e os aspectos translacionais relevantes à aplicação clínica do transplante de células-tronco na encefalopatia hipóxico-isquêmica.

Transplante de tecido neocortical fetal

Em 1996, Elsayed et al.[15] realizaram o primeiro estudo sobre o uso de recursos celulares no tratamento da HI experimental. Nessa investigação, os autores avaliaram os efeitos do transplante intracerebral de um bloco de tecido neocortical fetal realizado 7 dias após a indução da HI em filhotes de ratos. Embora tenham demonstrado que os transplantes obtiveram uma implantação satisfatória em 63% dos casos, o estudo não demonstrou efeitos terapêuticos significativos sobre a atrofia cerebral. Adicionalmente, o respectivo trabalho não realizou uma avaliação funcional dos animais transplantados. Outro grupo de pesquisa também realizou transplante intracerebral de tecido neocortical fetal 3 dias após a indução da HI[16]. Em vez de usar um bloco tecidual, os autores utilizaram uma suspensão celular a fim de facilitar o procedimento de transplante. Os resultados demonstraram uma melhora da coordenação e assimetria motora nos animais tratados. No entanto, mesmo com a identificação de 72% dos transplantes após 10-12 semanas pós-implantação, os autores observaram ausência de restabelecimento da citoarquitetura cortical.

Transplante de células-tronco neurais

As células-tronco neurais (CTN) são células com capacidade de autorrenovação e potencialidade restrita para gerar células de linhagens neuronais e gliais. Tais células podem ser isoladas de diferentes regiões do sistema nervoso embrionário ou extraídas de duas regiões específicas do cérebro adulto: a zona subventricular dos ventrículos laterais e a zona subgranular do giro denteado hipocampal[7,9]. Estudos mostram que as CTN podem migrar e sobreviver em áreas cerebrais lesadas, assim como podem ser induzidas a se diferenciar *in vivo* e *in vitro* em neurônios, oligodendrócitos e astrócitos, indicando uma possível alternativa de reposição dos tipos celulares afetados na HI[9,17].

Depois de isoladas, as CTN podem proliferar *in vitro*, em resposta à presença de fatores de crescimento específicos, formando agrupamentos celulares denominados de neuroesferas. Basicamente, essas estruturas são formadas por CTN multipotentes e progenitores neurais com desenvolvimento mais comprometido[9,18,19]. Alguns estudos têm demonstrado que astrócitos da zona subventricular que expressam uma proteína glial também podem ser considerados como CTN[20].

Nesse contexto, Zheng et al.[21] demonstraram que células-tronco astrocíticas multipotentes provenientes da zona subventricular de camundongos migram para a região cortical e periventricular isquêmica e expressam sinais de diferenciação neuronal e astrocítica nos animais HI submetidos ao transplante intracerebral. Em contrapartida, quando o transplante foi efetuado em animais sadios, as células permaneceram no local em que foram injetadas e mantiveram o seu perfil celular astrocítico. Esses dados indicam que o tecido cerebral HI apresenta processos fisiológicos capazes

Tabela 1 - Revisão das publicações sobre terapia celular em hipóxia-isquemia experimental no período neonatal

Referência	Modelo de HI (animal, idade e duração da hipóxia)	Tipo celular	Transplante (concentração, via, tempo e imunossupressão)	Resultados funcionais*	Resultados morfológicos, celulares e moleculares*	Migração celular	Diferenciação celular
Elsayed et al.[15]	Ratos Long-Evans black-hooded; P7-8; 60, 120 ou 120-150 min	Tecido neocortical fetal murino (13º dia embrionário)	Bloco tecidual de 1-2 mm^3; ic (TI); 7 dias após HI; SI	NA	Nenhum efeito sobre a atrofia cerebral. Presença de conectividade axonal entre o transplante e as áreas adjacentes corticais do tecido receptor	Transplantes com desenvolvimento satisfatório (63%) e pobre (19%), localizados no córtex ou em áreas adjacentes 6 semanas pT	NA
Jansen et al.[16]	Ratos Wistar; P7; 150 min	Tecido neocortical fetal murino (16º dia embrionário)	5 x 10^4 células/µL; ic (TI); 3 dias após HI; SI	Melhora da coordenação (3-8 semanas pT) e assimetria motora (9 semanas pT)	Ausência de restabelecimento da citoarquitetura cortical; o transplante expressou marcadores neuroquímicos e ausência de marcação glial; presença de astrócitos circundantes ao transplante. (9-11 semanas pT)	Identificação de 72% dos transplantes localizados em áreas adjacentes ao corpo caloso do córtex sensório-motor 9-11 semanas pT	NA
Park et al.[27]	Camundongos CD-1; P7; 120 min	CTN murinas (clone C17.2) em *scaffold* de PGA; CTN-PGA)	1 ou 2 complexos CTN-PGA (1 x 10^7 células/mL; 100-200 µL); ic (TI); 7 dias após HI; SI	↓ rotação unilateral	Redução do dano cerebral; formação de interconexões entre CTN/PGA e o tecido receptor; neovascularização; ↓ infiltração de monócitos e cicatriz astroglial; restabelecimento de projeções neuronais de longa distância	O transplante apresentou uma implantação satisfatória na cavidade infartada cerebral 2-6 semanas pT	Células transplantadas apresentaram marcação de neurônio (5%) e oligodendrócito, na penumbra cortical da lesão, 2 semanas pT
Imitola et al.[23]	Camundongos C57Bl/6; P7; 120 min	Cinco linhagens de CTN humanas e murinas	5 x 10^5 células/mL; ic (TC); 3 dias após HI; ciclosporina	NA	CTN expressam CXCR4; ↑ expressão de SDF-1α na região cerebral infartada (células astrocíticas e endoteliais); interação da via SDF-1α/CXCR4	Correlação positiva entre a presença de células transplantadas na região isquêmica e a expressão de SDF-1α	Células transplantadas com marcação neuronal na penumbra cortical da lesão
Katsuragi et al.[32]	Ratos Wistar; P14; 120 min	Células BHK-GDNF encapsuladas	1 cápsula (1 x 10^8 células/mL); ic (TI); 2 dias pré-HI; SI	NA	↑ GDNF sérico; ↓ incidência e severidade do dano neuronal (7 dias pT)	Células BHK-GDNF mantiveram-se viáveis 7 dias pT	NA
Katsuragi et al.[33]	Ratos Wistar; P9; 120 min	Células BHK-GDNF encapsuladas	1 cápsula (1 x 10^8 células/mL); ic (TI); 2 dias pré-HI; SI	Melhora no desempenho cognitivo a partir da 6ª semana pT	Redução do dano cerebral 17 semanas pT	NA	NA
Zheng et al.[21]	Ratos Sprague-Dawley; P7; 120 min	Células-tronco astrocíticas multipotentes da zona subventricular murina	5 x 10^4 células/µL; ic (TI); 24 h após HI; SI	NA	NA	Detecção das células transplantadas na região cortical e periventricular isquêmica em até 21 dias pT	Células transplantadas apresentaram marcação de astrócito e neurônio 3–21 dias pT
Meier et al.[36]	Ratos Wistar; P7; 80 min	CTCUH	1 x 10^7 células/ 500 µL; ip; 24 h após HI; SI	Melhora no desempenho locomotor 21 dias pT	Nenhum efeito sobre a atrofia cerebral em 21 dias pT	Presença das células transplantadas ao redor da lesão cerebral 21 dias pT	Ausência de células transplantadas com marcação para astrócito e neurônio 21 dias pT

BDNF = fator neurotrófico derivado do cérebro; BHK-GDNF = células renais de filhotes de hamster (BHK) transfectadas com DNA complementar de fator neurotrófico derivado de linhagem celular glial (GDNF); CPAM = células progenitoras adultas multipotentes; ChABC = condroitinase ABC; CTCUH = células-tronco de cordão umbilical humano; CTM = células-tronco mesenquimais; CTN = células-tronco neurais; CXCR4 = receptor de quimiocina CXC 4; FGF-2 = fator de crescimento de fibroblasto 2; GDNF = fator neurotrófico derivado de linhagem celular glial; HI = hipóxia-isquemia; ic = via intracerebral; ica = via intracardíaca; icv = via intracerebroventricular; ip = via intraperitoneal; iv = via intravenosa; NA = não avaliado; NGF = fator de crescimento neural; NT3 = neurotrofina -3; P = dia pós-natal; PGA = ácido poliglicólico; pT = pós-transplante; SDF-1 = fator derivado do estroma 1; T-PX = transplante no dia pós-natal X; SI = sem imunossupressão; TC = transplante contralateral; TI = transplante ipsilateral; VEH = veículo.

* Animais tratados *versus* animais controle.

↑ = aumento.

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Yasuhara et al.[58]	Ratos Sprague-Dawley; P7; 150 min	CPAM de medula óssea murina	2 x 10^5 células/3 µL; ic (TI); 7 dias após HI; ciclosporina A	Melhora da assimetria, mas não da coordenação motora, 14 dias pT	NA	Detecção das células transplantadas nas regiões hipocampais de CA2 e CA3 14 dias pT	NA
Park et al.[22]	Camundongos CD-1; P7; 138 min	CTN murinas (clone C17.2)	0,4-1,6 x 10^5 células/4 µL; ic; 1 (TI), 3 (TI ou TC), 7 (TI), 14 (TI) ou 35 (TI) dias após HI; SI	NA	NA	TC: migração celular através do corpo caloso e outras comissuras inter-hemisféricas para a área infartada; TI: as células permaneceram no local da lesão, com melhor implantação quando transplantadas entre 3 e 7 dias após HI	TI *versus* TC (3 dias após HI): células transplantadas apresentaram marcação de neurônio (~5 *versus* 0%), oligodendrócito (~4 *versus* ~1%), astrócito (~23 *versus* ~15%) e células progenitores neurais (~18 *versus* ~6%) no neocórtex (1 mês pT)
Park et al.[29]	Camundongos CD-1; P7; 120-180 min	CTN murinas (clone C17.2) com superexpressão de NT-3 (CTN-NT3)	3 x 10^5 células/8 µL (ic, TI em 2 locais) e 1,0 x 10^5 células/ 2 µL (icv, TC); 3 dias após HI; SI	NA	Expressão aumentada de NT-3	Presença das células nos locais de transplante 2-4 semanas pT	Células transplantadas com marcação neuronal (colinérgico, GABAérgico e glutamatérgico) na penumbra (81,4%) e área infartada (10-20%), e para oligodendrócito (0,4%) e glia (1%); células transplantadas com marcação neuronal no hemisfério contralateral (~90%) (2-4 semanas pT)
Ma et al.[24]	Camundongos C57Bl/6; P7; 90 min	Células-tronco embrionárias murinas após diferenciação neural *in vitro*	1 x 10^4 células/µL; icv (TI); 2-3 dias após HI; SI	Melhora da memória espacial 2 e 8 meses pT	↑ células neuronais na região hipocampal de CA1 8 meses pT	Detecção das células transplantadas no hipocampo e córtex cerebral 2 e 8 meses pT	Células transplantadas apresentaram marcação de neurônio, mas não de astrócito, 8 meses pT
Dayer et al.[30]	Ratos Wistar; P3; 30 min	Células progenitoras neurais murinas com superexpressão de FGF-2	4 x 10^4 células/ 0,5 µL; ic (TI); 1 e 3 dias após HI; SI	NA	Preservação do estado de imaturidade e proliferação celular é inversamente proporcional à expressividade de FGF-2 nas células transplantadas	Detecção das células transplantadas no córtex isquêmica infragranular e na margem entre córtex e corpo caloso 2 semanas pT	A grande maioria das células transplantadas apresentou marcação de neurônio imaturo; em menor número, houve marcação para astrócito, oligodendrócito e neurônio

BDNF = fator neurotrófico derivado do cérebro; BHK-GDNF = células renais de filhotes de hamster (BHK) transfectadas com DNA complementar de fator neurotrófico derivado de linhagem celular glial (GDNF); CPAM = células progenitoras adultas multipotentes; ChABC = condroitinase ABC; CTCUH = células-tronco de cordão umbilical humano; CTM = células-tronco mesenquimais; CTN = células-tronco neurais; CXCR4 = receptor de quimiocina CXC 4; FGF-2 = fator de crescimento de fibroblasto 2; GDNF = fator neurotrófico derivado de linhagem celular glial; HI = hipóxia-isquemia; ic = via intracerebral; ica = via intracardíaca; icv = via intracerebroventricular; ip = via intraperitoneal; iv = via intravenosa; NA = não avaliado; NGF = fator de crescimento neural; NT3 = neurotrofina -3; P = dia pós-natal; PGA = ácido poliglicólico; pT = pós-transplante; SDF-1 = fator derivado do estroma 1; T-PX = transplante no dia pós-natal X; SI = sem imunossupressão; TC = transplante contralateral; TI = transplante ipsilateral; VEH = veículo.

* Animais tratados *versus* animais controle.

↑ = aumento.

↓ = redução.

Tabela 1 - Revisão das publicações sobre terapia celular em hipóxia-isquemia experimental no período neonatal *(continuação)*

Referência	Modelo de HI (animal, idade e duração da hipóxia)	Tipo celular	Transplante (concentração, via, tempo e imunossupressão)	Resultados funcionais*	Resultados morfológicos, celulares e moleculares*	Migração celular	Diferenciação celular
Yasuhara et al.[57]	Ratos Sprague-Dawley; P7; 150 min	CPAM de medula óssea murina	2 x 10^5 células/3 µL; iv ou ic (TI); 7 dias após HI; SI	Melhora da coordenação e assimetria motora 14 dias pT (iv e ic)	Redução da morte neuronal na região hipocampal de CA3 14 dias pT (iv e ic)	Detecção das células transplantadas nas regiões hipocampais isquêmicas de CA2 e CA3 14 dias pT (iv e ic). Localizavam-se também em vasos sanguíneos no hipocampo (iv)	Células transplantadas apresentaram marcação neuronal 14 dias pT (iv e ic)
Sato et al.[28]	Ratos Sprague-Dawley; P7; 120 min	CTN murinas (14º dia embrionário) ou CTN+ChABC (25 mU)	2,5 x 10^5 células/ 5 µL; icv (TI); 24 h após HI; SI	NA	↓ dano cerebral (CTN+ChABC < CTN = VEH); ↑ peso cerebral (CTN+ChABC > CTN = VEH) (7 dias pT)	Presença das células transplantadas na lesão isquêmica e áreas adjacentes 7 dias pT	NA
de Paula et al.[38]	Ratos Wistar; P7; 120 min	CTCUH	1 x 10^7 células/ 100 µL; iv; 24 h após HI; SI	Nenhum efeito sobre o déficit da memória espacial 3 semanas pT	Nenhum efeito sobre a atrofia cerebral 3 semanas pT	Detecção de poucas células transplantadas 24 h, 1 e 3 semanas pT	NA
Yasuhara et al.[40]	Ratos Sprague-Dawley; P7; 150 min	CTCUH ou CTCUH+M (manitol 1.1 mol/L)	1,5 x 10^4 células/ 200 µL; iv; 7 dias após HI; SI	Melhora da coordenação e assimetria motora 7 e 14 dias pT (CTCUH < CTCUH+M)	↑ níveis cerebrais de GDNF, BDNF e NGF 3 dias pT (CTCUH < CTCUH+M); ↑ densidade dendrítica na região hipocampal de CA1 14 dias pT	Presença de poucas células transplantadas no hipocampo isquêmico 14 dias pT	NA
Pimentel-Coelho et al.[37]	Ratos Lister-Hooded; P7; 90 min	CTCUH	2 x 10^6 células/ 200 µL; ip; 3 h após HI; SI	Melhora no desenvolvimento dos reflexos sensório-motores 4 dias pT	↓ morte neuronal e expressão de caspase 3 no estriado isquêmico 2 dias pT; ↓ ativação microglial no córtex 7 dias pT	Presença de poucas células transplantadas no córtex e estriado isquêmico 2 dias pT	NA
Jenny et al.[31]	Ratos Wistar; P3; 30 min	Células progenitoras neurais murinas com superexpressão de FGF-2	2–5 x 10^4 células/ 1 µL; ic (TI); 4 dias após HI; SI	NA	Formação de agrupamentos celulares com superexpressão de FGF-2 em áreas perivasculares	Presença das células transplantadas em áreas de lesão cortical e próximas a vasos sanguíneos 7 dias pT	Células transplantadas apresentaram marcação de neurônio imaturo 7 dias pT
van Velthoven et al.[56]	Camundongos C57Bl/6; P9; 45 min	CTM de medula óssea murina	1 x 10^5 células/2 µL; ic (TI); 3 dias (T-P12) ou 10 dias (T-P19) após HI; SI	Melhora da assimetria motora em 7 e 18 dias pT (T-P12), ou em 11 e 18 pT (T-P19)	T-P12: ↓ lesão cerebral (18 dias pT); ↑ neuro- e oligodendrogênese, e ↓ proliferação microglial no hipocampo e córtex isquêmico (7 e 18 dias pT); ↑ proliferação de astrócitos no hipocampo, e ↓ no córtex isquêmico (7 dias pT). T-P19: ↓ lesão cerebral (18 dias pT)	Menos de 1% das células em proliferação no hemisfério isquêmico originaram-se daquelas transplantadas	NA
Lee et al.[55]	Ratos Sprague-Dawley; P7; 210 min	CTM de medula óssea humana	1 x 10^6 células/µL; ica; 3 dias após HI; SI	Apesar de nenhum efeito sobre o déficit na coordenação motora, houve ↓ assimetria motora 20 dias pT	Nenhum efeito sobre a atrofia cerebral 6 semanas pT	Detecção das células transplantadas igualmente distribuídas em ambos os hemisférios cerebrais 6 semanas pT	Células transplantadas apresentaram marcação de astrócito e microglia (> nº), neurônio e oligodendrócito (< nº), igualmente distribuídas em ambos os hemisférios cerebrais 6 semanas pT

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Referência	Modelo de HI (animal, idade e duração da hipóxia)	Tipo celular	Transplante (concentração, via, tempo e imunossupressão)	Resultados funcionais*	Resultados morfológicos, celulares e moleculares*	Migração celular	Diferenciação celular
Xia et al.[41]	Ratos Sprague-Dawley; P7; 150 min	CTM de sangue de cordão umbilical humano	5 x 10^4 células/µL; ic (TI); 3 dias após HI; ciclosporina	Melhora da função neurológica 14, 21 e 28 dias pT	Atenuação do dano cerebral 28 dias pT	Presença das células transplantadas no córtex e dispersão para hipocampo 7 dias pT	Células transplantadas apresentaram marcação de astrócito, mas não de neurônio, 7 dias pT
Daadi et al.[25]	Ratos Sprague-Dawley; P7; 90 min	CTN humana de células-tronco embrionárias	5 x 10^4 células/µL; ic (TI em 3 locais); 24 h após HI; SI	Melhora da coordenação e assimetria motora 28-30 dias pT	Nenhum efeito sobre a atrofia cerebral; superexpressão de genes envolvidos na neuro-/gliogênese e suporte neurotrófico no hemisfério isquêmico; ↑ brotamento axonal para o hemisfério ipsilateral; ↑ terminais axonais no córtex sensório-motor, corpo caloso, estriato e tálamo ipsilateral; ↑ células microgliais no estriado isquêmico, mas não no córtex (1 mês pT)	Permanência das células transplantadas, com dispersão para o estriato isquêmico, em 1 mês pT	Células transplantadas apresentaram marcação de CTN, neurônio e astrócito 1 mês pT

BDNF = fator neurotrófico derivado do cérebro; BHK-GDNF = células renais de filhotes de hamster (BHK) transfectadas com DNA complementar de fator neurotrófico derivado de linhagem celular glial (GDNF); CPAM = células progenitoras adultas multipotentes; ChABC = condroitinase ABC; CTCUH = células-tronco de cordão umbilical humano; CTM = células-tronco mesenquimais; CTN = células-tronco neurais; CXCR4 = receptor de quimiocina CXC 4; FGF-2 = fator de crescimento de fibroblasto 2; GDNF = fator neurotrófico derivado de linhagem celular glial; HI = hipóxia-isquemia; ic = via intracerebral; ica = via intracardíaca; icv = via intracerebroventricular; ip = via intraperitoneal; iv = via intravenosa; NA = não avaliado; NGF = fator de crescimento neural; NT3 = neurotrofina -3; P = dia pós-natal; PGA = ácido poliglicólico; pT = pós-transplante; SDF-1 = fator derivado do estroma 1; T-PX = transplante no dia pós-natal X; SI = sem imunossupressão; TC = transplante contralateral; TI = transplante ipsilateral; VEH = veículo.
* Animais tratados *versus* animais controle.
↑ = aumento.
↓ = redução.

de atrair células-tronco astrocíticas para o local de lesão, assim como de induzir a neurodiferenciação.

Em um elegante trabalho, Park et al.[22] apresentaram interessantes observações do comportamento de CTN quando transplantadas no cérebro de camundongos neonatos submetidos à HI. Quando transplantadas no hemisfério contralateral à lesão HI, as CTN migraram através do corpo caloso e de outras comissuras inter-hemisféricas para a área infartada. Já quando o transplante foi feito no hemisfério ipsilateral, as células permaneceram no local da lesão. Em ambos os casos, as CTN diferenciaram-se no sítio isquêmico, apresentando marcação para neurônio, oligodendrócitos, astrócitos e células progenitoras neurais. Além disso, esse mesmo grupo de pesquisa elucidou os mecanismos inflamatórios envolvidos na migração da CTN frente à lesão HI[23].

Células-tronco embrionárias da camada interna do blastocisto de embriões também apresentam a capacidade de diferenciar-se em neurônios através de protocolos de cultura específicos, podendo ser consideradas fontes celulares alternativas para o tratamento de lesões cerebrais. Dois estudos utilizaram células-tronco embrionárias neurodiferenciadas em modelo de HI. No primeiro deles, demonstrou-se que o transplante de células-tronco embrionárias neurodiferenciadas *in vitro* resultou na melhora da memória espacial em ratos HI[24]. No entanto, embora tenha ocorrido aumento de células neuronais no hipocampo isquêmico, não há evidências claras de que o sucesso nos desfechos encontrados esteja relacionado à repopulação neuronal, à diferenciação ou à liberação de fatores tróficos pelas células administradas. Em outro trabalho recentemente publicado, verificou-se o restabelecimento da coordenação e simetria motora de ratos HI após transplante de células-tronco embrionárias humanas neurodiferenciadas. No entanto, os autores constataram ausência de efeito reparador sobre o dano estrutural do cérebro[25].

O ambiente isquêmico remanescente pode influenciar na restauração tecidual em decorrência da agressividade do processo HI. As lesões HI podem se associar à volumosa necrose tecidual, resultando em um cisto porencefálico. Tal situação oferece um microambiente prejudicado para o abrigo de células transplantadas devido à carência de aporte sanguíneo e matriz extracelular adequada. Através de estudos na área da engenharia tecidual, uma nova abordagem de tratamento tem sido proposta com o uso de matrizes (*scaffolds*) biodegradáveis dentro da cavidade

infartada, a fim de intensificar a formação de conexões entre o enxerto e o tecido hospedeiro[9,26]. Utilizando essa abordagem terapêutica, Park et al.[27] demonstraram uma implantação satisfatória dos *scaffolds* associada à redução da cavidade cerebral infartada e ao déficit motor em ratos submetidos à HI. Surpreendentemente, constatou-se a formação de interconexões entre o implante e o tecido receptor, além do restabelecimento de projeções neuronais de longa distância anteriormente prejudicadas pelo processo HI.

Outros compostos também podem ser associados à terapia celular com o objetivo de prover suporte ao desenvolvimento de CTN em modelo de HI. Um exemplo disso é a utilização de condroitinase ABC (ChABC) associada ao transplante intracerebroventricular de CTN em ratos neonatos submetidos à HI que resultou na redução acentuada do dano cerebral[28]. Devido à capacidade de promover plasticidade cerebral, os autores sugerem que a ChABC teria facilitado a adesão e migração de CTN para áreas isquêmicas.

Considerando CTN como possíveis vetores de fatores tróficos, alguns trabalhos utilizam células-tronco geneticamente modificadas para expressar níveis aumentados dessas substâncias. Utilizando CTN que superexpressam neurotrofina-3 (CTN/NT-3) no cérebro de ratos HI, os pesquisadores observaram aumento da porcentagem de diferenciação neuronal de 5 para 10-20% na área infartada e > 80% na penumbra, quando comparado à linhagem de CTN não geneticamente modificada. Os autores sugerem que a produção da NT3 pelas CTN possa ter atuado de maneira autócrina e parácrina, ligando-se às células adjacentes do tecido hospedeiro e estimulando a neurogênese endógena[22,29]. Outros estudos também têm demonstrado que CTN que superexpressam fatores tróficos específicos migram para a região lesada e formam nichos de células imaturas e proliferativas disponíveis para o reparo celular após HI[30]. Adicionalmente, identificou-se que o ambiente perivascular é fundamental para a manutenção dessas células em um estado ativo de imaturidade e proliferação[31]. Estudos também mostram que a utilização de um vetor celular para liberar determinados fatores tróficos reduz a incidência e extensão do dano cerebral e cognitivo em estudos experimentais de HI[32,33].

Transplante de células-tronco de cordão umbilical

Células-tronco de cordão umbilical humano (CTCUH) têm sido usadas em alguns estudos experimentais de lesão HI em decorrência de sua capacidade de diferenciação em neurônios e células gliais *in vitro* e *in vivo*. No entanto, há poucos estudos usando células de sangue de cordão umbilical não humano em modelos animais de doenças neurológicas, havendo divergências do uso concomitante de imunossupressores[34,35].

O primeiro estudo a testar o efeito do transplante de CTCUH em ratos HI foi realizado por Meier et al.[36]. Os autores demonstraram que a injeção intraperitoneal de CTCUH 24 h após a lesão HI em roedores neonatos resultou em melhora do padrão de marcha dos animais. Pode-se também constatar que as células-tronco migraram para a região da lesão cerebral, porém não se observou redução da atrofia cerebral e transdiferenciação das células transplantadas. Utilizando a mesma via de administração dessas células, um grupo de pesquisadores demonstrou que a injeção intraperitoneal de CTCUH, 3 h após a lesão HI, tem a capacidade de melhorar os reflexos primitivos desses animais[37]. Além disso, os autores demonstraram redução da morte celular no estriado e um efeito anti-inflamatório no córtex. No entanto, foi possível verificar a presença de poucas células transplantadas no estriado e córtex isquêmicos dos animais tratados.

No mesmo ano, nosso grupo de pesquisa publicou um estudo sobre os efeitos da administração intravenosa de CTCUH em HI experimental[38]. No entanto, nossos resultados demonstraram apenas uma tendência para a redução dos déficits comportamentais e morfológicos nos ratos tratados 30 dias após o transplante. Além disso, poucas células transplantadas foram identificadas no cérebro dos animais tratados. Aspectos como dose, tempo de seguimento para a avaliação e via de administração podem ter influenciado nos desfechos do estudo.

Paralelamente, também desenvolvemos um modelo experimental de hipóxia-isquemia em suínos neonatos para o estudo da terapia celular em animais de médio porte[39]. Os suínos recém-nascidos que receberam CTCUH através da artéria carótida comum apresentaram melhores escores neurológicos quando comparados aos animais apenas submetidos ao modelo de HI ou ao grupo transplantado via artéria umbilical. Adicionalmente, foi possível identificar a presença das células transplantadas apenas em alguns animais do grupo transplantado por via artéria carótida.

Um interessante estudo demonstrou que a injeção intravenosa de CTCUH, 7 dias após a lesão HI, melhorou a coordenação e assimetria motora dos ratos tratados[40]. Além disso, foram detectados níveis aumentados de fatores de crescimento no hemisférico cerebral ipsilateral à lesão 3 dias pós-transplante. Nesse estudo, os autores também incluíram um grupo de animais HI que receberam a mesma dose de CTCUH associado ao uso de manitol, substância que aumenta a permeabilidade da barreira hematoencefálica. Os resultados desse grupo experimental demonstraram que o manitol potencializou ainda mais os efeitos funcionais e a expressão dos fatores tróficos, em comparação ao grupo apenas transplantado com CTCUH.

A viabilidade terapêutica de células-tronco mesenquimais, provenientes de cordão umbilical humano em modelo experimental de HI, também foi recentemente analisada[41]. O transplante foi efetuado 3 dias após HI através da via intracerebral. Os animais tratados apresentaram uma melhora progressiva da função neurológica, associada à atenuação do dano cerebral. Além disso, verificou-se a presença das células transplantadas no córtex, com dispersão em direção ao hipocampo, 7 dias pós-transplante, apresentando diferenciação astrocítica, mas não neuronal.

Em contexto clínico, pesquisadores da Universidade Duke iniciaram estudo fase I com transplante autólogo de células-tronco de cordão umbilical em recém-nascido a termo com encefalopatia HI de grau moderado a severo (NCT00593242). As crianças serão acompanhadas para avaliação de neurodesenvolvimento e submetidas a exames de ressonância mag-

nética. Até o presente momento, o ensaio clínico encontra-se na fase de recrutamento de pacientes compatíveis com os critérios de seleção do estudo.

Transplante de células-tronco de medula óssea

Estudos utilizando células-tronco da medula óssea também têm sido amplamente utilizados em modelos animais de lesão isquêmica cerebral, como em ratos adultos[42-45] e, mais recentemente, em ensaios clínicos, demonstrando resultados promissores[46,47].

O sangue da medula óssea contém, pelo menos, duas populações celulares com um grande potencial para o uso clínico: as células-tronco hematopoiéticas e as células-tronco mesenquimais (CTM)[34]. A descoberta de que células-tronco hematopoiéticas também possuíam a capacidade de se transdiferenciar em linhagem neuronal ampliou o espectro de utilização dessa fonte de células imaturas[48,49]. Especificamente, as CTM são derivadas do estroma medular e são definidas conforme a presença de marcadores de superfície específicos, comportamento *in vitro* e potencial de diferenciação[50]. Esse tipo celular apresenta uma elevada capacidade de expressar fenótipos neuronais *in vitro*[51,52] e *in vivo*[53,54].

Recentemente, dois grupos de pesquisa apresentaram seus resultados utilizando CTM de medula óssea em modelo de HI experimental. Lee et al.[55] utilizaram a via intracardíaca para o transplante 72 h após a lesão. Os autores observaram a presença dos implantes celulares igualmente distribuídos em ambos os hemisférios cerebrais 6 semanas após o transplante. Tais células expressaram mais frequentemente marcadores de astrócito e microglia, além de marcadores de neurônio e oligodendrócitos, em menor número. Adicionalmente, observou-se melhora da assimetria motora nos ratos HI 40 dias após o transplante, embora não tenha sido constatada a reparação do dano isquêmico tecidual.

Já em outro estudo, demonstrou-se que a injeção intracerebral de CTM, 72 h após lesão, aumentou a proliferação e a diferenciação neuronal no hemisfério isquêmico[56]. Os desfechos histológicos e motores também foram favoráveis 10 e 21 dias após o tratamento. No entanto, menos de 1% dos novos neurônios eram derivados das células transplantadas. O estudo sugere que as CTM tenham favorecido a formação endógena de novas células neuronais a partir da redução da atividade inflamatória microglial.

Utilizando uma fração purificada das CTM, denominada de células progenitoras adultas multipotentes (CPAM), Yasuhara et al.[57] demonstraram que a administração intracerebral ou intravenosa de CPAM de medula óssea de camundongo, 7 dias após HI, resultou em melhora motora dos animais transplantados. Curiosamente, houve equivalência na recuperação do déficit motor entre os animais que receberam transplante alogênico (entre animais geneticamente diferentes, porém da mesma espécie) e aqueles que receberam transplante singênico (entre animais geneticamente idênticos). Associada à perda neuronal diminuída, detectou-se também a presença de CPAM nas regiões hipocampais isquêmicas e a marcação neuronal 14 dias pós-transplante[57,58].

Mecanismos de ação envolvidos na terapia celular em hipóxia-isquemia experimental

Compreender os mecanismos de ação envolvidos no processo de neuroregeneração pelas células-tronco é fundamental para otimizar os benefícios clínicos da terapia celular. Diversos fatores (resumidos na Tabela 2) podem ser responsáveis pelos resultados positivos apresentados em estudos recentes de HI experimental. No entanto, apesar dos benefícios significativos demonstrados nesses trabalhos, as variáveis responsáveis pelo sucesso do transplante de células-tronco em HI ainda não foram estabelecidas.

Transdiferenciação celular

A primeira hipótese estudada para justificar os efeitos benéficos da terapia celular em doenças neurológicas foi a da transformação direta de células-tronco em neurônios maduros, processo denominado de transdiferenciação. No entanto,

Tabela 2 - Principais mecanismos de ação das células-tronco na asfixia perinatal

Mecanismo de ação	Descrição
Transdiferenciação celular	Transformação direta de células-tronco (diversas fontes) em neurônios maduros
Liberação de fatores tróficos	Células-tronco podem funcionar como vetores para a produção e/ou liberação de fatores neurotróficos
Estimulação da neurogênese endógena	É possível que as células-tronco transplantadas possam potencializar esses mecanismos intrínsecos de reparação
Modulação do processo inflamatório	Células-tronco podem inibir a ativação de diversas células imunes e, consequentemente, aumentar a neurogênese e a produção de fatores tróficos
Estimulação da angiogênese	Tem-se observado a transdiferenciação das células-tronco em novos vasos (angiogênese), o aumento da vascularização nas áreas de penumbra e a formação indireta de novos vasos através da liberação de fatores de crescimento
Indução da neuroplasticidade	Aumento nas conexões aferentes e eferentes entre o local da lesão e as regiões cerebrais, restaurando as atividades sinápticas locais através da sinaptogênese

os estudos com HI têm mostrado que a recuperação funcional dos animais tratados tem ocorrido independente da presença de células-tronco com expressão de marcadores neuronais nas áreas isquêmicas. A transdiferenciação de células-tronco adultas de origem mesodérmica em células derivadas da camada ectodérmica ainda permanece em debate. Em estudos *in vitro*, a transdiferenciação é detectada através da positividade de marcadores específicos de neurônios e células gliais. No entanto, a caracterização de propriedades eletrofisiológicas dessas células transdiferenciadas ainda está pouco documentada[59-61]. Em decorrência desses achados, outros mecanismos têm sido propostos[62].

Liberação de fatores tróficos

Os fatores tróficos constituem uma família de polipeptídeos essenciais para a sobrevivência e a diferenciação de neurônios normais em desenvolvimento, além de terem um papel importante na neuroproteção de neurônios maduros sob condições patológicas[63]. As células-tronco podem servir como veículos de moléculas específicas, funcionando como vetores para a produção e/ou liberação de fatores neurotróficos[29,64]. Interagindo com seus receptores, é possível que as células-tronco possam liberar fatores de crescimento e citocinas, inibindo processo de apoptose, aumentando a angiogênese e/ou estimulando a diferenciação de células precursoras endógenas[12,40]. Dados obtidos no estudo de Borlongan et al.[65] reportaram um aumento de 15% na produção de fatores tróficos no sangue circulante de ratos adultos submetidos à isquemia cerebral após transplante de CTCUH. Recentemente, o mesmo grupo demonstrou resultados similares após o transplante de células-tronco em um modelo de HI neonatal em ratos[40]. Embora exista a liberação endógena de fatores tróficos pelo tecido neuronal frente a lesões isquêmicas, esse mecanismo compensatório é insuficiente para promover a regeneração tecidual e/ou recuperação funcional. Portanto, os autores desses estudos sugerem que o aumento de fatores tróficos específicos seja, provavelmente, em decorrência do transplante de células-tronco.

Estimulação da neurogênese endógena

Diversos estudos demonstraram aumento da neurogênese endógena frente às lesões HI neonatais[66,67]. No entanto, é possível que as células-tronco transplantadas possam potencializar esses mecanismos intrínsecos de reparação. No estudo de van Velthoven et al.[56], verificou-se que o transplante de células-tronco em animais HI reduziu a proliferação microglial e aumentou a neurogênese. Os autores sugerem que as células transplantadas tiveram a capacidade de modular a resposta inflamatória pós-HI, favorecendo o processo de neurogênese endógena.

Modulação do processo inflamatório

Apesar de benéfico para o recrutamento e migração de células-tronco até o local da lesão, o processo inflamatório determinado por lesões cerebrais parece ser restritivo para a diferenciação celular[23]. Nesse contexto, estudos atuais têm demonstrado que diferentes fontes de células-tronco podem inibir a ativação de diversas células imunes. Pluchino et al.[68] mostraram que células-tronco neurais promoveram a neuroproteção através de citocinas anti-inflamatórias e de moléculas imunomodulatórias. Em outro estudo, a injeção intravenosa de CTCUH aumentou a sobrevivência do tecido neuronal e reduziu a infiltração de leucócitos e a expressão de proteínas pró-inflamatórias no cérebro isquêmico[69].

No SNC, as células microgliais constituem a primeira linha de defesa imunológica e compreendem um dos principais processos inflamatórios que contribuem ao dano cerebral. Essa população celular libera uma variedade de citocinas e fatores de crescimento e reagem rapidamente no tecido cerebral lesado para promover a fagocitose e apresentar antígenos para as células T. No entanto, a ativação microglial está comumente associada à diminuição da produção de fatores tróficos e com redução da neurogênese[9,63,70]. Pode-se observar que, em dois estudos de terapia celular em HI experimental, o transplante de células-tronco reduziu a expressão de microglia no córtex e hipocampo isquêmico e, consequentemente, melhorou os desfechos funcionais nos animais tratados[37,56]. Em contrapartida, Daadi et al.[25] verificaram aumento de proliferação de células microgliais no estriato de animais HI transplantados com CTN. Esses dados sustentam a ideia de que as células microgliais desempenham um papel pró- e anti-inflamatório, dependendo do seu estado de ativação e fenótipo funcional[71].

Estimulação da angiogênese

Alguns estudos com lesões isquêmicas em ratos adultos têm mostrado que há um aumento da vascularização nas áreas de penumbra poucos dias após o transplante de células-tronco[72,73]. Além disso, tem-se observado a transdiferenciação das células-tronco transplantadas em novos vasos (angiogênese)[34,74]. É provável que também ocorra um evento indireto das células na formação dos vasos sanguíneos. Chen et al.[75] mostraram que células-tronco do estroma da medula óssea promoveram angiogênese na região isquêmica através de um aumento dos níveis endógenos de fator de crescimento endotelial vascular. Até o presente momento, existe apenas um estudo indicando neovascularização no tecido cerebral de animais HI transplantados com CTN[27]. Os autores sugerem que tenha ocorrido a elaboração de sinais angiogênicos pós-transplante, propiciando a formação de um parênquima novo e vascularizado no local do cisto porencefálico.

Indução da neuroplasticidade

Eventos neuroplásticos incluem um aumento nas conexões aferentes e eferentes entre o local da lesão e as regiões cerebrais, restaurando as atividades sinápticas locais através da sinaptogênese[34]. Recentemente, tem-se observado a expressão de proteínas sinápticas em ratos adultos com lesão cerebral isquêmica que receberam transplante de células-tronco, indicando a formação de contatos sinápticos[76,77]. Confirmando tal possibilidade, um estudo recente demonstrou que células-tronco neurais, transplantadas via intracerebral de ratos HI, aumentou o número de terminais axonais no córtex sensório-motor, corpo caloso, estriato e tálamo ipsilaterais, assim como brotamento axonal acentuado em direção ao hemisfério lesado[25].

Aspectos translacionais da terapia celular em asfixia perinatal

A pesquisa translacional pode ser definida como um processo que parte da medicina baseada em evidências em direção a soluções sustentáveis para problemas de saúde da comunidade[78]. Os estudos pré-clínicos constituem o primeiro passo no desenvolvimento de novas terapias. No entanto, a transferência dos conhecimentos adquiridos através de estudos *in vitro* e em modelos animais de HI para o uso clínico da terapia celular requer atenção para as diferenças entre as espécies e a análise crítica dos resultados experimentais. Além disso, aspectos ainda pouco elucidados (Tabela 3), tais como, a via de administração, o número de células transplantadas, o tipo de célula utilizada e o momento de intervenção pós-lesão são variáveis importantes que poderão influenciar significativamente a resposta clínica. Portanto, após o esclarecimento de questões fundamentais para a segurança do tratamento, é possível que estudos clínicos de fase I possam ser iniciados para avaliar essa abordagem terapêutica inovadora.

A escolha da via de administração pode ser um fator crucial para o sucesso da terapia celular quando se considera que o benefício do tratamento dependa da migração e localização das células transplantadas na região cerebral comprometida. Atualmente, a melhor rota de transplante de células-tronco para o tratamento de neuropatologias ainda não está definida. No entanto, concorda-se que a rota ideal seria aquela que apresentasse alta eficiência e especificidade, com o mínimo de efeitos adversos. Estudos em modelos animais de HI têm utilizado uma variedade de métodos de administração, incluindo a via intraperitoneal, intravenosa e intracerebral.

Tabela 3 - Aspectos relevantes para o uso clínico da terapia celular em asfixia perinatal

Aspecto	Vantagens	Desvantagens
Via de administração		
Venosa	Menos invasiva e mais segura	Necessidade de sinais quimiotáticos Células podem migrar para outros órgãos Necessidade de avaliar efeitos adversos
Arterial	Maior direcionamento das células-tronco para os locais lesados Técnica de rotina	Risco de microembolismos e isquemias
Intracraniana	Facilita migração das células-tronco no sítio da lesão isquêmica	Mais invasiva, risco de lesões
Intraperitoneal	Técnica prática, simples e menos invasiva	Células necessitam viajar longas distâncias Poucos estudos
Fonte e tipo celular		
Embrião ou feto	Elevado poder de diferenciação e proliferação	Entraves ético-religiosos Dificuldade de obtenção Inviável para o uso em fase aguda de lesão
Cordão umbilical (mononucleares)	Facilidade de obtenção Elevada quantidade de células Possibilidade de transplante autólogo	Pouca caracterização das células-transplantadas
Medula óssea (mononucleares)	Elevada quantidade de células Possibilidade de transplante autólogo	Pouca caracterização das células-transplantadas Técnica dolorosa e inviável para recém-nascidos
Células-tronco neurais	Células da mesma origem embrionária Amplamente estudadas Padrão-ouro para o tratamento de lesões cerebrais	Migração restrita Morte celular Dificuldade de obtenção e uso imediato
Tempo pós-transplante		
Agudo	Atua de forma neuroprotetora Presença de sinais quimiotáticos e permeabilidade vascular Resultados pré-clínicos consistentes	Limita ao uso de tipos celulares pouco processados (cultura) Inviável para casos já estabelecidos
Crônico	Possibilidade do uso em pacientes com sequelas estabelecidas	Ausência de sinais quimiotáticos apropriados Ausência de permeabilidade vascular Escassez de estudos

Através do transplante intracerebral, é possível atingir diretamente uma área cerebral específica. Apesar da precisão da técnica, o procedimento é invasivo e diversas aplicações seriam necessárias para abranger uma lesão isquêmica, resultando em um comprometimento cerebral ainda maior[79]. Injeções intracerebroventriculares também constituem uma abordagem intracraniana de administração das células-tronco. No entanto, apesar de permitir uma distribuição celular difusa, essa via não é capaz de atingir lesões afastadas dos ventrículos e esbarra nas mesmas questões que o transplante intracerebral. Além disso, as células transplantadas podem permanecer aderidas nas paredes dos ventrículos e causar hidrocefalia obstrutiva[79,80]. Embora a maioria dos estudos experimentais de terapia celular em HI neonatal utilize o transplante intracerebral, é mais provável que outras vias de administração menos invasivas sejam estabelecidas para a prática clínica. Em contexto experimental demonstrou-se equivalência na recuperação motora e morfológica do transplante intravenoso e intracerebral de células-tronco em modelo animal de HI neonatal[57].

Uma recente metanálise demonstrou que transplante celular pela via intravenosa é capaz de melhorar os desfechos de modelos animais de neuropatologias, sendo que a inibição do processo apoptótico é a principal modificação molecular cerebral pós-transplante[81]. O procedimento de administração intravenosa é menos invasivo, simples e seguro, possibilitando ampla distribuição das células transplantadas para áreas cerebrais HI[38,40,57]. Além disso, permite que estas entrem em contato com sinais quimiotáticos provenientes da lesão cerebral e sejam seletivamente acumuladas no tecido alvo[79,82]. No entanto, apenas um número reduzido de células-tronco atinge o local de lesão cerebral por permanecerem retidas nos pulmões, rins, fígado e baço. Em decorrência disso, toxicidade e crescimento ectópico em outros órgãos necessitam ser avaliados antes do uso clínico[80].

Uma maneira de evitar a circulação corporal sistêmica seria optar pelo transplante das células-tronco por um acesso intra-arterial. Dados recentes mostram migração igualmente distribuída e desfechos neurológicos favoráveis através do uso dessa abordagem terapêutica em modelo de HI em ratos[55]. Essa rota desvia a captação pelos órgãos sistêmicos, permitindo que uma concentração maior de células-tronco atinja o sítio isquêmico, uma vez que os vasos tenham reperfusão. Caso os vasos estejam permanentemente ocluídos, as células transplantadas permanecerão distribuídas apenas na região da penumbra[79]. Entretanto, estudos recentes reportam alta taxa de mortalidade de animais transplantados pela via intra-arterial, sugerindo a formação de oclusões microvasculares e isquemias[79,80,83]. Na prática médica, o método de acesso intra-arterial é comumente utilizado em técnicas intervencionistas de cateterismo vascular. Em um ensaio clínico brasileiro de terapia celular em pacientes com isquemia crônica (NCT00473057), foram observados resultados promissores pós-transplante autólogo de células-tronco da medula óssea utilizando a via intra-arterial[46].

A via intraperitoneal também foi utilizada para transplante celular em HI neonatal, demonstrando migração celular da cavidade peritoneal até as regiões de dano cerebral[36,37]. Os resultados do estudo sugerem a presença de sinais quimiotáticos precisos e a necessidade de quebra da barreira hematoencefálica para que ocorra migração celular em distâncias tão longas. Apesar da praticidade do procedimento, necessita-se um número maior de estudos que dêem suporte para esta via de transplante alternativa.

A escolha do intervalo de tempo entre o estabelecimento da lesão HI e a intervenção também é importante, pois ao longo do tempo, o ambiente cerebral adapta-se drasticamente frente a uma lesão HI. Em decorrência disso, a maioria dos estudos utilizando a administração sistêmica de células-tronco em HI utiliza estágios agudos de lesão. Células-tronco injetadas precocemente podem auxiliar na preservação e na sobrevivência do tecido neuronal. Adicionalmente, van Velthoven et al.[56] sugerem que o transplante celular seja realizado entre 2 e 3 dias pós-HI, momento em que o cérebro apresenta maior capacidade de proliferação celular e ativação de mecanismos de reparo endógeno. No trabalho de Park et al.[22], a janela terapêutica para transplante celular eficaz abrangeu 3-7 dias pós-HI, devido à elevada atividade metabólica, bioquímica e molecular deste período que poderia facilitar a migração das células transplantadas[22]. Já no caso de transplantes tardios, é possível que as células-tronco possam estimular a liberação de fatores tróficos para restaurar as funções perdidas[80]. No entanto, apesar da melhora funcional observada em ratos adultos que receberam células-tronco um mês após isquemia cerebral[84], não há estudos que demonstram benefícios terapêuticos em modelos animais de HI crônica, limitando a aplicabilidade clínica em lesões hipóxico-isquêmicas estabelecidas em decorrência da ausência de sinais quimiotáticos e permeabilidade vascular apropriados.

A permeabilização da barreira hematoencefálica também pode facilitar a entrada de células-tronco ou de fatores neurotróficos no SNC, especialmente quando forem utilizadas vias periféricas de transplante. Entretanto, recentemente, demonstrou-se que o número de células-tronco no hipocampo isquêmico dos animais HI transplantados pela via intravenosa, não diferiu significativamente quando comparados os animais que receberam apenas células-tronco com aqueles em que foi associado o manitol[40]. Em contrapartida, os animais transplantados e administrados com manitol apresentaram maior concentração cerebral de alguns fatores tróficos. Também se verificou melhora acentuada dos desfechos comportamentais e histológicos com o uso de células-tronco e manitol, possivelmente através do efeito parácrino dessas células. Essas evidências indicam a importância da permeabilização da barreira hematoencefálica em terapia celular, mas também questionam se a neuroproteção é realmente dependente da migração e diferenciação celular.

O tipo e fonte celular mais adequados para o tratamento das lesões HI neonatais ainda necessitam ser definidos[34]. O uso de CTN é considerado padrão-ouro para o tratamento de doenças neurológicas. No entanto, os resultados de muitos estudos demonstram que morte celular, migração restrita e rápida diferenciação em células gliais limitam a aplicabilidade das CTN no reparo neuronal. Além disso, um dos maiores obstáculos para o uso clínico desse tipo celular em lesões cerebrais neonatais é a sua dificuldade de obtenção. É provável que tecidos de biópsia ou de material extraído de tecido *post-mortem* sejam insuficientes para a produção de quantidades

significativas dessas células[85]. As questões ético-religiosas envolvidas na extração de material embrionário e fetal e as preocupações com os riscos de resposta imunológica ou de formação de tumores malignos também inibem a aplicabilidade clínica dessas células. A neurodiferenciação *in vitro* para futura utilização dessas células em recém-nascidos com HI adiciona preocupação com os potenciais riscos de contaminação, reação imunológica aos fatores adicionados ao meio de cultivo celular e dificuldade de uso em transplantes agudos. Desvantagens similares relacionadas a segurança e dificuldade de uso imediato também são apresentadas pelas células-tronco mesenquimais da medula óssea e cordão umbilical, amplamente usadas em HI experimental.

Em contrapartida, o processamento de células-tronco mononucleares provenientes do sangue de cordão umbilical e da medula óssea é relativamente simples e rápido. No contexto da área neonatal, as células-tronco mononucleares derivadas de sangue de cordão umbilical apresentam algumas vantagens em relação à aspiração da medula óssea. A obtenção de células-tronco de sangue de cordão umbilical não oferece nenhum risco e desconforto para o recém-nascido, podendo ser transplantadas após coleta autóloga. Além disso, o sangue do cordão umbilical pode ser usado terapeuticamente no período perinatal ou criopreservado para o uso tardio[62,86]. No entanto, estudos adicionais necessitam ser realizados para caracterizar as populações celulares e avaliar efetividade e segurança[80,87].

Avaliação da dose-resposta das células-tronco em modelos animais também necessita ser realizada para embasar o seu uso clínico. Segundo Janowski et al.[81], há uma associação dose-resposta entre o número de células injetadas e os efeitos do tratamento de neuropatologias em estudos experimentais. No entanto, a dose ideal de células-tronco é, em parte, dependente da via de administração, assim como do tipo celular usado, do momento de intervenção e a quantidade de células transplantadas que atingem a lesão cerebral. Do ponto de vista clínico, desfechos relacionados à segurança do transplante celular, tais como a formação tecidual ectópica e anormalidades comportamentais, devem ser incorporados na metodologia das investigações e no seguimento dos pacientes. Também é importante considerar metodologias seguras e efetivas para o monitoramento não invasivo da migração das células transplantadas e, consequentemente, estabelecer uma compreensão adequada das respostas do paciente ao tratamento proposto.

Conclusões

A pesquisa básica vem apresentando resultados promissores na área da lesão cerebral neonatal. Apesar da variabilidade dos métodos empregados, a maioria dos estudos citados nessa revisão indica que as células-tronco podem apresentar propriedades neuroprotetoras, resultando em melhores desfechos funcionais nos animais tratados. Certamente, o sucesso da terapia celular em roedores pode ser considerado o primeiro passo no desenvolvimento de uma abordagem terapêutica para um futuro uso clínico. No entanto, cabe ressaltar que as doenças cerebrais humanas apresentam mecanismos mais complexos de dano e regeneração do que em animais de experimentação. Portanto, questões relacionadas à dose e ao tipo celular, à via de administração e ao momento adequado de intervenção necessitam ser definidas. Embora não determinante para a aplicação clínica, investigações adicionais sobre os mecanismos de ação das células-tronco em HI também constituem uma ferramenta importante na determinação da segurança e efetividade do transplante.

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Capítulo III

1 ARTIGO ORIGINAL I

NAP prevents acute cerebral oxidative stress and protects against long-term brain injury and cognitive impairment in a model of neonatal hypoxia-ischemia

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NAP prevents acute cerebral oxidative stress and protects against long-term brain injury and cognitive impairment in a model of neonatal hypoxia–ischemia

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ABSTRACT

Hypoxia–ischemia (HI) is a common cause of neonatal brain damage with lifelong morbidities in which current therapies are limited. In this study, we investigated the effect of neuropeptide NAP (NAPVSIPQ) on early cerebral oxidative stress, long-term neurological function and brain injury after neonatal HI. Seven-day-old rat pups were subjected to an HI model by applying a unilateral carotid artery occlusion and systemic hypoxia. The animals were randomly assigned to groups receiving an intraperitoneal injection of NAP (3 μg/g) or vehicle immediately (0 h) and 24 h after HI. Brain DNA damage, lipid peroxidation and reduced glutathione (GSH) content were determined 24 h after the last NAP injection. Cognitive impairment was assessed on postnatal day 60 using the spatial version of the Morris water maze learning task. Next, the animals were euthanized to assess the cerebral hemispheric volume using the Cavalieri principle associated with the counting point method. We observed that NAP prevented the acute HI-induced DNA and lipid membrane damage and also recovered the GSH levels in the injured hemisphere of the HI rat pups. Further, NAP was able to prevent impairments in learning and long-term spatial memory and to significantly reduce brain damage up to 7 weeks following the neonatal HI injury. Our findings demonstrate that NAP confers potent neuroprotection from acute brain oxidative stress, long-term cognitive impairment and brain lesions induced by neonatal HI through, at least in part, the modulation of the glutathione-mediated antioxidant system.

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Introduction

Perinatal hypoxia–ischemia (HI) is a leading cause of acute child mortality and lifelong sequelae that includes cerebral palsy, epilepsy, mental retardation and motor and learning impairments (Volpe, 2008). Presently, all clinically available therapies are ineffective at reducing the neurodevelopmental disabilities found in the surviving infants. Although many neuroprotective treatments have been discovered, only hypothermia has shown a potential benefit in clinical trials (Perlman, 2006). Thus, the development of innovative therapeutic strategies to treat HI brain injury and its associated side effects is necessary in the clinical setting.

The pathophysiology of HI involves a cascade of deleterious biochemical events, such as energy failure, acidosis, glutamate excitotoxicity, immune/inflammatory activation, mitochondrial dysfunction, oxidative stress and necrosis/apoptosis (Perlman, 2006). In particular, oxidative damage begins in the early stages following the HI insult and leads to an increase in reactive species production and changes in the activity of enzymatic and non-enzymatic antioxidants, resulting in lipid peroxidation, protein oxidation and DNA fragmentation (Buonocore et al., 2001). Moreover, as compared with the adult brain, the immature brain is highly susceptible to oxidative damage because of its high concentrations of unsaturated fatty acids, high rate of oxygen consumption, poorly developed scavenging systems and high availability of redox active iron (Ferriero, 2001). Therefore, oxidative stress is thought to be one of the major factors that induce neuronal cell death in the immature brain.

The 8-amino-acid NAP (NAPVSIPQ or davunetide) is the smallest active motif of the VIP-responsive glial protein, activity-dependent neuroprotective protein (ADNP), and it has demonstrated a variety of neuroprotective effects in both cell cultures and animal models of neuropathologies (Bassan et al., 1999; Gozes et al., 2005). Several mechanisms have been proposed for NAP, but studies have indicated a potent antioxidative activity *in vitro* (Busciglio et al., 2007; Offen et al., 2000; Steingart and Gozes, 2006; Steingart et al., 2000) and *in vivo* (Spong et al., 2001). Recently, we demonstrated that NAP dose-

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dependently prevented oxidative stress in the hippocampus of neonatal rats subjected to hypoxia-induced seizures (Greggio et al., 2009). Studies have suggested that the increase in ADNP levels in the injured brains is part of an endogenous neuroprotection mechanism (Zaltzman et al., 2004). Thus, NAP treatment could provide additional protection from the neonatal HI-induced brain injury. In this vein, Kumral et al. (2006) demonstrated that NAP prevents neuronal cell death, apoptosis and nitric oxide production in a newborn rat model of HI (Kumral et al., 2006). Although NAP is effective at reducing acute neuronal degeneration, it should also prevent the chronic functional and histological alterations that result from the HI insult. However, the long-term neuroprotective effects of NAP in neonatal HI are unknown. The purpose of the present study was to investigate the impact of NAP treatment on early DNA damage, lipid peroxidation and redox status in the cerebral cortex and hippocampus of neonatal rats after the HI insult. Further, we examined the ability of NAP to recover the spatial memory deficits in the adult rats that were subjected to the neonatal HI. Finally, we also verified whether NAP administration could prevent the chronic HI-induced brain tissue damage.

Materials and methods

Animals

Pregnant Wistar rats were housed individually, and the presence of pups was checked daily. The day of birth was considered day 0, and on postnatal day (PND) 1, the litters were culled to 8 rat pups per dam. The animals were maintained in the same temperature and humidity-controlled holding facility (22–24 °C) under a 12/12 light/dark cycle (light onset at 07:00) with free access to food and water. The rats were weaned on PND 21, and only males were selected for this study. Efforts were made to minimize the animal suffering and to reduce the number of animals used. All analyses were performed in a blinded set-up, and all experimental procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Ethics Committee of Pontifícia Universidade Católica do Rio Grande do Sul, RS, Brazil (CEUA 10/00172). A schedule of the surgical procedure, treatment and tests of the animals is shown in Table 1.

Neonatal hypoxia–ischemia model

For hypoxic–ischemic lesion induction, we adapted the Levine model for use on neonatal rats (Rice et al., 1981). Wistar rats on PND 7 were briefly anesthetized with halothane, and a longitudinal incision was made along the midline of the neck. The right common carotid artery was identified, isolated from the vagus nerve and permanently occluded using 7.0 surgical silk sutures. The entire surgical procedure was completed within 15 min. After surgery, the animals were returned to their dams and allowed to recover for 2–4 h. The rats were removed from the litter and placed in a custom-made airtight acrylic chamber that was partially immersed in a water bath at 37 °C. They were left in the hypoxia chamber for 2 h, with a constant flow of humidified 8% oxygen balanced with nitrogen and at a temperature of 37–38 °C. Intra-chamber O_2 concentration was measured using a commercially available oxygen meter (Model DG-400, Instrutherm, SP, Brazil). Following the hypoxic exposure, all of the pups were returned to their dams for recovery. The sham-operated animals were anesthetized with halothane, and the right common carotid artery was exposed but did not receive ligation or hypoxia.

Table 1
Timeline of experimental procedures.

PND 7	Neonatal rats subjected to hypoxic–ischemic brain injury and subsequent intraperitoneal injection of NAP or vehicle
PND 8	Second intraperitoneal injection of NAP or vehicle
PND 9	Oxidative stress analysis of hippocampus and cerebral cortex (DNA damage, lipid peroxidation and GSH content)
PND 60	Spatial version of the Morris water maze learning task
PND 66	Cerebral hemispheric volume assessment

NAP administration and cerebral sample processing

Neuropeptide NAP, with a >95% purity and an identity that was assessed using a high-performance liquid chromatography and mass spectrometry analysis, was purchased from Sigma Genosys (Woodlands, TX). NAP was dissolved in 10% DMSO in PBS (0.13 M NaCl, 0.003 M KCl, 0.01 M Na_2HPO_4 and 0.002 M KH_2PO_4) and stored as 1 mM aliquots. In addition, NAP was stable in solution and could be aliquoted and frozen for later use without loss of activity. The final peptide solution was freshly prepared from stock solutions that were diluted in distilled sterile PBS. For rat pups treated with NAP, an intraperitoneal injection of the NAP solution was administered at 3 μg/g of body weight (0.1 mL) immediately (0 h) and 24 h after the HI insult. In this study, randomly selected animals were assigned to 3 different experimental groups: sham-operated animals (sham group), vehicle-treated HI animals (VEH group) and NAP-treated HI animals (NAP group). For the oxidative stress studies, a set of animals ($n=4$ for each experimental group) was euthanized 24 h after the last NAP administration. The brains were quickly removed and kept in ice-cold saline, and hippocampal and cerebral cortex samples from the ischemic hemisphere (ipsilateral to the carotid ligation) were dissected into Petri dishes placed on ice. A portion from each structure was gently homogenized in 0.3 mL of ice cold Hanks' solution for use in the alkaline comet assay, while the rest of the samples were stored at −80 °C for the biochemical analysis.

Determination of DNA damage

The alkaline comet assay was performed, with minor modifications, as described by Singh (Greggio et al., 2009; Singh et al., 1988). Aliquots of the hippocampus or cortex homogenate (20 μL) were mixed with 0.75% warm, low-melting point agarose (Invitrogen, Carlsbad, CA, USA) and immediately spread onto a microscope slide pre-coated with a layer of 1% normal melting point agarose (Invitrogen, Carlsbad, CA, USA). This mixture was then evenly distributed across the microscope slide, which was followed by the application of a coverslip, and allowed to set at 4 °C for 5 min prior to the removal of the coverslips. The slides were then incubated in ice cold lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 1% Triton X-100 and 10% DMSO, pH 10.0; Gibco BRL, Grand Island, NY, USA), protected from light and stored at 4 °C for at least 4 days to remove the cell proteins, but leaving the DNA as 'nucleoids'. The slides were then transferred to a tank containing the freshly prepared electrophoresis solution (300 mM NaOH, 1 mM EDTA, pH>13.0) and stored for 20 min at 4 °C prior to being transferred to a horizontal electrophoresis tank in a chilled incubator and covered in fresh electrophoresis solution (pH>13.0). Electrophoresis was performed for 20 min at 25 V and 300 mA (0.90 V/cm). All of the steps above were conducted under a yellow light or in the dark to prevent additional DNA damage. The slides were then neutralized (0.4 M Tris, pH 7.5), washed in bi-distilled water and stained using a silver nitrate staining protocol as described elsewhere (Nadin et al., 2001). After drying at room temperature, the gels were analyzed using an optical microscope. For the visual score analysis, the slides were coded and scored blindly. In total, 100 cells (50 cells from each of the 2 replicate slides) were analyzed. When selecting cells for quantification, the edges and cells around air bubbles were avoided. The cells were visually scored according to the tail length into 5 classes: class 0: undamaged, without a tail; class 1: with a tail shorter than the diameter of the head (nucleus); class 2: with a tail length 1–2× the diameter of the head; class 3: with a tail longer than 2× the diameter of the head and class 4: comets with no

heads. A value of the damage index (DI) was assigned to each comet according to its class. The DI is based on the length of migration and the amount of DNA in the tail, and it is considered a sensitive DNA measurement. The DI ranged from 0 (completely undamaged: 100 cells×0) to 400 (with maximum damage: 100 cells×4), which is represented as arbitrary units (AU). The damage frequency (DF) was calculated based on the number of cells with tails as compared with those with no tail and represented as a percentage (%). The international guidelines and recommendations for the comet assay suggest that visual scoring of comets is a well-validated evaluation method (Burlinson et al., 2007). The vehicle was used as the negative control, and hippocampal and cortical cells exposed to 150 μM H_2O_2 (Sigma-Aldrich, St. Louis, MO, USA) for 5 min at 4 °C were used as the positive control.

Determination of lipid peroxidation

The amount of lipid peroxidation was determined by the reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA), a product formed by lipid peroxidation during an acid-heating reaction. The assays were performed according to Salgo and Pryor, with minor modifications (Salgo and Pryor, 1996). Briefly, the samples were mixed after lysis with Tris–HCl (15 mM for 1 h). Next, 2 mL of 0.4 mg/mL TCA (Sigma-Aldrich, St. Louis, MO, USA) and 0.25 M HCl was added to the lysate, which was then incubated with 6.7 mg/mL TBA (Sigma-Aldrich, St. Louis, MO, USA) for 15 min at 100 °C. The mixture was centrifuged at 750×*g* for 10 min. Because TBA reacts with other products of lipid peroxidation in addition to MDA, thiobarbituric acid reactive species (TBARS) levels were expressed as MDA equivalents (nmol/mg protein), which were determined by absorbance at 532 nm. The 1,1,3,3-tetramethoxypropan (TMP) (Sigma-Aldrich, St. Louis, MO, USA) was used as the standard. The results were normalized by protein content (Lowry et al., 1951).

Determination of GSH levels

The reduced glutathione (GSH) levels were determined by photometric determination of 5-thio-2-nitrobenzoate (TNB), which was produced from sulfhydryl reagent 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) (Sigma-Aldrich, St. Louis, MO, USA) in a kinetic assay according to Akerboom and Sies (1981), with minor modifications. The samples were mixed in 0.1 M sodium phosphate with 5 mM EDTA pH 8.0 and sonicated to obtain the cell homogenate. An equal volume of 2 M $HClO_4$ with 4 mM EDTA was added to the cell extract, and the precipitated proteins were sedimented by centrifugation at 8000×*g* for 10 min at 4 °C. The supernatant was neutralized with 2 M KOH and 0.3 M MOPS, and the insoluble residue was removed by centrifugation under the same conditions. For the spectrophotometric determination, 910 μL of the cell extract supernatant or of the standard glutathione solution in the same phosphate-EDTA buffer was mixed with 50 μL of 4 mg/mL NADPH in 0.5% (w/v) $NaHCO_3$, 20 μL of 6 U/mL glutathione reductase (Sigma-Aldrich, St. Louis, MO, USA) in phosphate-EDTA buffer, and 20 μL of 1.5 mg/mL DTNB in 0.5% $NaHCO_3$. The increase in absorbance was measured at 412 nm. The total glutathione content was normalized by protein content (Lowry et al., 1951). For oxidized glutathione (GSSG) determination, 4-vinylpyridine (Sigma-Aldrich, St. Louis, MO, USA) was added to a final concentration of 0.1% (v/v) and then incubated for 1 h at room temperature. At this concentration, 4-vinylpyridine was able to react with all GSH without interfering with the GSSG determination. The GSH levels were determined based on the total glutathione and GSSG concentration results.

Spatial version of the Morris water maze learning task

An additional cohort of animals ($n=9$ for each experimental group) was tested in the spatial version of the Morris water maze (MWM) learning task at PND 60. The water maze is a black circular pool (200 cm in diameter) conceptually divided into 4 equal imaginary quadrants. The water temperature was 21–23 °C, and 2 cm beneath the surface of the water and hidden from the rat's view was a black circular platform (12 cm in diameter). It had a rough surface, which allowed the rats to climb onto it easily once detected. The swimming path of the animals was recorded using a video camera mounted above the center of the pool and analyzed using an analysis system. The water maze was located in a well lit white room with several posters and other distal visual stimuli hanging on the walls to provide spatial cues. The rats were handled 5 min per day for 3 days prior to the training. The training using the spaced training protocol was performed during 5 successive days. On each day, the rats received 8 consecutive training trials during which the hidden platform was kept in a constant location. A different starting location was used for each trial, which consisted of a swim followed by a 30-s platform sit. Any rat that did not find the platform within 60 s was guided to it by the experimenter that was blinded for each experimental group. Memory retention was evaluated in a 60-s probe trial performed in the absence of the escape platform 24 h after the last training session. The parameters measured were the time taken to find the platform location and the percent time spent in the target quadrant during the 60-s probe test (de Paula et al., 2009; Venturin et al., in press).

Cerebral hemispheric volume assessment

After MWM learning task (PND 66), the animals were deeply anesthetized with thiopental sodium (0.1 mL/100 g, i.p.) and perfused transcardially with saline followed by 4% paraformaldehyde at 4 °C. The brains were removed from the skulls, post-fixed in the same solution at room temperature for 24 h and cryoprotected by immersion in a 30% sucrose solution in phosphate buffer at 4 °C until they sank. The brains were then quickly frozen in isopentane that was cooled in liquid nitrogen (−70 °C). Coronal sections of the brain (50 μm) were cut using a cryostat (Shandon, United Kingdom) at 250 μm intervals, mounted on poly-L-lysine coated slides and stained with cresyl violet using the Nissl method. To analyze the brain volume, the unbiased estimation of hemispheric volume was obtained using the Cavalieri principle associated with the counting point method. Then, digitized images of the coronal sections overlaying millimetric graph paper (point grid) were obtained using a stereoscopic light microscope (DF Vasconcellos MU-M19, SP, Brazil) coupled to a high performance CCD camera and interfaced using Image Pro-Plus 6.1 (Media Cybernetics, Silver Spring, USA). All points that hit each brain section were counted and used to compute the volume. The volume was estimated using the following equation: $V=T.a/p.\Sigma P$, where V = volume estimation; T = distance between the analyzed sections; a/p = point area (1 mm^2) and ΣP is the sum of the points overlaid in the image (Alles et al., 2010; de Paula et al., 2009; Gundersen and Jensen, 1987). The volume estimations were performed in approximately 10 sections for each rat by a single experimenter that was blinded to the source of the images.

Statistical analysis

The statistical analysis was performed using PrismGraph 5.0 software (Graph-Pad Software, San Diego, CA). The data were expressed as mean ± standard error of the mean (S.E.M.). Comparisons between the experimental groups were made using one-way analysis of variance (ANOVA) followed by the Newman–Keuls post hoc test or using two-way ANOVA followed by Bonferroni post hoc test, as appropriate. A statistical significance level of $\alpha=0.05$ and $p<0.05$ was applied to all tests.

Results

NAP prevents acute HI-induced DNA damage, lipid peroxidation and disruption of GSH homeostasis in the hippocampus and cerebral cortex of rat pups

To analyze whether NAP can block brain DNA damage and lipid peroxidation generated by HI, an intraperitoneal double injection of NAP (3 μg/g) was given to rat pups immediately (0 h) and 24 h after the insult. Because HI lesions occur primarily in the cerebral cortex and hippocampus ipsilateral to the carotid occlusion (Rice et al., 1981), we collected these two tissues 24 h after the last NAP injection (48 h post-HI) and processed it using the alkaline comet assay, TBARS and GSH analysis. Our results demonstrated a significant increase in the level of DNA breaks in the cortex (Figs. 1A and B) and hippocampus (Figs. 1E and F) of the VEH animals. The DNA-damaging effects induced by HI insult comprehend not only a high frequency of affected cells (Figs. 1A and E, $p<0.01$ *vs.* sham) but also a significant severity of the lesions (Figs. 1B and F, $p<0.001$ *vs.* sham). In addition, the same level of damage from the HI was observed on membrane lipids in cortical (Fig. 1C, $p<0.001$ *vs.* sham) and hippocampal (Fig. 1G, $p<0.001$ *vs.* sham) samples from the VEH animals. To further elucidate the mechanism of oxidative damage involved in the HI insult, GSH was depleted in the ipsilateral cortex (Fig. 1D, $p<0.05$ *vs.* sham) and hippocampus (Fig. 1H, $p<0.001$ *vs.* sham) of the VEH animals. Interestingly, the brain samples from the NAP and sham groups were similar in the DF (Fig. 1A for cortex, Fig. 1E for hippocampus), DI (Fig. 1B for cortex, Fig. 1F for hippocampus) and lipid peroxidation (Fig. 1C for cortex, Fig. 1G for hippocampus). In contrast, the NAP group was significantly different than the VEH animals for all variables analyzed ($p<0.001$). Moreover, we observed a concomitant beneficial impact of NAP administration on cortical (Fig. 1D, $p<0.05$ *vs.* VEH) and hippocampal (Fig. 1H, $p<0.001$ *vs.* VEH) GSH homeostasis in HI rat pups.

NAP protects against impairments in learning and long-term spatial memory in adult rats subjected to neonatal HI

A set of animals was trained in the MWM to verify the effect of NAP in cognitive function following HI. The training trials for the tests began on PND 60 (53 days post-HI). The mean escape latencies to the hidden platform were shortened as training progressed, and the groups showed a different rate of change over time [$F_{(2,\ 120)}=0.64$, $p=0.73$, Fig. 2A]. The Bonferroni post hoc test showed a significant difference ($p<0.001$) between the VEH and sham animals throughout the entire 5-day training session. These groups indicate a severe learning disability following the HI insult because the damaged structure, hippocampus, plays a major role in spatial memory. Whereas the NAP-treated HI animals had a learning performance similar to the sham animals, except on the second day of training ($p<0.05$), the NAP group showed a significant reduction in the escape latency than the VEH animals ($p<0.05$ for session days 1–4; $p<0.001$ for session day 5). The probe test was performed 24 h after the last training session in the absence of the escape platform. The VEH animals showed significantly longer latencies in the correct quadrant where the platform was previously located compared to the sham ($p<0.01$) and NAP groups ($p<0.05$) (Fig. 2B). The Newman–Keuls post hoc test demonstrated that there was no difference between the sham and NAP groups. As shown in Fig. 2C, the VEH animals spent less time swimming in the target quadrant than the NAP ($p<0.05$) and sham animals ($p<0.01$). There was no significant difference between the sham and NAP animals. These data indicate an inhibition of NAP on the long-term neonatal HI-induced impairments of learning and spatial memory.

NAP blocks long-term brain injury in adult rats subjected to neonatal HI

Based on the ability of NAP to reduce brain oxidative stress and cognitive impairment in the HI rats, we next examined whether NAP

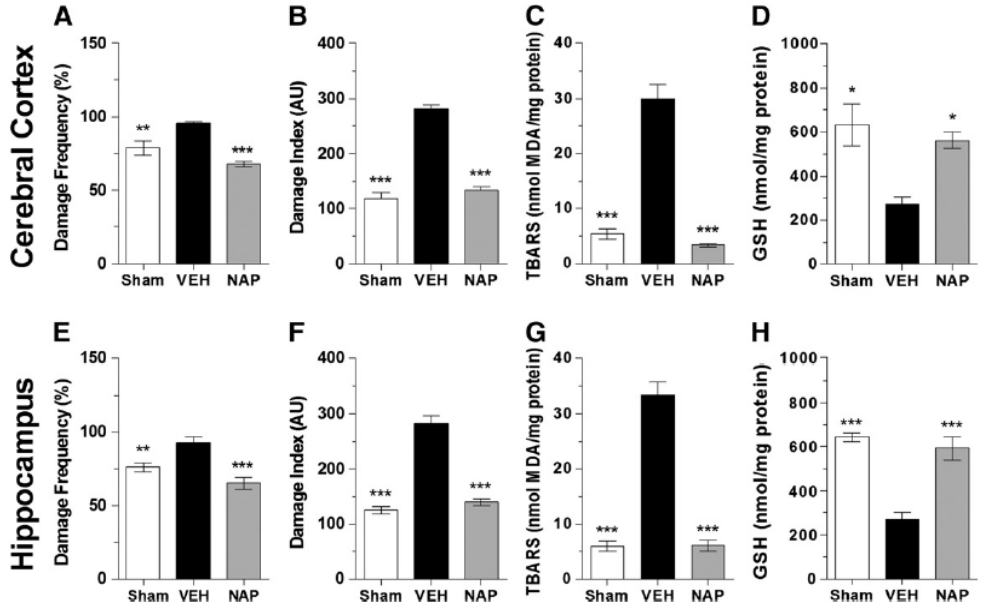

Fig. 1. The acute effects of NAP treatment on genotoxicity, lipid peroxidation and GSH content in the brain tissue from the neonatal rats subjected to HI. The top line of each graph (A–D) corresponds to the analyses of the cerebral cortex and the bottom line (E–H) corresponds to the hippocampus. DNA damage was assessed using the alkaline comet assay, and the data are expressed as percentage for DF (A and E) and arbitrary units for DI (B and F). Lipid peroxidation was assessed using the reaction of TBA with MDA during an acid-heating reaction (C and G), and the results are expressed as MDA equivalents (nmol/mg protein). The intracellular total glutathione was determined using the photometric determination (D and H), and the results are expressed as GSH content (nmol/mg protein). The values represent the mean ± S.D. ($n=4$ rats/group). $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ *vs.* VEH group in Newman–Keuls post hoc test after one-way ANOVA.

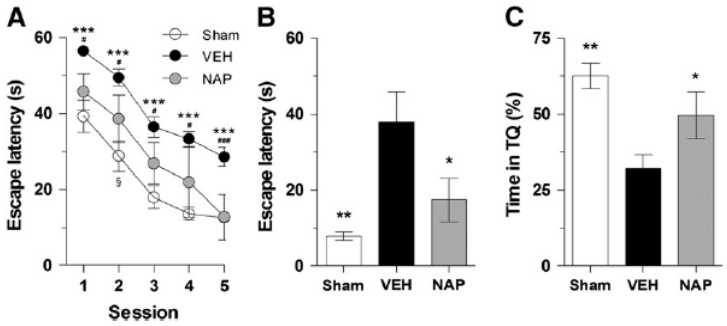

Fig. 2. The neuropeptide NAP protects against long-term learning and spatial memory impairments in the rats subjected to the neonatal HI. The spatial version of the MWM was used to assess cognitive function following the HI injury at PND 60. (A) The mean escape latencies to the hidden platform were obtained from a 5-day training session. Data are presented in blocks of 8 trials as mean ± S.E.M. ($n = 9$ per group). $^{\#}p<0.05$ and $^{\#\#\#}p<0.001$ *vs.* sham group, $^{*}p<0.05$ and $^{***}p<0.001$ *vs.* VEH animals, $^{\S}p<0.05$ *vs.* NAP animals in Bonferroni post hoc test after two-way ANOVA. (B) The latency to swim over the quadrant where the escape platform was located ($p = 0.0031$) and (C) the mean percent time spent in the target quadrant (TQ) ($p = 0.0033$) during a 60 s probe test was performed 24 h after the last MWM training session. Data are presented as mean ± S.E.M., $^{*}p<0.05$ and $^{**}p<0.01$ *vs.* VEH animals in Newman–Keuls post hoc test after one-way ANOVA.

could prevent the long-term morphological alterations in the brain volume of these animals. Therefore, the brain volumes were estimated using the Cavalieri principle associated with the counting point method. The morphological examination of cresyl violet-stained sections of rat brains from the sham group revealed no histological abnormalities (Fig. 3C) or volume differences between the cerebral hemispheres (Fig. 3A). The morphology of the VEH animals showed a significant decrease in the volume of the ipsilateral cerebral hemisphere following carotid occlusion (right) when compared to the contralateral hemisphere 60 days after the HI injury ($p<0.001$; Fig. 3A). In the severe HI model used in this study, the ligation of the right common carotid artery followed by exposure to low ambient oxygen in the 7-day-old rats produced a brain lesion in the cerebral cortex, subcortical and periventricular white matter, striatum and hippocampus. As a result, the profound loss of parenchyma throughout the ipsilateral cerebral hemisphere, in most rats, leads to the formation of a porencephalic cyst (Fig. 3D). However, the stereological evaluation of the brains from the NAP animals demonstrated that the volume of the ipsilateral cerebral hemisphere was similar to the contralateral side (Figs. 3A and E). Similarly, there were significant differences in the relative ratio between the left and right hemispheric volume between the experimental groups ($p = 0.0001$; Fig. 3B). The hemispheric volume ratios of the sham and NAP animals were significantly different from the VEH group ($p<0.001$). Further, there was no significant difference between the sham and NAP groups. Thus, these results demonstrate a long-term neuroprotective effect of NAP treatment on HI-induced brain injury.

Discussion

In this study, we demonstrated that NAP administration significantly decreased the cortical and hippocampal DNA damage, lipid peroxidation and GSH depletion 2 days after the neonatal HI insult. We further showed the reduction in the long-term neurological outcome and brain lesions up to 7 weeks after the NAP treatment on rat pups subjected to the HI. This study is the first *in vivo* demonstration that NAP exerts a prolonged and strong neuroprotective effect following HI insult through, at least partially, modulation of the intracellular glutathione system.

Because of the oxidative brain damage formation in the acute stages after HI (Cai et al., 2009; Takizawa et al., 2009; Weis et al., 2011), we administered NAP immediately after the HI insult (0 h). A second NAP injection (24 h post-HI) was administered because of the severity and continuous formation of brain damage that is characteristic of the Rice–Vannucci model. The NAP dose used in the present study (3 μg/g) was chosen based on its protective effects in hypoxic–ischemic brain injury (Kumral et al., 2006). Moreover, the intraperitoneal administration of NAP was considered appropriate because NAP can cross the blood–brain barrier (Gozes et al., 2005) and was also appropriate for use in neonatal rats (Greggio et al., 2009; Kumral et al., 2006).

It has been demonstrated that the abnormal production and/or impaired clearance of reactive species play important roles in the

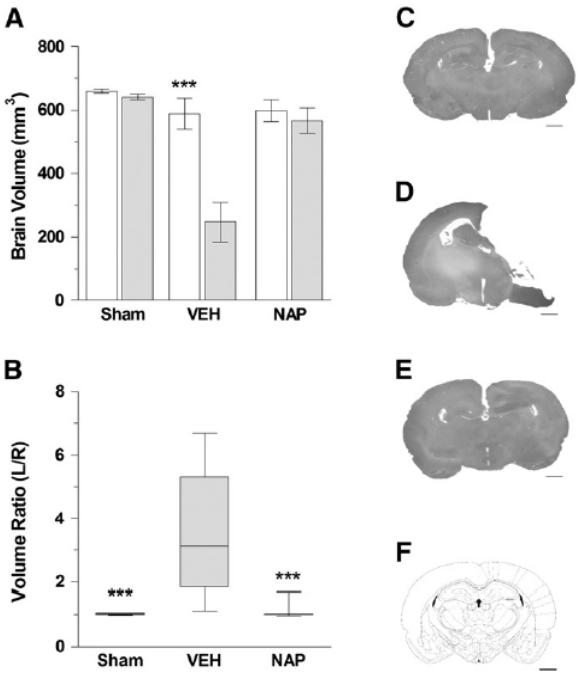

Fig. 3. The neuropeptide NAP prevents long-term brain lesions in adult rats that were subjected to the neonatal HI. The brain volume was estimated using the Cavalieri principle associated with the counting point method 2 months after HI injury. Data are presented as mean ± S.D. ($n = 9$ per group). (A) The estimated brain volumes of the left (contralateral, white columns) and right hemispheres (ipsilateral, gray columns). $^{***}p<0.001$ *vs.* right hemisphere volume in Newman–Keuls post hoc test after one-way ANOVA. (B) The hemispheric volume ratio was determined by the division of the left hemisphere by the right hemisphere. $^{***}p<0.001$ *vs.* VEH group in Newman–Keuls post hoc test after one-way ANOVA. (C–E) Digitized images of cresyl violet-stained coronal sections of the rat brains that corresponded to the coronal sections from the sham (C), VEH (D) and NAP (E) groups. (F) A schematic drawing obtained from the Paxinos and Watson's atlas (interaural 5.70 mm; bregma −3.30 mm). Calibration bars = 1 mm.

pathogenesis of HI injury and the functional deficits that occur in both the experimental (Bagenholm et al., 1997; Lafemina et al., 2006) and clinical settings (Kumar et al., 2008; Shi et al., 2000). The immature central nervous system is highly sensitive to free radicals, which affects the cellular components. Thus, previous studies have demonstrated that DNA fragmentation occurs in the early stages following the HI injury in the neonatal rat brain using several different methodological approaches (Cai et al., 2009; Takizawa et al., 2009; Zhu et al., 2000). In our study, we detected DNA damage, such as primary repairable DNA single and double-strand breaks and alkali-labile sites, 48 h post-HI in the cerebral cortex and hippocampus of neonatal rats. Notably, the amount of DNA breaks detected in the brain of NAP-treated HI animals was similar to the sham animals. Recently, it was demonstrated that NAP dose-dependently inhibited DNA damage and oxidative adducts formation that was induced by neonatal hypoxia-induced seizures in the immature rat hippocampus using the alkaline comet assay (Greggio et al., 2009). Moreover, the effect of NAP in maintaining the brain DNA integrity was demonstrated in animal models of neonatal HI (Kumral et al., 2006), cerebral palsy (Sokolowska et al., 2011) and focal irreversible ischemia (Leker et al., 2002) using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling reaction. The TBARS formation is one of the most commonly used biomarker for the measurement of membranes injured by lipid peroxidation. Augmentation of TBARS levels, such as by MDA, has been detected in the immature rat brain at different times following HI (Bagenholm et al., 1997; Cai et al., 2009; Weis et al., 2011) and in the plasma of infants with hypoxic–ischemic encephalopathy (Kumar et al., 2008; Schmidt et al., 1996). In this study, we demonstrated an increase in the cortical and hippocampal TBARS levels in the rat brain 48 h post-HI. In contrast, NAP treatment prevented brain lipid peroxidation in NAP-treated HI animals. Consistent with these results, we previously demonstrated the NAP effect on preventing the lipid peroxidation reaction in another neonatal animal model (Greggio et al., 2009). The GSH depletion is involved in the redox homeostasis imbalance triggered by the HI insult in several brain regions of the neonatal rat (Dafre et al., 2003; Sheldon et al., 2007) as well as in the plasma of asphyxiated newborns (Schmidt et al., 1996). Due to the involvement of the glutathione system in the NAP-associated neuroprotective mechanism (Greggio et al., 2009; Offen et al., 2000; Spong et al., 2001), we investigated the neuroprotective role of this pathway. We verified an inhibition of the HI-induced GSH depletion in the brain of NAP-treated animals 48 h after the insult. Interestingly, the NAP treatment of HI rat pups was effective at reducing the amount of DNA breaks and lipid peroxidation in the brain and increasing GSH levels, which is consistent with previous studies (Greggio et al., 2009). Although we have not explored other mechanisms of action of NAP, it was reported that polyADP-ribosylation is involved in the neurotrophic activity of NAP (Visochek et al., 2005). Poly(ADP-ribose) polymerase-1 (PARP-1) activation by NAP could facilitate DNA repair and enable rapid gene expression. In addition, it has been demonstrated that PARP-1 activation is important for repairing single-strand DNA damage following sublethal global ischemia (Nagayama et al., 2000). Therefore, GSH stimulation and polyADP-ribosylation activation may have influenced the brain DNA and lipid membranes integrity recovery following the neonatal HI insult.

PolyADP-ribosylation, the same process responsible for repairing DNA damage, also contributes to spatial memory acquisition in the MWM paradigm (Satchell et al., 2003) and to long-term memory formation in mammals (Goldberg et al., 2009). Therefore, NAP could stimulate polyADP-ribosylation to induce transcriptional regulation, protein synthesis and/or ribosylation of other factors that are important in memory formation. Consistent with this idea, NAP, through distinct routes and modes of administration, enhanced spatial memory acquisition in the MWM paradigm in several animal models, such as normal and aged animals (Alcalay et al., 2004; Gozes et al., 2002; Toso et al., 2006a), apolipoprotein E knockout (Bassan et al., 1999; Brenneman et al., 2004; Rotstein et al., 2006) and ADNP-deficient mice (Vulih-Shultzman et al., 2007), fetal alcohol syndrome (Brenneman et al., 2004; Incerti et al., 2010; Toso et al., 2007b; Vink et al., 2005) and Down syndrome (Incerti et al., 2011). Our interest in training HI rats in MWM was to determine whether NAP treatment would affect the impaired cognitive outcomes induced by the neonatal HI. In this study, we demonstrated that the postnatal NAP treatment inhibited learning and long-term spatial memory deficits induced by the neonatal HI brain injury. However, it is not likely that NAP would evoke long-lasting effects on cognitive function up to 7 weeks directly through polyADP-ribosylation alone. Studies have demonstrated that the expression of N-methyl-aspartate (NMDA) and γ-aminobutyric acid (GABA) receptors is modulated after pre- or postnatal NAP + SAL administration (SALLRSIPA is structurally and functionally similar to NAP) in the prevention of alcohol- (Incerti et al., 2010; Toso et al., 2006b) and Down syndrome-induced learning deficits (Incerti et al., 2011; Vink et al., 2009). In contrast, postnatal oral treatment with NAP + SAL resulted in enhanced learning in aged mice without altering NR2A, NR2B, GABA-$_A$β3 and GABA-$_A$α5 subunits expression (Toso et al., 2007a). Another possibility includes the reversal of vasoactive intestinal peptide and activity-dependent neuroprotective protein dysregulation by NAP in fetal alcohol syndrome (Incerti et al., 2010) and Down syndrome (Incerti et al., 2011) models. Despite the well-documented neuroprotective actions of NAP in these studies, the molecular mechanisms and signaling pathways are still not fully characterized.

Previous studies have demonstrated that NAP reduces brain lesions in a cerebral ischemia model (Leker et al., 2002), closed head injury (Beni-Adani et al., 2001) and cerebral palsy (Sokolowska et al., 2011). For the neonatal HI, it has been reported that intraperitoneal NAP administration preserves the neuronal density in the immature rat hippocampus 72 h post-HI (Kumral et al., 2006). We demonstrated that NAP is able to prevent severe brain lesions up to 7 weeks after the neonatal HI. The activity of NAP in promoting cell survival could be partly justified by the reduction of proapoptotic protein p53 (Gozes et al., 2004), the control of cGMP and nitric oxide production (Ashur-Fabian et al., 2001), immunomodulation (*i.e.*, tumor necrosis factor-α) (Beni-Adani et al., 2001), inhibition of caspase 3 activation (Leker et al., 2002) and polyADP-ribosylation stimulation (Visochek et al., 2005). Recently, it has been shown that NAP can stimulate pathways that have been implicated in neuronal survival, differentiation and synaptic plasticity, such as the mitogen-activated protein kinase family of signal transduction proteins and phosphatidylinositol-3-kinase/Akt pathways (Pascual and Guerri, 2007; Sokolowska et al., 2011), phosphorylation of the Fyn kinase and the scaffold protein Crk-associated substrate (Chen and Charness, 2008).

Thus, with the experimental design and methodology used in the present study, we suggest that the positive effects on spatial memory and brain volume may be attributed to the ability of NAP to decrease the accumulation of oxidative damage by modulating GSH and, consequently, maintain the brain cellular integrity involved in learning and memory processes. Further studies are necessary to delineate additional pathways underlying the mechanisms behind the learning and brain injury recovery in the neonatal HI model as well as to confirm the beneficial effects of NAP in the human neonate.

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2 ARTIGO ORIGINAL II

Intra-arterial transplantation of human umbilical cord blood mononuclear cells in neonatal hypoxic-ischemic rats is feasible and restores long-term learning and spatial memory impairments.

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Regular Article for Neurobiology of Disease

Intra-arterial transplantation of human umbilical cord blood mononuclear cells in neonatal hypoxic-ischemic rats is feasible and restores long-term learning and spatial memory impairments

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Abstract

Based on preclinical findings, cellular therapy has become a promising therapeutic approach for neonatal hypoxia-ischemia (HI). However, before translation into the clinical setting, a noninvasive and effective route of cell delivery must be determined. Intra-arterial (IA) delivery is an attractive route of cellular administration but has not been used in neonatal HI rats. In this study, we investigated the feasibility of IA transplantation of human umbilical cord blood (HUCB) mononuclear cells for the treatment of long-term learning dysfunction and brain lesions after neonatal HI. Seven-day-old rats were subjected to a HI model by permanent occlusion of the right common carotid artery and by systemic hypoxia. The animals received HUCB mononuclear cells into the left common carotid artery 24 h after HI insult. After 63 days post-HI, intra-arterially transplanted HUCB mononuclear cells significantly improved learning and long-term spatial memory impairments when evaluated by the Morris water maze paradigm. There was no effect of cellular administration or HI insult on body weight or on motor coordination and balance when evaluated by the accelerating rotarod test. Cellular transplantation by the IA route did not restore neonatal HI-induced brain damage according to stereological volume assessment. Furthermore, HUCB mononuclear cells were tracked in the injured brain and peripheral organs of HI transplanted-rats by nested polymerase chain reaction analysis at different time points. Our findings contribute to the translational knowledge of cell based-therapy in neonatal HI and demonstrate for the first time that IA transplantation into rat pups is a feasible route for cellular delivery and prevents long-term cognitive deficits induced by experimental neonatal HI.

Keywords: neonatal hypoxia-ischemia, human umbilical cord blood mononuclear cells, intra-arterial transplantation, rodent model, memory, motor function, brain lesion.

Highlights

- The intra-arterial transplantation in HI neonatal rats is a feasible delivery route for cellular therapy;
- Intra-arterial transplantation of HUCB cells rescues long-term learning and spatial memory impairments in HI rats;
- Intra-arterial transplantation of HUCB cells or vehicle have no impact in the body weight and motor function in HI rats;
- Intra-arterially transplanted HUCB cells do not prevent brain injury in adult rats previously subjected to neonatal HI;
- HUCB cells are detected in the ischemic brain after intra-arterial transplantation and also in peripheral organs until at least 30 days later.

Introduction

Neonatal hypoxia ischemia (HI) remains an important cause of mortality and is associated with a high incidence of life-long disabilities (Volpe, 2008). Unfortunately, the clinical treatment of HI is quite limited, and no available intervention can effectively hinder the long-term sequelae of HI insult (Johnston et al., 2011). In the preclinical context, there is increasing evidence that cell based-therapy exhibits a neuroprotective effect against HI brain injury and subsequent deleterious outcomes (Bennet et al., 2012; Liao et al., 2012; van Velthoven et al., 2012a). According to the Baby STEPS (Baby Stem cell Therapeutics as an Emerging Paradigm in Stroke) consortium, the design of preclinical studies should closely approximate clinical trials to favor the translational potential of cell therapy in neonatal HI (Borlongan and Weiss, 2011). Additionally, the experimental design should consider and determine the cell type, dose, timing of administration and the best delivery route. Despite these recommendations, intraparenchymal injection is the most commonly used technique for cell delivery into the rodent HI brain (de Paula et al., 2012; Pimentel-Coelho et al., 2012). Intracerebroventricular administration precludes systemic dissemination but fails due to the limited cell numbers that can be injected. The same trade-off can be observed with the intracerebral (IC) route, where cells can be delivered directly to the target, but they are nonuniformly distributed which may cause additional brain injury. As an alternative to the aforementioned methods, we and others have used intravenous (IV) delivery to transplant cells into neonatal HI animals (Bae et al., 2012; de Paula et al., 2012; de Paula et al., 2009; Yasuhara et al., 2008; Yasuhara et al., 2010). However, the IV route does not yield a large number of cells reaching the brain, and the majority of transplanted cells are trapped within the filtering organs such as the lungs, liver, spleen

and kidneys (Fischer et al., 2009; Lappalainen et al., 2008). Non-conventional cell delivery methods, such as intraperitoneal (Pimentel-Coelho et al., 2010; Rosenkranz et al., 2012; Wasielewski et al., 2012), intracardiac (Lee et al., 2010) and intranasal (Donega et al., 2013) delivery, were also applied in the HI animal model. Therefore, it is reasonable to investigate less invasive and more effective ways to deliver stem cells for the treatment of neonatal HI.

The intra-arterial (IA) route has the advantage of selectively targeting a larger number of cells to an injured brain area, bypassing the filter of the peripheral organs, and permits a multiple treatment paradigm (Misra et al., 2012). Additionally, it was demonstrated that cell administration via IA delivery could spread cells uniformly throughout the ischemic brain (Li et al., 2010; Walczak et al., 2008). Although the IA transplantation of cells has numerous precedents in clinical trials (Barbosa da Fonseca et al., 2010; Battistella et al., 2011; Brazzini et al., 2010; Friedrich et al., 2011) and in animal models of adult HI (Andres et al., 2011; Guzman et al., 2008; Pendharkar et al., 2010; Rosenblum et al., 2012), stroke (Brenneman et al., 2010; Chua et al., 2011; Chung et al., 2009; Gutierrez-Fernandez et al., 2011; Jin et al., 2005; Kamiya et al., 2008; Lappalainen et al., 2008; Li et al., 2010; Li et al., 2001; Mitkari et al., 2012; Ohta et al., 2006; Shen et al., 2006; Vasconcelos-dos-Santos et al., 2012; Walczak et al., 2008; Zhang et al., 2012) and traumatic brain injury (Lu et al., 2001; Lundberg et al., 2009; Lundberg et al., 2012; Osanai et al., 2012), it has not been tested in neonatal HI rodents. Here we investigate for the first time the use of the IA route for the transplantation of human umbilical cord blood (HUCB) mononuclear cells in a neonatal rat HI model. Parameters such as long-term behavior impairment, body and cerebral weight, brain damage and cell migration were evaluated.

Materials and Methods

Animals

Pregnant Wistar rats were housed individually, and the presence of pups was checked daily. The day of birth was considered day 0, and on postnatal day (PND) 1 the litters were culled to 8 rat pups per dam. The animals were maintained in the same temperature and humidity-controlled holding facility (22–24 °C) under a 12 h/12 h light/dark cycle (light onset at 07:00) with free access to food and water. The rats were weaned on PND 21, and only the males were selected for this study. Efforts were made to minimize animal suffering and to reduce the number of animals used. All analyses were performed in a blinded set-up, and all experimental procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Ethics Committee of Pontifícia Universidade Católica do Rio Grande do Sul, RS, Brazil (CEUA 09/00105). A schedule of the surgical procedure, the treatment and tests of the animals is shown in table 1.

Experimental groups

The animals were randomly assigned into five experimental groups: sham-operated rats (sham, n = 10), rats subjected to the HI model (HI, n = 11), HI rats intra-arterially administered with vehicle (VEH, n = 9) and HI rats transplanted with 1×10^6 (HI + 10^6, n = 10) or 1×10^7 (HI + 10^7, n = 10) HUCB mononuclear cells into the left carotid artery 24 h post-HI. The rat pups from each litter were randomly divided among the five experimental groups to avoid “litter effects” on the study outcome. An additional cohort of HI animals (n = 12) was injected with 1×10^7 HUCB mononuclear cells for cellular detection by nested PCR analyses.

Neonatal hypoxia-ischemia model

For hypoxic-ischemic lesion induction, we adapted the Levine model for neonatal rats (Rice et al., 1981). Wistar rats on PND 7 were briefly anesthetized with halothane, and a longitudinal incision was made along the midline of the neck. The right common carotid artery was identified, isolated from the vagus nerve and permanently occluded using 7.0 surgical silk sutures. The entire surgical procedure was completed within 15 min. After surgery, the animals were returned to their dams and allowed to recover for 2–4 h. The rats were then removed from the litter and placed in a custom-made airtight acrylic chamber that was partially immersed in a 37 °C water bath. They were left in the hypoxia chamber for 2 h, with a constant flow of humidified 8% oxygen balanced with nitrogen and maintained at a temperature of 37–38 °C. The intra-chamber O_2 concentration was measured using a commercially available oxygen meter (Model DG-400, Instrutherm, SP, Brazil). Following the hypoxic exposure, all of the pups were returned to their dams for recovery. The sham-operated animals were anesthetized with halothane, and the right common carotid artery was exposed but did not receive ligation or hypoxia.

HUCB mononuclear cells preparation and intra-arterial transplantation

After obtaining informed consent, HUCB cells were collected ex-utero from healthy volunteers immediately after full-term delivery using sterile syringes containing 5.000 UI of heparin. We used a proportion of one donated umbilical cord to 4–7 transplanted rats, depending on the amount of available mononuclear cells. Blood samples were kept at a temperature of 4 °C during the transport and storage procedures, and all units were processed within 24 h of collection. For the separation of mononuclear cells, the obtained material was diluted in RPMI-1640 medium (1:1) (Gibco, Grand Island, NY,

USA). The cells were resuspended and fractionated on a density gradient generated by centrifugation, over a Ficoll-Paque solution with a density of 1.077 g/L (Histopaque 1077, Sigma Aldrich, St. Louis, MO, USA), at 400 × g for 30 min at 25 °C. The mononuclear fraction over the Ficoll-Paque layer was collected and washed twice with Dulbecco's Phosphate Buffered Saline (DPBS) (Gibco, Grand Island, NY, USA). The cell density was determined with a Neubauer-counting chamber, and the number of viable cells was determined using the Trypan Blue 0.4% exclusion method (de Paula et al., 2012; de Paula et al., 2009). Twenty-four hours after HI insult, animals received HUCB mononuclear cells (1×10^6 or 1×10^7 cells resuspended in 50 µl of phosphate buffered saline) or vehicle delivered into the left common carotid artery using an ultrafine 34 gauge microneedle (outer-Ø 0.20 mm, inner-Ø 0.10 mm; Nodegraf Co., Ltd., Tokyo, Japan), with preserved flow within the carotid artery during cell injection. For this procedure, animals were anesthetized with halothane, the previous neck suture was carefully opened, and the left carotid artery was isolated from adjacent tissue to facilitate the intra-arterial injection. Thereafter, the skin was once again closed with a suture, and the animals were returned to their dams for recovery.

Spatial version of the Morris water maze learning task

The five experimental groups were tested in the spatial version of the Morris water maze (MWM) learning task beginning at PND 65. The water maze is a black circular pool (200 cm in diameter) conceptually divided into 4 equal imaginary quadrants. The water temperature was 21–23 °C, and a black circular platform (12 cm in diameter) was hidden from the rat's view 2 cm beneath the surface of the water. The platform had a rough surface, which allowed the rats to climb onto it easily once it was detected. The swimming path of the animals was recorded using a video camera mounted above the

center of the pool and was analyzed using an analysis system (ANY-maze software, version 4.3, Stoelting Co., Wood Dale, IL, USA). The water maze was located in a well-lit white room with several posters and other distal visual stimuli hanging on the walls to provide spatial cues. The rats were handled 5 min per day for 3 days prior to the training. The spaced training protocol was performed for 5 successive days. On each day, the rats received 8 consecutive training trials during which the hidden platform was kept in a constant location. A different starting location was used for each trial, which consisted of a swim followed by a 30-s platform sit. Any rat that did not find the platform within 60 s was guided to it by the experimenter. Memory retention was evaluated in a 60-s probe trial performed in the absence of the escape platform 24 h after the last training session. The parameters measured were the time taken to find the platform location, the percent time spent in the target quadrant, the number of crossings over the former target position and the mean swimming speed (de Paula et al., 2012; Greggio et al., 2011; Venturin et al., 2011).

Accelerated rotarod performance

At PND 71, the rotarod test was performed to measure motor coordination and balance in the rats. A fully automated apparatus equipped with a sensor that detected the fall and automatically stopped the timer was used (EFF 411, Insight, SP, Brazil). The test was performed using an accelerating speed paradigm. One day prior to the test, all animals were habituated to the apparatus using a protocol consisting of three trials of 3 min each at a low constant speed of 16 rpm. The rats that fell from the treadmill were again placed on it until they were able to stay on it for 3 min. After completion of the habituation training, the speed of the rotarod was set to gradually increase from 4 to 37 rpm over 6 min across ten phases (phase 1-2: 16 rpm; phase 3-4: 20 rpm; phase 5-6: 25

rpm; phase 7-8: 28 rpm; phase 9-10: 37 rpm). Upon reaching the maximum speed, the animals were kept in place for an additional minute (maximum latency of 420 s), after which they were put back into their home cage. The test session consisted of five trials separated by 15-minute intervals. The mean of the latencies to fall from the treadmill and the mean of the phase in which the fall occurred in the 5-trial test were calculated (Balduini et al., 2000; Daadi et al., 2010; Park et al., 2011).

Stereological volume assessment of the cerebral hemispheres

After the accelerated rotarod test (PND 72), the animals were weighed, deeply anesthetized with thiopental sodium (0.1 mL/100 g, i.p.) and then perfused transcardially with saline followed by 4% paraformaldehyde at 4 °C. The brains were removed from the skulls, weighed and post-fixed in the same solution at room temperature for 24 h and cryoprotected by immersion in a 30% sucrose solution in phosphate buffer at 4 °C until they sank. The brains were then quickly frozen in isopentane that was cooled in liquid nitrogen (− 70 °C). Coronal sections of the brain (50 μm) were cut using a cryostat (Leica CM1850, Leica Microsystems, Wetzlar, Germany) in 250 μm intervals, mounted on gelatin-coated slides and stained with cresyl violet using the Nissl method. To analyze the brain volume, an unbiased estimation of the hemispheric volume was obtained using the Cavalieri principle associated with the counting point method. Subsequently, images of the coronal sections overlaying millimetric graph paper (point grid) were obtained using a stereoscopic light microscope (DF Vasconcellos MU-M19, SP, Brazil) coupled to a high performance CCD camera and interfaced using Image Pro-Plus 6.1 (Media Cybernetics, Silver Spring, USA). All points that hit each brain section were counted and used to compute the volume. The volume was estimated using the following equation: $V = T.a/p.\Sigma P$, where V = volume

estimation; T = distance between the analyzed sections; a/p = point area (1 mm^2) and ΣP is the sum of the points overlaid on the image. The volume estimations were performed on 10 sections for each rat by a single experimenter. The parameters measured were the cerebral hemispheric volume and the percent tissue loss of the rat brains (Alles et al., 2010; de Paula et al., 2012).

Nested polymerase chain reaction (PCR) analysis

An additional group of HI rats (n = 12) was euthanized, and samples were collected at 1, 3, 6, 12 and 24 h, and 7 and 30 days after IA transplantation of HUCB mononuclear cells (n = 2 rats for each time point). The samples collected were the right and left cerebral hemispheres, lungs, liver and spleen. DNA was obtained from the samples using the phenol/chloroform method. PCR analysis was performed to identify the presence of the administered HUCB mononuclear cells in the samples of transplanted animals using complementary primers to the human β-actin gene sequence. We used the forward primer 5′-TCCCTGTACGCCTCTGGCCATA-3′ and the reverse primer 5′-CCTTCTGCATCCTGTTGGTGATGCTA-3′ complimentary to the human β-actin DNA sequence and reamplified with the forward primer 5′-TGACTGGCCGGAACCTGACT-3′ and the reverse primer 5′-GGTGATGACCTGGCCATTGGG-3′ using the nested PCR technique, resulting in fragments of 535 and 209 bp, respectively. The positive control (DNA from human peripheral blood) and negative control (without any DNA) samples were assayed along with the experimental samples in every reaction. Amplified products were detected by gel electrophoresis (2% agarose containing ethidium bromide) for 30 min, at a voltage of 100 V and amperage of 400 mA. The gels were visualized under an ultraviolet transilluminator (3UV™), and the images were captured using photodocumentation

equipment connected to Quantity One software (Bio-Rad, CA, USA) (de Paula et al., 2012).

Statistical analysis

The statistical analysis was performed using PrismGraph 5.0 software (Graph-Pad Software, San Diego, CA). The data were expressed as the means ± standard error of the mean (S.E.M.). Comparisons between the experimental groups were made by using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test or by using two-way ANOVA followed by Bonferroni's post hoc test, as appropriate. A statistical significance level of $\alpha = 0.05$ and $p < 0.05$ was applied to all tests.

Results

Intra-arterial transplantation of 1 x 10^7 HUCB mononuclear cells rescues long-term spatial memory impairments in rats previously subjected to neonatal hypoxia-ischemia

To assess the effects of IA transplantation of HUCB mononuclear cells on long-term spatial memory deficits, we employed the MWM paradigm. The mean escape latencies to the hidden platform decreased as training progressed, and the experimental groups showed a distinct performance over time [$F_{(4, 225)} = 2.824$, $p = 0.0003$, Fig. 1A]. As shown in Table 2, two-way ANOVA followed by Bonferroni's post hoc test revealed a significant difference between the HI and sham groups throughout the entire 5-day training session. The HI group animals intra-arterially administered with either vehicle or 1 x 10^6 HUCB mononuclear cells had similar learning performances when compared with the HI only group and were significantly different from the sham rats. Conversely, the animals subjected to neonatal HI injury and transplanted with 1 x 10^7 HUCB mononuclear cells learned differently from the sham rats but performed better than the HI, Veh and HI+10^6 groups. The probe test was performed 24 h after the last training session in the absence of the escape platform. One-way ANOVA followed by Dunnett's post hoc test showed that the escape latency to swim over the previous position of the escape platform was longer in the HI (42.45 ± 6.73 s, $p < 0.01$), HI+10^6 (38.66 ± 6.67 s, $p < 0.05$) and Veh (45.24 ± 5.36 s, $p < 0.01$) groups when compared with the sham group (14.96 ± 2.91 s) (Fig. 1B). Only the HI+10^7 group had a significantly shorter time required to localize the platform position (27.53 ± 6.93 s, $p > 0.05$ versus sham). In addition, the HI and Veh animals spent less time swimming in the target quadrant that previously contained the escape platform (28.88 ± 5.35% and 29.55 ± 4.77%, respectively) compared with the sham-operated rats (57.22 ± 4.18%) (Fig. 1C). Neither

HI group, transplanted with 1 x 10^6 or 1 x 10^7 HUCB mononuclear cells, had any significant difference when compared with the sham group for the time spent in the target quadrant (41.57 ± 4.86% and 48.38 ± 7.03%, respectively). With respect to the number of crossings over the former target position, all groups displayed significantly lower values than the sham animals (6.10 ± 0.78) (Fig. 1D). However, this difference was more evident in the HI (1.54 ± 0.45, $p > 0.001$), Veh (1.77 ± 0.66, $p > 0.001$) and HI+10^6 (2.60 ± 0.87, $p > 0.01$) animals than in the HI+10^7 group (3.30 ± 0.65, $p > 0.05$). No statistically significant differences were observed for swimming speed among the experimental groups, demonstrating that the results in the MWM probe test were not confounded by any HI-induced motor impairment (Fig. 1E). Additionally, because the Veh and HI rats had the same performance on the training session and the probe tests, we can assume that the IA administration procedure is safe in neonatal rats. Therefore, the results obtained from the MWM task indicate that the IA transplantation of 1 x 10^7 HUCB mononuclear cells rescues the learning and memory impairments induced by neonatal HI.

Intra-arterial transplantation of HUCB mononuclear cells or vehicle does not induce impairments in motor coordination and balance in HI rats

In the present study, the rotarod test was performed to measure motor coordination and balance in rats. Using an accelerated rotarod protocol, there were no statistically significant differences between the HI group and the sham rats in the mean time spent on the treadmill (Fig. 2A) and mean phase in which the fall occurred (Fig. 2B). One-way ANOVA followed by Dunnett's test showed that the HUCB-treated and Veh animals performed equally in both variables analyzed in the rotarod test when compared with the sham group. These results are in agreement with the normal motor function

observed in the MWM probe test and the evaluation of the swimming speed of the rats. After the rotarod test, the animals were weighed and no significant differences were observed among the experimental groups (Fig. 3). Additionally, the mortality rate was the same between the control and the transplanted animals. Again, the data indicate that intra-carotid transplantation of HUCB mononuclear cells or administration of vehicle does not induce any long-lasting detrimental effect in HI rats.

Intra-arterially transplanted HUCB mononuclear cells are not able to prevent brain injury in adult rats previously subjected to neonatal HI

After verifying the ability of IA transplantation of HUCB mononuclear cells to prevent cognitive impairments in HI rats, we next examined whether this treatment could also block the long-term brain morphological alterations. Therefore, the cerebral hemispheric volumes were estimated using a stereological method based on the Cavalieri principle and the counting point method. Morphological examination of cresyl violet-stained sections of rat brains from the sham group revealed no histological abnormalities or volume differences between the cerebral hemispheres (542.4 ± 10.17 *versus* 535.6 ± 9.13 mm^3) (Fig. 4A). Conversely, absolute hemispheric volume analysis showed significant atrophy of the hemisphere ipsilateral to the carotid occlusion (right side) when compared with the contralateral hemisphere (551.1 ± 10.27 *versus* 385.4 ± 57.49 mm^3, $p < 0.05$) (Fig. 4A) 65 days after the HI injury. Similarly, the HI+10^6 and Veh groups also had a significantly reduced right hemisphere volume when compared with the left hemisphere (510.2 ± 18.43 *versus* 352.0 ± 66.95 mm^3, $p < 0.05$; and 570.7 ± 20.73 *versus* 396.1 ± 65.10 mm^3, $p < 0.05$; respectively) (Fig. 4A). However, no statistically significant difference was observed between the right and left hemispheres in HI rats intra-arterially transplanted with 1 x 10^7 HUCB mononuclear cells (505.2 ±

13.47 versus 362.6 ± 67.25 mm^3) (Fig. 4A). In a different analysis approach, we examined cerebral atrophy in terms of percentage of brain tissue loss (Fig. 4B). One-way ANOVA followed by Bonferroni's post hoc test revealed no significant difference between the sham (1.22 ± 0.32%) and HI (30.21 ± 10.47%), HI+10^6 (33.99 ± 11.57%), HI+10^7 (29.73 ± 12.48%) or Veh (32.02 ± 10.29%) groups. In the neonatal HI model used in this study, the profound loss of parenchyma throughout the ipsilateral cerebral hemisphere can lead to the formation of a porencephalic cyst. Based on visual and histological investigation, the percent of porencephalic cysts (~40%) was the same among transplanted and non-treated HI animals. Corroborating this evidence, one-way ANOVA followed by Dunnett's post hoc test demonstrated no significant difference in brain weight among the groups, but only when compared with sham animals ($p < 0.05$) (Fig. 4C). Taken together, these findings suggest that intra-arterially delivered HUCB mononuclear cells do not worsen brain lesions in rats subjected to a neonatal HI model. Furthermore, no brain damage was identified in the ipsilateral hemisphere due to HUCB transplantation, indicating the safety of the IA delivery method.

Human β-actin gene expression was detected in samples obtained from HI rats intra-arterially transplanted with HUCB mononuclear cells

We employed nested PCR analysis to verify whether the human β-actin gene from HUCB mononuclear cells could be detected in the brain and peripheral organs of HI rats transplanted with 1 x 10^7 HUCB mononuclear cells at different time points (1, 3, 6 and 24 h, 7 and 30 days after cellular transplantation). This investigation was performed to elucidate whether the observed beneficial cognitive effects were associated with the migration of cells to the injured brain and to monitor their spreading in the rodent body. The expression of the band corresponding to the human β-actin gene was initially

detected (1 h post-transplantation) in the left cerebral hemisphere, liver and lungs of HI rats that received cellular administration into the left carotid artery. At 3 h after cellular transplantation, human DNA was detected in the liver and lungs and not detected in the left hemisphere, but it was detected in the right injured brain hemisphere. However, at 6 h post-transplantation, the human β-actin gene was only amplified in the right hemisphere. We could not detect any expression for the human gene in the brain or systemic organs at 24 h after IA transplantation of HUCB mononuclear cells. At later analysis time points, we could detect human DNA in the left cerebral hemisphere and lungs (7 and 30 days post-transplantation), and in the right hemisphere (30 days post-transplantation). It was not possible to detect the expression of human β-actin gene in the spleen of intra-arterially transplanted HI rats at any time point analyzed.

Discussion

IA delivery of HUCB mononuclear cells is a promising strategy for neonatal HI treatment. Here we demonstrate for the first time that IA transplantation is a feasible delivery route for cellular therapy in neonatal rats subjected to a HI model. The intra-arterially delivered cells prevented learning and spatial memory impairments nine weeks after neonatal HI insult but did affect recovery from brain damage. Additionally we further showed that HI insult or IA cell transplantation had no long-term impact on body weight or motor function in rodents. HUCB mononuclear cells were rapidly identified in the ischemic brain after IA transplantation and also in several peripheral organs for at least 30 days after transplantation. Altogether the data indicate that the IA approach as a minimally invasive delivery method for cell based-therapy for neonatal HI.

Stem cells from a variety of sources such as neural stem/progenitor cells derived from either fetal or embryonic tissue, mesenchymal stem cells, HUCB and bone marrow cells, among others (Bennet et al., 2012; de Paula et al., 2010; Pimentel-Coelho et al., 2012; van Velthoven et al., 2012a), have been used in animal models of neonatal HI. The HUCB mononuclear cell fraction was chosen for this study because these cells are one of the most convenient and rich sources of therapeutic cells in pediatrics and can be used in the case of neonates affected by HI insult. Cord blood is easily collected after delivery and can be promptly used because it requires minimal ex vivo processing, or it can be cryopreserved for a longer period in cord blood banks (Liao et al., 2012). We have recently demonstrated that acute IV administration of HUCB mononuclear cells exerts a dose-dependent effect on long-term behavior and morphological outcomes in HI-injured rats (de Paula et al., 2012). Therefore, HUCB mononuclear cells represent a

promising candidate for neonatal HI therapy. Nevertheless, the ideal route for stem cell delivery is a key factor that needs to be addressed in the preclinical context to help in the design of future clinical trials for neonatal HI treatment (Borlongan and Weiss, 2011).

Different approaches were tested for cellular delivery in neonatal rats subjected to neonatal HI, namely IC (Daadi et al., 2010; Obenaus et al., 2011; van Velthoven et al., 2012b), IV (Bae et al., 2012; de Paula et al., 2012; de Paula et al., 2009; Yasuhara et al., 2008; Yasuhara et al., 2010), intraperitoneal (Pimentel-Coelho et al., 2010; Rosenkranz et al., 2012; Wasielewski et al., 2012), intranasal (Donega et al., 2013) and intracardiac (Lee et al., 2010) routes. However, the IA delivery route has not been used for cellular transplantation in immature rats following neonatal HI insult. The main advantage of IA transplantation over the other routes is the possibility of directing a larger number of cells to a specific injured brain area by a minimally invasive procedure. Moreover, this approach circumvents cellular entrapment by internal organs and allows for repetitive transplant regimens (Misra et al., 2012). In the present study, we intra-arterially delivered HUCB mononuclear cells in neonatal rats 24 h after HI insult. The cell doses and volumes were based on previously published studies (Gutierrez-Fernandez et al., 2011; Kamiya et al., 2008; Lappalainen et al., 2008; Walczak et al., 2008). We adapted the microneedle technique for cellular transplantation in neonatal rats. This technique uses the high rate of blood flow in the common carotid artery to dilute and carry the injected single-cell suspension (Chua et al., 2011). In contrast to studies that had shown that intra-carotid delivery of cells induces vascular occlusion and increased mortality in stroke animals (Li et al., 2010; Walczak et al., 2008), we found no differences in the mortality rate, body weight, motor function and left brain hemisphere integrity among the experimental groups, indicating the safety of microneedle-based injection method.

The IA transplantation was performed in the left common carotid artery, contralateral to the injured brain hemisphere, because we used Rice's model of neonatal HI in which the right common carotid artery is permanently occluded and does not allow reperfusion (Rice et al., 1981). Some studies suggest that transplanted cells migrate preferentially to the ischemic hemisphere (Lundberg et al., 2009) and that chemoattraction may recruit therapeutic cells through the interhemispheric vasculature, followed by transendothelial diapedesis and subsequent intraparenchymal migration to the HI brain lesion (Andres et al., 2011; Pendharkar et al., 2010; Rosenblum et al., 2012).

The spatial version of the MWM was used to assess long-term cognitive function of intra-arterially transplanted HI rats, as the hippocampus, which plays a major role in spatial memory, is the predominant region damaged after neonatal HI insult. In this study, our results revealed that the IA transplantation of HUCB mononuclear cells dose-dependently reduced learning and long-term spatial memory deficits induced by neonatal HI brain injury. We have previously demonstrated the same beneficial effect on cognitive function using the IV route for HUCB mononuclear cell delivery (de Paula et al., 2012). According to our studies, the cellular dose capable of promoting cognitive recovery in HI rats is lower with IA transplantation than with IV (1×10^7 vs 1×10^8 cells, respectively) transplantation. Ma and coworkers also demonstrated that 1×10^4 embryonic stem cell-derived cells improve the learning ability and memory of HI mice at 2 and 8 months after intracerebroventricular transplantation using the MWM paradigm (Ma et al., 2007). The accelerating rotarod test was utilized in our study as it can reveal the sensorimotor deficits caused by motor cortical dysfunction in rats. However, there was no significant impact of the neonatal HI model, IA administration of HUCB mononuclear cells or vehicle on balance and motor coordination in adult rats. The finding of normal locomotor activity in adult rats subject to neonatal HI insult may

be due to the spontaneous recovery of sensorimotor deficits because of plasticity of the immature brain (Balduini et al., 2000; de Paula et al., 2009; Lubics et al., 2005; Rojas et al., 2012). There is evidence for a positive effect of cell-based therapy on neonatal HI-induced sensorimotor impairment using the IC (Daadi et al., 2010; Jansen et al., 1997; Park et al., 2011; van Velthoven et al., 2010; Yasuhara et al., 2008) and IV (Yasuhara et al., 2008; Yasuhara et al., 2010) routes. With regard to IA transplantation, stem cells have been shown to recover sensorimotor deficits in experimental models of stroke (Kamiya et al., 2008; Ohta et al., 2006) and traumatic brain injury (Osanai et al., 2012). A positive correlation was demonstrated between the number of intra-arterially transplanted cells found in the brain and the degree of motor recovery in the adult HI model (Guzman et al., 2008). Conversely, two studies using the neonatal HI model failed to demonstrate motor recovery in the rotarod test by using IC (Yasuhara et al., 2006) and intra-cardiac (Lee et al., 2010) delivery methods. In our study, intra-carotid transplantation was unable to reduce brain damage independent of cognitive recovery in the HI rats. It is possible that IA transplantation of HUCB mononuclear cells enhances brain plasticity rather than neuroprotection. Similarly, others have also shown functional recovery without brain injury reduction through IC (Daadi et al., 2010; Jansen et al., 1997) and IA (Gutierrez-Fernandez et al., 2011; Guzman et al., 2008; Zhang et al., 2012) cellular transplantation. Interestingly, the greater brain engraftment of intra-arterially transplanted cells may not necessarily be associated with higher brain lesion recovery in the stroke model (Gutierrez-Fernandez et al., 2011; Li et al., 2010; Zhang et al., 2012). Intra-cardiac injection of mesenchymal stem cells neither restores brain infarction nor improves the performance on the rotarod test in neonatal HI (Lee et al., 2010). In contrast, IA delivery of therapeutic cells favored both histological and cognitive outcomes in adult HI (Andres et al., 2011) and stroke (Brenneman et al.,

2010; Kamiya et al., 2008; Ohta et al., 2006) models. Interestingly, in our previous work, the transplantation of 1 x 10^7 HUCB mononuclear cells decreased infarction volume in animals receiving IV but not IA treatment (de Paula et al., 2012). Indeed, direct comparisons between our data and other studies are difficult to make because of differences in the methodologies used, animal models, brain immaturity, cellular source and concentration, time of administration and behavioral protocols among others.

The monitoring of cell biodistribution is important when delivery strategies for cellular transplantation are studied. As a preliminary evaluation, we verified cellular migration to the injured brain hemisphere as early as 3 and 6 h post-transplantation and also at 7 and 30 days later. However, human cells were not detectable at 24 h after their IA administration into the neonatal rat brain. This evidence is questionable because different sets of animals were used for each time point analyzed. Furthermore, interindividual variability of the IA transplant efficiency and degree of HI brain damage might have affected cellular migration to the target injured brain (Li et al., 2010). We also detected human cells in the liver and lungs at 1 and 3 h post-IA transplantation, which is consistent with preclinical and clinical studies (Barbosa da Fonseca et al., 2010; Vasconcelos-dos-Santos et al., 2012). PCR analyses are commonly used to investigate cell migration in preclinical studies (de Paula et al., 2012; de Paula et al., 2009; Venturin et al., 2011). However, the drawback of the PCR technique lies in its inability to permit longitudinal studies in the same animals. Recently, new technologies such as scintigraphy and gamma counter (Vasconcelos-dos-Santos et al., 2012), small animal SPECT/CT (Lappalainen et al., 2008; Mitkari et al., 2012), small animal MRI (Gutierrez-Fernandez et al., 2011; Li et al., 2010; Pendharkar et al., 2010; Walczak et al., 2008) and optical imaging (Andres et al., 2011; Chua et al., 2011; Osanai et al., 2012; Pendharkar et al., 2010; Rosenblum et al., 2012) have become available for

dynamic studies of cell biodistribution over time in living animals that receive intracarotid cellular transplantation. It has been shown in animal models of ischemic stroke (Gutierrez-Fernandez et al., 2011; Kamiya et al., 2008; Lappalainen et al., 2008; Li et al., 2010; Pendharkar et al., 2010; Walczak et al., 2008; Zhang et al., 2012) and traumatic brain injury (Lundberg et al., 2009; Lundberg et al., 2012) that IA delivery results in a wider distribution and larger number of grafted cells in the injured brain than by IV injection. In contrast, a recent study showed that IA and IV delivery routes provide similar brain homing of bone marrow mononuclear cells in focal cerebral ischemia (Vasconcelos-dos-Santos et al., 2012). Notwithstanding, it was demonstrated that IA transplantation of stem cells after transient ischemia results in a high variability of cell homing in the rat brain (Brenneman et al., 2010; Lappalainen et al., 2008; Li et al., 2001; Walczak et al., 2008). An explanation for this variability could be the ratio of the cell diameter to the capillary size, which may affect the biodistribution of transplanted cells into cerebral tissue (Lappalainen et al., 2008; Walczak et al., 2008). Therefore, it is likely that intra-arterially injected cells become initially entrapped in the cerebrovasculature due to cellular adhesion and size and are then gradually recruited by chemotactic signals to the ischemic tissue (Mitkari et al., 2012; Osanai et al., 2012; Vasconcelos-dos-Santos et al., 2012).

The present study was not designed to investigate the mechanisms of action by which HUCB mononuclear cells exert their neuroprotective effects on HI neonatal rats. However, it should be noted that stem cells may promote functional and/or lesion recovery through a variety mechanisms in the immature brain. In contrast to the initial concept, studies indicate that engraftment and direct differentiation of the transplanted cells are not the key mechanisms of cell-based therapies. Currently, it has been postulated that stem cells exert therapeutic effects mediated by blood vessel

regeneration, greater survival of intrinsic cells, synaptic plasticity enhancement, anti-inflammatory action and immunomodulation, most likely via neurotrophic factors and cytokine secretion (Bennet et al., 2012; Carroll, 2012; Liao et al., 2012; Pimentel-Coelho et al., 2012; van Velthoven et al., 2012a). Although the mechanisms underlying the action of stem cell-based therapeutics are still debatable, some studies suggest that only the presence of transplanted HUCB cells within the systemic circulation is sufficient for functional recovery and brain repair (Borlongan et al., 2004; Yasuhara et al., 2010). Conversely, others point to the importance of stem cell engraftment into the brain for behavioral improvement (Guzman et al., 2008). It could therefore be hypothesized that the IA delivery route would allow the convergence of systemic effects and the focal action of HUCB mononuclear cells in the brain of HI animals.

To our knowledge, this is the first study to demonstrate the feasibility and protective effects of IA transplantation of HUCB mononuclear cells in an experimental model of neonatal HI. Major factors such as the particulars of the human cerebral vasculature and hemodynamics, the dosage and infusion regimen, the adjuvant interventions and the cell type and treatment timing will need to be addressed before translating the IA approach for the delivery of stem cells into clinical applications. It would be suitable to compare multiple routes of cell delivery using the same neonatal HI model paradigm, assessments of brain lesions and behavioral outcomes and cellular tracking by small animal imaging. Moreover, porcine animal models of neonatal HI should be encouraged to evaluate the safety issues of IA delivery. Therefore, we propose that the IA route might provide an effective alternative for cellular administration for pediatric patients with HI injury.

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Figure 1

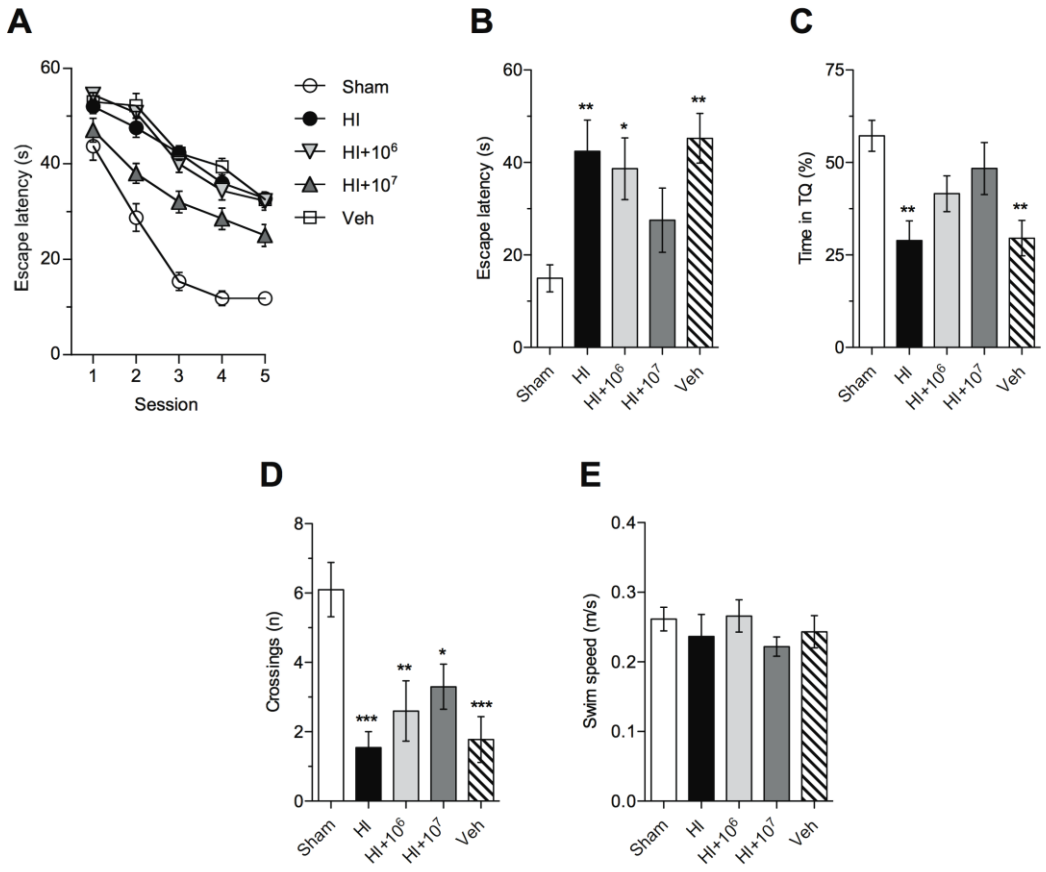

Fig. 1. Intra-arterial transplantation of 1 x 10^7 HUCB mononuclear cells reduced the injury-induced spatial memory impairment in hypoxic-ischemic rats. (A) The mean escape latencies to the hidden platform were obtained from a 5-day training session. Data are presented in blocks of 8 trials as the means ± S.E.M. (B) The HI+10^7 group showed a significantly shorter latency of swimming over the previous platform location when compared with sham-operated animals. (C) The HI+10^6 and HI+10^7 group groups spent a greater percentage of time searching the quadrant in which the platform had been submerged during training compared with the sham rats. (D) No significant differences were observed between the sham group and the HI+10^6 or HI+10^7 animals for the number of crossings over the former target position. (E) The swimming speed was not altered by the neonatal HI model or intra-arterial administration of HUCB

mononuclear cells or vehicle. The values are presented as the means ± S.E.M. Differences between groups were analyzed by one-way ANOVA followed by the Dunnett's post hoc test; $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ versus the sham group.

Figure 2

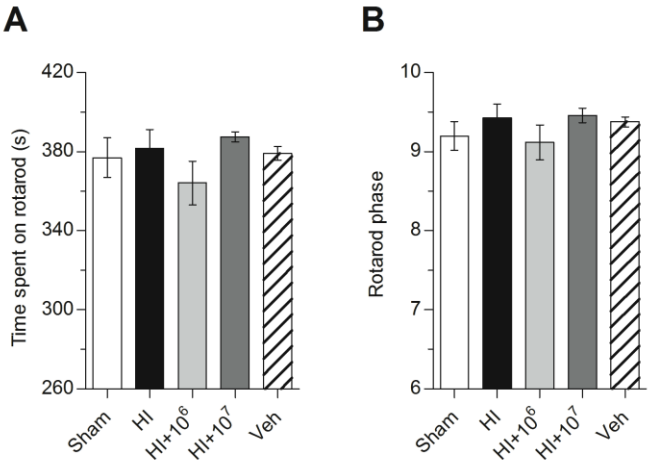

Fig. 2. Effect of intra-arterial transplantation of HUCB mononuclear cells or vehicle on motor coordination and balance in HI rats. There were no statistically significant differences between the HI group and the sham rats on the mean time spent on the treadmill (A) and mean phase in which the fall occurred (B). The values are presented as the means ± S.E.M. Differences between groups were analyzed by one-way ANOVA followed by the Dunnett's post hoc test.

Figure 3

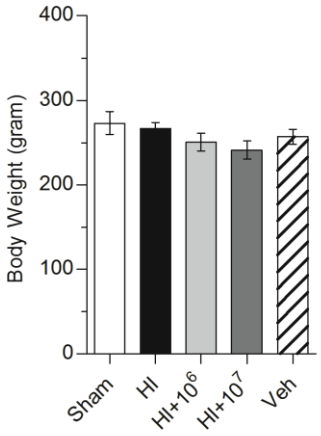

Fig. 3. Effect of intra-arterial transplantation of HUCB mononuclear cells or vehicle on the body weight of adult rats previously subjected to neonatal HI. At PND 71, animals were weighted before histological analysis, and they all displayed the same body weight. The values are presented as the means ± S.E.M. Differences between groups were analyzed by one-way ANOVA followed by the Dunnett's post hoc test.

Figure 4

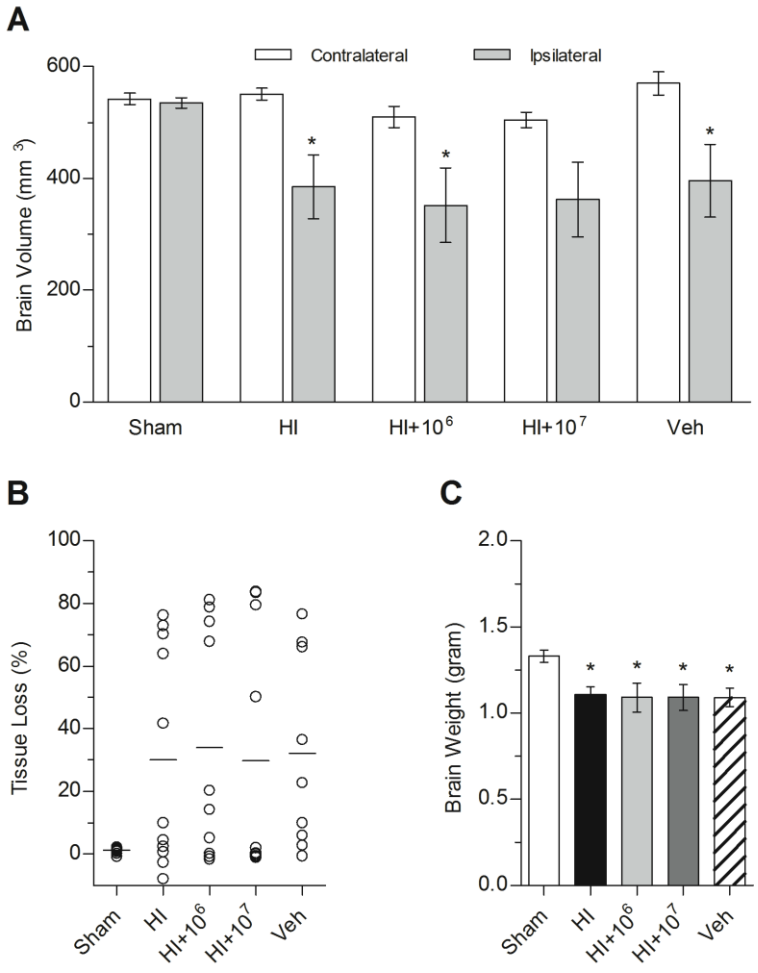

Fig. 4. Intra-arterial transplantation of HUCB mononuclear cells is not able to prevent long-term brain injury in adult rats subjected to neonatal HI. The brain volume was estimated using the Cavalieri principle associated with the counting point method 65 days after HI injury. Data are presented as the mean ± S.E.M. (A) The estimated brain volumes of the left (contralateral, white columns) and right hemispheres (ipsilateral, gray columns). $^{*}p < 0.05$ *vs.* left hemisphere volume in Bonferroni's post hoc test after one-way ANOVA. (B) Percentage of brain tissue loss demonstrated no statistically significant difference between the experimental groups in Bonferroni's post hoc test after one-way ANOVA. All values represent the mean ± S.E.M. (C) Brain weight measurement showed no significant difference among the HI groups, but all of these

groups had lighter brains than the sham group. $^{*}p < 0.05$ *vs.* sham in Dunnett's post hoc test after one-way ANOVA.

Table 1

Timeline of experimental procedures

PND 7	Neonatal rats subjected to the hypoxia-ischemia model
PND 8	Intra-arterial administration of HUCB mononuclear cells or vehicle
PND 65-69	Training for spatial version of the Morris water maze (MWM) learning task
PND 70	Probe test for the MWM and rotarod habituation
PND 71	Accelerated rotarod test
PND 72	Cerebral hemispheric volume assessment

Table 2

Morris water maze acquisition performance

Group	Training session				
	1	2	3	4	5
Sham	$43.7 \pm 2.9^{**}$	$28.8 \pm 2.9^{***}$	$15.4 \pm 1.9^{***}$	$11.8 \pm 1.5^{***}$	$11.8 \pm 1.1^{***}$
HI	$52.1 \pm 1.5^{\dagger\dagger}$	$47.6 \pm 2.0^{\dagger\dagger\dagger\S\S}$	$42.3 \pm 1.4^{\dagger\dagger\dagger\S\S\S}$	$36.0 \pm 0.9^{\dagger\dagger\dagger\S}$	$32.8 \pm 1.2^{\dagger\dagger\dagger\S}$
HI+10^6	$54.5 \pm 1.0^{\dagger\dagger\dagger\S}$	$50.7 \pm 0.9^{\dagger\dagger\dagger\S\S\S}$	$40.0 \pm 1.8^{\dagger\dagger\dagger\S}$	$34.4 \pm 2.0^{\dagger\dagger\dagger}$	$32.2 \pm 1.9^{\dagger\dagger\dagger\S}$
HI+10^7	47.1 ± 2.5	$38.0 \pm 2.1^{\dagger\dagger**}$	$32.0 \pm 2.3^{\dagger\dagger\dagger***}$	$28.5 \pm 2.2^{\dagger\dagger\dagger*}$	$25.0 \pm 2.3^{\dagger\dagger\dagger*}$
Veh	$53.0 \pm 1.9^{\dagger\dagger}$	$52.2 \pm 2.6^{\dagger\dagger\dagger\S\S\S}$	$42.3 \pm 1.5^{\dagger\dagger\dagger\S\S}$	$39.4 \pm 1.7^{\dagger\dagger\dagger\S\S\S}$	$32.5 \pm 1.6^{\dagger\dagger\dagger\S}$

Values represent the mean ± S.E.M.. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. HI group; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ and $^{\dagger\dagger\dagger}p < 0.001$ vs. sham group; $^{\S}p < 0.05$, $^{\S\S}p < 0.01$, $^{\S\S\S}p < 0.001$ vs. HI+10^7 group in Bonferroni post hoc test after two-way ANOVA.

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Capítulo IV

1 CONCLUSÕES

1.1 Tratamento com neuropeptídeo NAP em HI neonatal:

- O neuropeptídeo NAP bloqueou a formação aguda de danos ao DNA e peroxidação lipídica, além de modular o sistema glutationa, no hipocampo e córtex cerebral de ratos submetidos ao modelo de HI neonatal;
- A administração do neuropeptídeo NAP, logo após a indução de lesão hipóxico-isquêmica neonatal, impediu o desenvolvimento de déficits de aprendizado e de memória espacial em ratos adultos;
- O neuropeptídeo NAP teve ação protetora a longo prazo contra a atrofia cerebral característica do modelo de HI neonatal.

1.2 Transplante intra-arterial de CMSCUH em HI neonatal:

- O transplante intra-arterial de CMSCHU em ratos neonatos hipóxico-isquêmicos foi factível através da técnica de microagulha;
- A administração intracarotídica de CMSCHU ou veículo em ratos neonatos demonstrou-se segura, pois não agravou os desfechos do modelo de HI neonatal, tais como mortalidade, função motora, desempenho cognitivo e lesão cerebral;
- O transplante intra-arterial de CMSCHU, após o insulto hipóxico-isquêmico neonatal, preveniu de maneira dose-dependente o desenvolvimento de prejuízo na aprendizagem e na memória espacial de ratos adultos;
- Tanto a indução de HI neonatal como a administração intracarotídica de CMSCHU ou veículo não alteraram a coordenação motora e equilíbrio de ratos na fase adulta;
- O transplante intra-arterial de CMSCHU ou veículo e o modelo experimental de HI neonatal não tiveram influência sobre o peso corporal de ratos hipóxico-isquêmicos na fase adulta;
- As CMSCHU transplantadas intra-arterialmente não foram capazes de promover neuroproteção contra a atrofia cerebral e, consequentemente, restabelecimento do peso cerebral adequado de ratos adultos previamente submetidos ao modelo de HI neonatal;

– Pode-se detectar as CMSCHU tanto logo após o transplante intra-arterial – hemisfério cerebral esquerdo (1 h), hemisfério direito (3 e 6 h), fígado e pulmões (1 e 3 h) – como tardiamente – hemisfério esquerdo (7 e 30 dias), hemisfério direito (30 dias) e pulmões (7 e 30 dias). Não detectou-se CMSCHU no baço dos animais hipóxico-isquêmicos transplantados em nenhum dos tempos analisados.

Anexos

ANEXO A

Aprovação do Projeto de Pesquisa I pelo CEUA-PUCRS

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMITÊ DE ÉTICA PARA O USO DE ANIMAIS

Ofício 138/10 – CEUA

Porto Alegre, 19 de agosto de 2010.

Senhor Pesquisador:

O Comitê de Ética para o Uso de Animais apreciou e aprovou seu protocolo de pesquisa, registro CEUA 10/00172, intitulado: **"Terapia com neuropeptídeo NAP na lesão hipóxico-isquêmica neonatal: avaliação de aspectos bioquímicos, cognitivos e histológicos".**

Sua investigação está autorizada a partir da presente data.

Atenciosamente,

Profa. Dra. Anamaria Gonçalves Feijó
Coordenadora do CEUA – PUCRS

Ilmo. Sr.
Dr. Jaderson Costa da Costa
Incer
N/Universidade

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Campus Central
Av. Ipiranga, 6690 – Prédio 60, sala 314
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ANEXO B

Aprovação do Projeto de Pesquisa II pelo CEUA-PUCRS

PUCRS CEUA

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMITÊ DE ÉTICA PARA O USO DE ANIMAIS

Ofício 085/09 – CEUA

Porto Alegre, 04 de setembro de 2009.

Senhor Pesquisador:

O Comitê de Ética para o Uso de Animais apreciou e aprovou seu protocolo de pesquisa, registro CEUA 09/00105, intitulado: **"Efeito do transplante intra-arterial de células-tronco de cordão umbilical humano na hipóxia-isquemia experimental"**.

Sua investigação está autorizada a partir da presente data.

Atenciosamente,

Profa. Dr. Anamaria Feijó
Coordenadora do CEUA – PUCRS

Ilmo. Sr.
Prof. Dr. Jaderson Costa da Costa
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ANEXO C

Comprovante de submissão de artigo original II para a revista *Neurobiology of Disease*

Assunto:	Neurobiology of Disease: Submission Confirmation
De:	Neurobiology of Disease Editorial Office (nbd@elsevier.com)
Para:	jcc@pucrs.br; samuelgreggio@yahoo.com;
Data:	Quinta-feira, 17 de Janeiro de 2013 15:05

Title: Intra-arterial transplantation of human umbilical cord blood mononuclear cells in neonatal hypoxic-ischemic rats is feasible and restores long-term learning and spatial memory impairments
Corresponding Author: Dr Jaderson Costa DaCosta
Authors: Samuel Greggio, MsC; Simone de Paula, PhD; Pâmella N Azevedo; Gianina T Venturin, PhD;

Dear Dr DaCosta,

This is to confirm that the above-mentioned manuscript has been received for consideration in Neurobiology of Disease.

You will be able to check on the progress of your manuscript by logging on to the Elsevier Editorial System for Neurobiology of Disease as an author:
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Thank you for submitting your manuscript to Neurobiology of Disease. Should you have any questions, please feel free to contact our office.

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http://help.elsevier.com/app/answers/list/p/7923 Here you can search for solutions on a range of topics, find answers to frequently asked questions and learn more about EES via interactive tutorials. You will also find our 24/7 support contact details should you need any further assistance from one of our customer support representatives.

Kind regards,

Neurobiology of Disease

ANEXO D

Ata de Defesa de Tese n.º 036-P

Pontifícia Universidade Católica do Rio Grande do Sul
FACULDADE DE MEDICINA
PÓS-GRADUAÇÃO EM PEDIATRIA E SAÚDE DA CRIANÇA

ATA DE DEFESA DE TESE Nº 036-P

Ao primeiro dia do mês de março do ano de dois mil e treze, no Programa de Pós-graduação em Medicina/Pediatria e Saúde da Criança da Pontifícia Universidade Católica do Rio Grande do Sul o doutorando **SAMUEL GREGGIO** apresentou a tese de doutorado intitulada **"TERAPIAS INOVADORAS EM MODELO EXPERIMENTAL DE HIPÓXIA-ISQUEMIA NEONATAL: NEUROPEPTÍDEO NAP E CÉLULAS MONONUCLEARES DE SANGUE DE CORDÃO UMBILICAL HUMANO",** sob a orientação do Professor Doutor Jaderson Costa DaCosta, em sessão pública, no Auditório 1 da FAMED, 3º andar do Hospital São Lucas da PUCRS. A Comissão Examinadora foi presidida pelo Professor Doutor Jaderson Costa DaCosta e constituída pelos Professores Doutores: Rosália Mendez-Otero, Jociane de Carvalho Myskiw, Magda Lahorgue Nunes. A sessão foi aberta pelo Professor Doutor Jaderson Costa DaCosta, Professor do Programa de Pós-Graduação em Medicina/Pediatria e Saúde da Criança, que saudou os presentes e, inicialmente, deu ao doutorando as orientações sobre o processo de defesa de tese concedendo-lhe cinquenta minutos para expor o trabalho. Após a exposição, ao doutorando foi arguida pelos componentes da Comissão Examinadora, respondendo a cada examinador. Encerrada a arguição os examinadores consideraram o candidato ***APROVADO COM VOTOS DE LOUVOR.*** O presidente da banca comunicou a aprovação do doutorando, encerrando a sessão pública de apresentação para constar, lavrou-se esta ata que será assinada pelos integrantes da Comissão Examinadora, pelo professor orientador, pelo Coordenador do Programa de Pós-graduação em Medicina/Pediatria e Saúde da Criança, pelo doutorando e por mim, Carla Carmo de Melo Rothmann, secretária que a redigi.

Porto Alegre, 01 de março de 2013.

Profª. Drª. Rosália Mendez-Otero

Profª. Drª. Jociane de Carvalho Myskiw

Profª. Drª. Magda Lahorgue Nunes

Prof. Dr. Jaderson Costa DaCosta
Orientador

Prof. Dr. Renato Tetelbom Stein
Coordenador

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Apêndices

APÊNDICE A

Termo de Consentimento Livre e Esclarecido

Durante a gravidez, o oxigênio e nutrientes essenciais passam do sangue materno para o bebê através da placenta e do cordão umbilical. Após o parto, o sangue que permanece no cordão umbilical e na placenta é geralmente descartado. Este sangue contém um grande número de células-tronco, que são células jovens, que conseguem se reproduzir em células de seus respectivos tecidos. Pesquisas em andamento buscam utilizar essas células na regeneração de órgãos, como o coração e o cérebro, pois estas células podem se transformar em diversas outras, tais como, células sanguíneas, musculares e nervosas (neurônios).

O objetivo deste trabalho é avaliar se as células-tronco de cordão umbilical humano podem tratar a lesão cerebral de ratos que sofreram asfixia no período do nascimento. Para a coleta do sangue, após o nascimento, o cordão umbilical é pinçado (lacrado com uma pinça) e separado do bebê, cortando a ligação entre o bebê e a placenta. A quantidade de sangue (cerca de 70 - 100 ml) que permanece no cordão e na placenta é drenada para uma bolsa de coleta. Em seguida, já no laboratório de processamento, as células-tronco são separadas e preparadas para o congelamento. Durante este procedimento de coleta, não há nenhum risco para a mãe e para o bebê. As equipes de coleta atuam somente com o consentimento do obstetra, garantindo que nada interfira no parto.

Eu, _____ (responsável) fui informada dos objetivos da pesquisa acima de maneira clara e detalhada. Recebi informação a respeito dos procedimentos e esclareci minhas dúvidas. Sei que minha participação no estudo é voluntária e gratuita e que em qualquer momento poderei solicitar novas informações e modificar minha decisão se assim eu o desejar. O doutorando Samuel Greggio certificou-me de que todos os dados desta pesquisa referentes a mim e ao meu bebê serão confidenciais, bem como o seu tratamento não será modificado em razão desta pesquisa e terei liberdade de retirar meu consentimento de participação na pesquisa, em face destas informações. Concordo que as amostras de sangue de cordão umbilical e placenta obtidas serão utilizadas apenas com finalidade de pesquisa experimental.

Fui informada que caso existam danos à minha saúde, causados diretamente pela pesquisa, terei direito a tratamento médico e indenização conforme estabelece a lei. Também sei que caso existam gastos adicionais, estes serão absorvidos pelo orçamento da pesquisa. Caso tiver novas perguntas sobre este estudo, posso chamar pelo doutorando Samuel Greggio

nos telefones (51) 32763636 e (51) 82083636, ou através do e-mail (samuelgreggio@yahoo.com). Para qualquer pergunta sobre os meus direitos como participante deste estudo ou se penso que fui prejudicado pela minha participação, posso chamar pelo orientador da pesquisa, Dr. Jaderson Costa da Costa, ou pelo Comitê de Ética e Pesquisa da PUCRS pelo telefone (51) 33203345. Declaro que recebi cópia do presente Termo de Consentimento.

Data: ___/___/___

_____

Assinatura do Paciente

_____

Nome do Paciente

_____

Assinatura do Pesquisador

APÊNDICE B

Revisão das publicações sobre terapia celular em hipóxia-isquemia experimental no período neonatal

Referência	Modelo de HI (animal, idade e duração da hipóxia)	Tipo celular	Transplante (concentração via, tempo e imunossupressão)	Resultados funcionais*	Resultados morfológicos, celulares e moleculares*	Migração	Diferenciação
Elsayed et al. 1996	Ratos Long-Evans black-hooded; P7-8; 60, 120 ou 120-150 min	Tecido neocortical fetal murino (13º dia embrionário)	Bloco tecidual de 1-2 mm^3; ic (TI); 7 dias após HI; SI	NA	Nenhum efeito sobre a atrofia cerebral; presença de conectividade axonal entre o transplante e as áreas adjacentes corticais do tecido receptor.	Transplantes com desenvolvimento satisfatório (63%) e pobre (19%), localizados no córtex ou em áreas adjacentes 6 semanas pT	NA
Jansen et al. 1997	Ratos Wistar; P7; 150 min	Tecido neocortical fetal murino (16º dia embrionário)	5 x 10^4 células/µL; ic (TI); 3 dias após HI; SI	Melhora da coordenação (3-8 semanas pT) e assimetria motora (9 semanas pT)	Ausência de restabelecimento da citoarquitetura cortical; o transplante expressou marcadores neuroquímicos e ausência de marcação glial; presença de astrócitos circundantes ao transplante. (9-11 semanas pT)	Identificação de 72% dos transplantes localizados em áreas adjacentes ao corpo caloso do córtex sensório-motor 9-11 semanas pT	NA
Park et al. 2002	Camundongos CD-1; P7; 120 min	CTN murinas (clone C17.2) em *scaffold* de PGA; CTN-	1 ou 2 complexos CTN-PGA (1 x 10^7 células/mL; 100-200 µL); ic (TI); 7	↓ rotação unilateral	Redução do dano cerebral; formação de interconexões entre CTN/PGA e o tecido	O transplante apresentou uma implantação satisfatória na	Células transplantadas apresentaram marcação de

		PGA)	dias após HI; SI		receptor; neovascularização; ↓ infiltração de monócitos e cicatriz astroglial; restabelecimento de projeções neuronais de longa distância	cavidade infartada cerebral 2-6 semanas pT	neurônio (5%) e oligodendrócito, na penumbra cortical da lesão, 2 semanas pT
Imitola et al. 2004	Camundongos C57Bl/6; P7; 120 min	Cinco linhagens de CTN humanas e murinas	5×10^5 células/mL; ic (TC); 3 dias após HI; ciclosporina	NA	CTN expressam CXCR4; ↑ expressão de SDF-1α na região cerebral infartada (células astrocíticas e endoteliais); interação da via SDF-1α/CXCR4	Correlação positiva entre a presença de células transplantadas na região isquêmica e a expressão de SDF-1α	Células transplantadas com marcação neuronal na penumbra cortical da lesão
Guan et al. 2004	Ratos Wistar; P7; 120 min (O_2 NE)	CTM de medula óssea murina	4×10^6 células/500 µL; ip; 2 h após HI; SI (NE)	NA	NA	Detecção de CTM no hemisfério lesado 14 dias pT	Poucas CTM apresentaram marcação para células progenitoras neurais (nestina); nenhuma diferenciação em neurônios maduros e astrócitos 14 dias pT
Katsuragi et al. 2005a	Ratos Wistar; P14; 120 min	Células BHK-GDNF encapsuladas	1 cápsula (1×10^8 células/mL); ic (TI); 2 dias pré-HI; SI	NA	↑ GDNF sérico; ↓ incidência e severidade do dano neuronal (7 dias pT)	Células BHK-GDNF mantiveram-se viáveis 7 dias pT.	NA

Katsuragi et al. 2005b	Ratos Wistar; P9; 120 min	Células BHK-GDNF encapsuladas	1 cápsula (1 x 10^8 células/mL); ic (TI); 2 dias pré-HI; SI	Melhora no desempenho cognitivo a partir da 6ª semana pT	Redução do dano cerebral 17 semanas pT	NA	NA
Qu et al. 2005	Ratos Wistar; P7; 90 min (O_2 NE)	CTN de tecido fetal humano	5 x 10^5 células/5 µL; ic (TI); 3 dias após HI; SI	NA	NA	Detecção de CTN na zona subventricular, corpo caloso, córtex e hipocampo, principalmente no hemisfério lesado em 1, 2 e 4 semanas, e 3 meses pT	A maioria das CTN transplantadas diferenciou-se em neurônios e astrócitos em 1, 2 e 4 semanas, e 3 meses pT
Zheng et al. 2006	Ratos Sprague-Dawley; P7; 120 min	Células-tronco astrocíticas multipotentes da zona subventricular murina	5 x 10^4 células/µL; ic (TI); 24 h após HI; SI	NA	NA	Detecção das células transplantadas na região cortical e periventricular isquêmica em até 21 dias pT	Células transplantadas apresentaram marcação de astrócito e neurônio 3–21 dias pT
Meier et al. 2006	Ratos Wistar; P7; 80 min	CTCUH	1 x 10^7 células/500 µL; ip; 24 h após HI; SI	Melhora no desempenho locomotor 21 dias pT	Nenhum efeito sobre a atrofia cerebral em 21 dias pT	Presença das células transplantadas ao redor da lesão cerebral 21 dias pT	Ausência de células transplantadas com marcação para astrócito e neurônio 21 dias pT
Yasuhara et al. 2006	Ratos Sprague-Dawley; P7; 150 min	CPAM de medula óssea murina	2 x 10^5 células/3 µL; ic (TI); 7 dias após HI;	Melhora da assimetria, mas não da	NA	Detecção das células transplantadas nas	NA

			ciclosporina A	coordenação motora, 14 dias pT		regiões hipocampais de CA2 e CA3 14 dias pT	
Park et al. 2006	Camundongos CD-1; P7; 138 min	CTN murinas (clone C17.2)	0,4-1,6 x 10^5 células/4 µL; ic; 1 (TI), 3 (TI ou TC), 7 (TI), 14 (TI) ou 35 (TI) dias após HI; SI	NA	NA	TC: migração celular através do corpo caloso e outras comissuras inter-hemisféricas para a área infartada; TI: as células permaneceram no local da lesão, com melhor implantação quando transplantadas entre 3 e 7 dias após HI.	TI *versus* TC (3 dias após HI): células transplantadas apresentaram marcação de neurônio (~5 *versus* 0%), oligodendrócito (~4 *versus* ~1%), astrócito (~23 *versus* ~15%) e células progenitores neurais (~18 *versus* ~6%) no neocórtex (1 mês pT)
Park et al. 2006	Camundongos CD-1; P7; 120-180 min	CTN murinas (clone C17.2) com superexpressão de NT-3 (CTN-NT3)	3 x 10^5 células/8 µL (ic, TI em 2 locais) e 1,0 x 10^5 células/2 µL (icv, TC); 3 dias após HI; SI	NA	Expressão aumentada de NT-3	Presença das células nos locais de transplante 2-4 semanas pT	Células transplantadas com marcação neuronal (colinérgico, GABAérgico e glutamatérgico) na penumbra (81,4%) e área infartada (10-20%), e para oligodendrócito

							(0,4%) e glia (1%); células transplantadas com marcação neuronal no hemisfério contralateral (~90%) (2-4 semanas pT)
Ma et al. 2007	Camundongos C57Bl/6; P7; 90 min	Células-tronco embrionárias murinas após diferenciação neural *in vitro*	1 x 10^4 células/µL; icv (TI); 2-3 dias após HI; SI	Melhora da memória espacial 2 e 8 meses pT	↑ células neuronais na região hipocampal de CA1 8 meses pT	Detecção das células transplantadas no hipocampo e córtex cerebral 2 e 8 meses pT	Células transplantadas apresentaram marcação de neurônio, mas não de astrócito, 8 meses pT
Dayer et al. 2007	Ratos Wistar; P3; 30 min	Células progenitoras neurais murinas com superexpressão de FGF-2	4 x 10^4 células/0,5 µL; ic (TI); 1 e 3 dias após HI; SI	NA	Preservação do estado de imaturidade e proliferação celular é inversamente proporcional à expressividade de FGF-2 nas células transplantadas	Detecção das células transplantadas no córtex isquêmica infragranular e na margem entre córtex e corpo caloso 2 semanas pT	A grande maioria das células transplantadas apresentou marcação de neurônio imaturo; em menor número, houve marcação para astrócito, oligodendrócito e neurônio
Yasuhara et al. 2008	Ratos Sprague-Dawley; P7; 150	CPAM de medula óssea	2 x 10^5 células/3 µL; iv ou ic (TI); 7	Melhora da coordenação e assimetria	Redução da morte neuronal na região hipocampal de CA3 14	Detecção das células transplantadas nas	Células transplantadas apresentaram

	min	murina	dias após HI; SI	motora 14 dias pT (iv e ic)	dias pT (iv e ic)	regiões hipocampais isquêmicas de CA2 e CA3 14 dias pT (iv e ic). Localizavam-se também em vasos sanguíneos no hipocampo (iv).	marcação neuronal 14 dias pT (iv e ic)
Sato et al. 2008	Ratos Sprague-Dawley; P7; 120 min	CTN murinas (14º dia embrionário) ou CTN+ChABC (25 mU)	2,5 x 10^5 células/5 µL; icv (TI); 24 h após HI; SI	NA	↓ dano cerebral (CTN+ChABC < CTN = VEH); ↑ peso cerebral (CTN+ChABC > CTN = VEH) (7 dias pT)	Presença das células transplantadas na lesão isquêmica e áreas adjacentes 7 dias pT	NA
de Paula et al. 2009	Ratos Wistar; P7; 120 min	CTCUH	1 x 10^7 células/100 µL; iv; 24 h após HI; SI	Nenhum efeito sobre o déficit da memória espacial 3 semanas pT	Nenhum efeito sobre a atrofia cerebral 3 semanas pT	Detecção de poucas células transplantadas 24 h, 1 e 3 semanas pT	NA
Pimentel-Coelho et al. 2009	Ratos Lister-Hooded; P7; 90 min	CTCUH	2 x 10^6 células/200 µL; ip; 3 h após HI; SI	Melhora no desenvolvimento dos reflexos sensório-motores 4 dias pT	↓ morte neuronal e expressão de caspase 3 no estriado isquêmico 2 dias pT; ↓ ativação microglial no córtex 7 dias pT	Presença de poucas células transplantadas no córtex e estriado isquêmico 2 dias pT	NA
Jenny et al. 2009	Ratos Wistar; P3; 30 min	Células progenitoras neurais murinas com superexpressão de FGF-2	2–5 x 10^4 células/1 µL; ic (TI); 4 dias após HI; SI	NA	Formação de agrupamentos celulares com superexpressão de FGF-2 em áreas perivasculares	Presença das células transplantadas em áreas de lesão cortical e próximas a vasos sanguíneos 7 dias	Células transplantadas apresentaram marcação de neurônio imaturo 7 dias pT

						pT	
van Velthoven et al. 2010a	Camundongos C57Bl/6; P9; 45 min	CTM de medula óssea murina	1 x 10^5 células/2 µL; ic (TI); 3 dias (T-P12) ou 10 dias (T-P19) após HI; SI	Melhora da assimetria motora em 7 e 18 dias pT (T-P12), ou em 11 e 18 pT (T-P19)	T-P12: ↓ lesão cerebral (18 dias pT); ↑ neuro- e oligodendrogênese, e ↓ proliferação microglial no hipocampo e córtex isquêmico (7 e 18 dias pT); ↑ proliferação de astrócitos no hipocampo, e ↓ no córtex isquêmico (7 dias pT). T-P19: ↓ lesão cerebral (18 dias pT);	Menos de 1% das células em proliferação no hemisfério isquêmico originaram-se daquelas transplantadas	NA
Yasuhara et al. 2010	Ratos Sprague-Dawley; P7; 150 min	CTCUH ou CTCUH+M (manitol 1.1 mol/L)	1,5 x 10^4 células/200 µL; iv; 7 dias após HI; SI	Melhora da coordenação e assimetria motora 7 e 14 dias pT (CTCUH < CTCUH+M)	↑ níveis cerebrais de GDNF, BDNF e NGF 3 dias pT (CTCUH < CTCUH+M); ↑ densidade dendrítica na região hipocampal de CA1 14 dias pT	Presença de poucas células transplantadas no hipocampo isquêmico 14 dias pT	NA
Lee et al. 2010	Ratos Sprague-Dawley; P7; 210 min	CTM de medula óssea humana	1 x 10^6 células/µL; ica; 3 dias após HI; SI	Apesar de nenhum efeito sobre o déficit na coordenação motora, houve ↓ assimetria motora 20 dias pT	Nenhum efeito sobre a atrofia cerebral 6 semanas pT	Detecção das células transplantadas igualmente distribuídas em ambos os hemisférios cerebrais 6 semanas pT	Células transplantadas apresentaram marcação de astrócito e microglia (> nº), neurônio e oligodendrócito (< nº), igualmente distribuídas em

							ambos os hemisférios cerebrais 6 semanas pT
Xia et al. 2010	Ratos Sprague-Dawley; P7; 150 min	CTM de sangue de cordão umbilical humano	5 x 10^4 células/µL; ic (TI); 3 dias após HI; ciclosporina	Melhora da função neurológica 14, 21 e 28 dias pT	Atenuação do dano cerebral 28 dias pT	Presença das células transplantadas no córtex e dispersão para hipocampo 7 dias pT	Células transplantadas apresentaram marcação de astrócito, mas não de neurônio, 7 dias pT
Daadi et al. 2010	Ratos Sprague-Dawley; P7; 90 min	CTN humana de células-tronco embrionárias	5 x 10^4 células/µL; ic (TI em 3 locais); 24 h após HI; SI	Melhora da coordenação e assimetria motora 28-30 dias pT	Nenhum efeito sobre a atrofia cerebral; superexpressão de genes envolvidos na neuro-/gliogênese e suporte neurotrófico no hemisfério isquêmico; ↑ brotamento axonal para o hemisfério ipsilateral; ↑ terminais axonais no córtex sensório-motor, corpo caloso, estriato e tálamo ipsilateral; ↑ células microgliais no estriado isquêmico, mas não no córtex (1 mês pT)	Permanência das células transplantadas, com dispersão para o estriato isquêmico, em 1 mês pT	Células transplantadas apresentaram marcação de célula-tronco neural, neurônio e astrócito 1 mês pT
Rosenkranz et al. 2010	Ratos Wistar; P7; 80 min (8%)	CTCUH	1 x 10^7 células/500 µL; ip; 24 h após	NA	CTCUH expressavam CXCR4; ↑ expressão	Ação do eixo SDF-1/CXCR4	NA

			HI; SI		de SDF-1 (astrócitos reativos) no hemisfério lesado 2 e 14 dias após HI (CTCUH > HI)	sob "homing" celular; detecção de CTCUH no hemisfério lesado 2 e 14 dias após HI	
van Velthoven et al. 2010b	Camundongos C57Bl/6J; P9; 45 min (10%)	CTM de medula óssea murina	1 x 10^5 células/2 µL; ic (TI); 3 dias (T-P12), 10 dias (T-P19), ou 3+10 dias (T-P12+19) após HI; SI	Melhora da coordenação e assimetria motora 10, 21 e 28 dias após HI (T-P12 < T-P12+19)	↑ proliferação celular no hipocampo e córtex isquêmico 21 e 28 dias após HI (T-P12 = T-P19 = T-P12+19); neuro- e oligodendrogênese no hipocampo e córtex isquêmico 21 e 28 dias após HI (T-P12 = T-P12+19); ↓ perda de massa cerebral branca e cinzenta 21 e 28 dias após HI (T-P12 < T-P12+19); regeneração do trato corticoespinhal 28 dias após HI (T-P12+19); neurito- e sinaptogênese 28 dias após HI (T-P12 < T-P12+19); CTM ↑ expressão de IL-10, IL-1β, MT3 e NGF; ↓ expressão de BMP2, Inhbb e TGFβ1	Presença de poucas células transplantadas 28 dias após HI	NA
van Velthoven et al. 2010c	Camundongos C57Bl/6J; P9;	CTM de medula óssea	5 x 10^5 células/12 µL; in; 10 dias após	Melhora da assimetria	↓ perda de massa cerebral branca e	Detecção de células	Nenhuma evidência de

	45 min (10%)	murina	HI; SI	motora 21 e 28 dias após HI	cinzenta 28 dias após HI; CTM ↑ expressão de CXCR4, FGF2, SDF-1, NGF e NT3; ↓ expressão de IL-1β e TGFβ1; nenhum efeito sobre a expressão de BDNF e IL-6	transplantadas no hipocampo (ipsilateral), bulbo olfatório e zona subventricular (ambos hemisférios) 28 dias após HI	diferenciação das CTMs transplantadas em neurônios maduros, astrócitos e microglias
van Velthoven et al. 2011	Camundongos C57Bl/6J; P9; 45 min (10%)	CTM de medula óssea murina	1 x 10^5 células/2 µL; ic (TI); 3 dias (T-P12), 10 dias (T-P19), ou 3+10 dias (T-P12+19) após HI; SI	NA	↓ atrofia cerebral (T-P12 = T-P19 < T-P12+19) 28 dias após HI; T-P19 induziu expressão gênica associados à sobrevivência e proliferação celular 13 dias após HI; T-P12+19 induziu expressão gênica associados à proliferação celular, diferenciação e integração de redes neurais 13 dias após HI	Detecção de CTMs transplantadas no hemisfério ipsilateral 10 (T-P19 = T-P12+19), 13 (~22%, T-P19 < T-P12+19) e 28 dias após HI (~1%, T-P19 < T-P12+19)	NA
Park et al. 2011	Ratos Sprague-Dawley; P5; oclusão bilateral; 120 min (8%)	Progenitores neurais provenientes de CTM de tecido placentário humano	2 x 10^5 células/4 µL; ic (estriado bilateral); 2 semanas após HI; ciclosporina A	Melhora progressiva da coordenação motora entre 2, 4, 6 e 8 semanas pT	NA	Detecção de células transplantadas 8 semanas pT	Diferenciação em neurônios maduros, astrócitos e células produtoras de dopamina 8 semanas pT

Obenaus et al. 2011	Ratos Sprague-Dawley; P10; 90 min (8%)	CTN murina	5 x 10^5 células/5 µL; ic (estriado [Te] e ventrículo [Tv]); TC; 3 dias após HI; SI	NE	↑ proliferação (273%) de CTN nas primeiras 4 semanas pT	CTNs detectáveis em até 58 semanas pT; CTNs migraram (100-125 µm/dia) para a lesão HI (Tv > Te); pico migratório em 10-12 dias pT; corrente migratória presente no corpo caloso	Diferenciação em neurônio (~5%), oligodendrócitos (~4%) e astrócitos (~30%)
Geißler et al. 2011	Ratos Wistar; P7; 80 min (8%)	CTCUH	1 x 10^7 células/500 µL; ip; 24 h após HI; SI	Melhora sensório-motora e da assimetria motora 40 dias pT	Preservação da representação cortical da pata traseira esquerda; normalização do tamanho do campo receptivo e processamento temporal cortical do hemisfério esquerdo (41 dias pT)	CTCUHs migraram para o hemisfério isquêmico, sendo detectadas na periferia da lesão HI, 41 dias pT	NA
Liu et al. 2011	Ratos Sprague-Dawley; P7; 120 min (8%)	CTM ou de-CTM de medula óssea murina	1-2 x 10^5/5 µL; icv (TI); 5 dias após HI; SI	Melhora da memória e aprendizado durante 2 meses pT (de-CTM > CTM)	Angiogênese (marcador endotelial CD-31) 7 dias pT (de-CTM > CTM)	Células migraram para a periferia da lesão HI 3 (CTM e de-CTM) e 7 (de-CTM) dias pT	Neuro-diferenciação pelos marcadores NF-M e MAP-2 (3 dias pT: CTM e de-CTM; 7 dias pT: de-CTM); provável

							diferenciação/ fusão de de-CTMs em/a células endoteliais 7 dias pT
de Paula et al. 2012	Ratos Wistar; P7; 120 min (8%)	CTCUH	1 x 10^6, 1 x 10^7, 1 x 10^8 células/100 µL; iv; 24 h após HI; SI	Melhora da memória espacial (10^8) 8 semanas pT	↓ atrofia cerebral (VEH = 10^6; 10^7 < 10^8) 8 semanas pT	Detecção de células transplantadas no córtex e hipocampo de ambos os hemisférios (10^6, 10^7 e 10^8) 7 dias pT	NA
Bae et al. 2012	Ratos Sprague-Dawley; P7; 90 min (8%)	CTCUH	1 x 10^7 células/200 µL; iv; 24 h após HI; ciclosporina A	Melhora da assimetria motora 5 semanas pT associada ao n.º de neurônios neocórtex; melhora cognitiva 10 semanas pT; ↓ ansiedade e depressão 9 semanas pT	↑ neurônios maduros no neocórtex contralateral 10 semanas pT; ↑ microglia no estriado ipsilateral 1 semana pT; ↑ neurônios imaturos na zona subventricular 1 e 10 semanas pT; ↑ progenitores neurais no hemisfério ipsilateral 1 semana pT; CTCUH expressam altos níveis de IL-6, IL-8, IP-10, RANTES, GRO-α, G-CSF, MCP-1 e MCP-3	Detecção de células transplantadas na região periventricular (núcleo septal e estriado) do hemisfério ipsilateral 1 semana pT; possível influência da expressão de GRO-α e MIP-1α sobre a migração das células transplantadas	Co-marcação para células progenitoras neurais (nestina) e neurônios imaturos (doblecortina) 1 semana pT
Wasielewski et	Ratos Wistar;	CTCUH	1 x 10^7 células (ip:	Melhora da	↓ da expressão de	Detecção de	NA

al. 2012	P7; 80 min (8%)		500 µL; it: 20 µL); 24 h após HI; SI	assimetria motora (43 dias pT), força muscular (13 e 43 dias pT) e espasticidade distal (13 e 43 dias pT) (ip = it)	Cx43, GFAP e SHP-1 (1, 13 e 43 dias pT); ↓ da infiltração de microglia ativada/macrófagos (13 e 43 dias pT) (ip = it)	células transplantadas nas áreas de ativação microglial 13 dias pT (ip = it)	
van Velthoven et al. 2012	Camundongos C57Bl/6J; P9; 45 min (10%)	CTM de medula óssea murina	1×10^5 células/2 µL; ic (TI); 3+10 dias (T-P12+19) após HI; SI	↓ assimetria motora 10, 21 e 28 dias após HI	↓ atrofia cerebral; ↓ reestruturação axonal no hemisfério contralateral; ↑ conectividade axonal no hemisfério ipsilateral; ↓ dano ao trato corticoespinhal (28 dias após HI)	NA	NA
Rosenkranz et al. 2012	Ratos Wistar; P7; 80 min (8%)	CTCUH	1×10^7 células/500 µL; ip; 24 h após HI; SI	NA	↓ expressão de caspase-3 (14 dias após HI); ↑ sobrevivência neuronal (apenas em 2 dias após HI, mas não aos 14 dias); ↑ angiogênese (Tie-2, apenas em 2 dias após HI, mas não aos 14 dias) e integridade da BHE (ocludina, 14 dias após HI); ↑ expressão de VEGF, mas não de BDNF, no hemisfério ipsilateral (apenas em	NA	NA

					2 dias após HI, mas não aos 14 dias)		
Yamagata et al. 2012	Camundongos ICR; P5; 20 min (8%)	Células-tronco humanas de polpa de dente decíduo esfoliado (CTPD), fibroblasto de pele humana (FPH), meio de cultura condicionado de CTPD (MC-CTPD) e de FPH (MC-FPH)	CTPDD e FPH: 2 x 10^5 células/2 µL, ic, 24 h após HI, ciclosporina A; MC: 2 µL, ic, 24 h após HI; SI	Melhora da função motora e ↑ sobrevida (CTPD e MC-CTPD)	↓ atrofia cerebral, ↓ apoptose de neurônios (córtex, corpo caloso e and hipocampo) e oligodendrócitos (corpo caloso), ↑ escore neuropatológico (CTPD e MC-CTPD) (3 dias após HI); ↑ expressão de IL-6 e IL-10, ↓expressão de TNF-α e IL-1β (CTPD) (2 dias após HI)	NA	Praticamente nenhuma diferenciação das CTPD transplantadas em neurônios, oligodendrócitos e astrócitos (8 semanas pT)
Donega et al. 2013	Camundongos C57Bl/6; P9; 45 min (10%)	CTM de medula óssea murina	0,25 x 10^6, 0,5 x 10^6 e 1 x 10^6 células/3 µL; in; 3 dias (T-P12), 10 dias (T-P19), 17 dias (T-P26) ou 3+10 dias (T-P12+19) após HI; SI	Melhora da assimetria motora 3, 4, 5 semanas após HI (0,5 e 1 x 10^6; T-P12 e T-P19 = T-P12+19), e 8 semanas após HI (0,5 x 10^6; T-P19 = T-P12+19); ↑ função cognitva 7 semanas após HI (0,5 x 10^6; T-P19 = T-	↓ perda de massa cerebral branca e cinzenta 5 semanas após HI (0,5 e 1 x 10^6; T-P12 e T-P19 = T-P12+19), e 9 semanas após HI (0,5 x 10^6; T-P19 = T-P12+19)	Detecção de CTMs no hipocampo ipsilateral (T-P12) e ao redor da lesão (T-P19) 24 h após transplante; poucas CTMs detectadas para T-P26; ausência de células no hemisfério contralateral	NA

			P12+19)				
Greggio et al. 2013 (*submetido*)	Ratos Wistar; P7; 120 min (8%)	CTCUH	1 x 10^6 e 1 x 10^7 células/50 µL; ia; 24 h após HI; SI	Melhora da memória espacial (10^7); nenhum efeito sobre o equilíbrio e coordenação motora (9 semanas pT)	Nenhum efeito sobre a atrofia cerebral 9 semanas pT	Detecção de células transplantadas no hemisfério cerebral esquerdo (1 h, 7 e 30 dias pT), direito (3 e 6 h, 30 dias pT), fígado (1 e 3 h pT) e pulmões (1 e 3 h pT)	NA

* animais tratados *versus* animais controle; ↑: aumento; ↓: redução.

BDNF: fator neurotrófico derivado do cérebro; **BHK-GDNF**: células renais de filhotes de hamster (BHK) transfectadas com DNA complementar de fator neurotrófico derivado de linhagem celular glial (GDNF); **BMP2**: proteína morfogenética óssea 2; **ChABC**: condroitinase ABC; **CPAM**: células progenitoras adultas multipotentes; **CTCUH**: células-tronco de cordão umbilical humano; **CTM**: células-tronco mesenquimais; **CTN**: células-tronco neurais; **Cx43**: conexina 43; **CXCR4**: receptor de quimiocina CXC 4; **De-CTM**: células-tronco mesenquimais de-diferenciadas; **FGF-2**: fator de crescimento de fibroblasto 2; **G-CSF**: fator estimulante de colônia de granulócitos; **GDNF**: fator neurotrófico derivado de linhagem celular glial; **GFAP**: proteína glial fibrilar ácida; **GRO-α**: proteína regulatória do crescimento-alfa; **HI**: hipóxia-isquemia; **ia**: via intra-arterial; **ic**: via intracerebral; **icv**: via intracerebroventricular; **IL-1β**: interleucina-1β; **IL-10**: interleucina-10; **IL-6**: interleucina-6; **IL-8**: interleucina-8; **in**: via intranasal; **Inhbb**: inibitina β-B; **ip**: via intraperitoneal; **IP-10**: proteína 10 induzível por interferon gama; **it**: via intratecal; **iv**: via intravenosa; **MAP-2**: proteína associada a microtúbulo-2; **MCP-1**: proteína quimotática de monócitos-1; **MCP-3**: proteína quimotática de monócitos-3; **MT3**: metalotionina-3; **NA**: não avaliado; **NE**: não especificado; **NF-M**: neurofilamento-M; **NGF**: fator de crescimento neural; **NT3**: neurotrofina-3; **P**: dia pós-natal; **PGA**: ácido poliglicólico; **pT**: pós-transplante; **RANTES**: regulado na ativação, expresso e secretado por células T normais; **SDF-1**: fator derivado do estroma 1; **SHP-1**: proteína tirosina fosfatase-1; **T-PX**: transplante no dia pós-natal X; **SI**: sem imunossupressão; **TC**: transplante contralateral; **TGFβ1**: fator de crescimento transformante-β1; **TI**: transplante ipsilateral; **VEH**: veículo.

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APÊNDICE C

ARTIGO ORIGINAL

NAP prevents hippocampal oxidative damage in neonatal rats subjected to hypoxia-induced seizures

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NAP prevents hippocampal oxidative damage in neonatal rats subjected to hypoxia-induced seizures

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ABSTRACT

Neonatal seizures in which hypoxic-ischemic encephalopathy is the main triggering etiology have a challenging diagnosis and limited efficacy of treatment. NAP (NAPVSIPQ) has shown extensive neuroprotective and antioxidant capacity *in vitro* and *in vivo*. To evaluate its neuroprotective role in the context of seizures associated with perinatal hypoxia, we assessed the integrity of DNA and lipid membranes as well as the redox status in the hippocampus of 10-day-old rats exposed to hypoxia-induced seizures (HS) with and without NAP treatment. Rats were exposed to transient global hypoxia (12 min exposure to 5–7% O_2 was able to induce electrographic seizures) or room air with subsequent intraperitoneal NAP (0.03, 0.3 or 3 μg/g) or vehicle administration. Results showed elevated DNA damage immediately after the insult until 72 h post-HS, while oxidized bases were only detected 3, 6 and 24 h later. In addition, thiobarbituric acid reactive species peaked at 6 h in parallel with decreased levels of reduced glutathione between 3 and 72 h post-HS insult. Our findings expand on the knowledge about the time course of HS-induced oxidative damage and demonstrate for the first time that a single NAP injection dose-dependently prevents HS-induced oxidative damage to DNA and lipid membranes, in correlation with modulation of the glutathione system. Hence, NAP may represent a promising therapeutic strategy for avoiding HS-induced oxidative damage.

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Introduction

The immature brain is more susceptible to seizures in the first year of life (Volpe, 2001), particularly during the neonatal period, in which the incidence reaches 1–5 per 1000 live births (Hauser, 1994; Sheth et al., 1999). Hypoxic-ischemic encephalopathy remains the leading cause of refractory neonatal seizures (Volpe, 2001). While postnatal hypoxia alters key developmental programs of the newborn brain (Curristin et al., 2002), early-life seizures can also cause functional alterations in neuronal networks (Ben-Ari and Holmes, 2006). Both situations render the brain prone to an increased risk of epilepsy later in life and an increased incidence of cognitive deficits, such as cerebral palsy and mental retardation (Sanchez and Jensen, 2001; Volpe, 2001). Unfortunately, current anticonvulsants are potentially neurotoxic to the immature brain and are often ineffective in the treatment of neonatal seizures (Bittigau et al., 2002; Sankar and Painter, 2005). Moreover, even when the outward clinical manifestations are suppressed by drugs, electroencephalographic (EEG) recordings may show ongoing seizure discharges (Connell et al., 1989).

Previous studies have implicated a role for reactive oxygen and nitrogen species in the generation of hypoxic injury and the progression of post-hypoxic reoxygenation (Mishra et al., 2000, 2006). Seizures can also induce oxidative damage to susceptible subcellular targets (Liang et al., 2000; Patel et al., 2008). At present, there are no studies about neonatal hypoxia-induced seizures (HS) in this context. One aim of gaining new knowledge about the role of oxidative stress in HS is the development of therapeutic strategies to protect the vulnerable developing brain. Recently, a novel octapeptide termed NAP (NAPVSIPQ), derived from the VIP-responsive glial protein activity-dependent neuroprotective protein (ADNP) (Bassan et al., 1999; Gozes, 2007), demonstrated an extensive neuroprotective capacity in several animal models and against multiple toxins (Beni-Adani et al., 2001; Gozes et al., 2005; Leker et al., 2002; Rotstein et al., 2006; Shiryaev et al., 2009). In particular, NAP protects PC12 and neuronal cells against oxidative stress *in vitro* (Busciglio et al., 2007; Gozes et al., 2004; Offen et al., 2000; Steingart and Gozes, 2006; Steingart et al., 2000) and *in vivo* (Spong et al., 2001). These studies suggest that NAP may be a promising drug candidate for the

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treatment of HS, in which oxidative damage leads to the loss of neuronal cell integrity. However, until now, the putative neuroprotective effects of NAP have not been explored in neonatal rats previously exposed to HS.

To evaluate the extent to which hippocampal DNA integrity and redox status are compromised by HS, and whether NAP treatment can act in a protective capacity on HS-generated oxidative stress, we used a well-established rodent model of HS. In this model, 10-day-old rat pups are exposed to transient global hypoxia, causing them to exhibit spontaneous electrographic seizures concomitantly with cerebral tissue oxygen saturation reduction. The HS rats present decreased seizure thresholds throughout adulthood, thus mimicking clinical aspects of neonatal hypoxic encephalopathy (Jensen, 1995). We first investigated the temporal patterns of early oxidative DNA damage formation, reduced glutathione (GSH) content and lipid peroxidation in hippocampal tissue of HS rats at distinct time points post-HS. Once these parameters were established, we examined whether NAP administration after HS insult could prevent the formation of oxidative biomarkers.

Materials and methods

Animals

Pregnant Wistar rats were purchased from the colony of the Federal University of Rio Grande do Sul (Porto Alegre, Brazil). Females were housed individually and the presence of pups was checked for daily. The day of birth was considered day 0, and on postnatal day (PND) 1, the litters were culled to eight rat pups per dam. Animals were maintained in the same temperature and humidity-controlled holding facility (22–24 °C) under a 12:12 light/dark cycle (light onset at 7:00 AM), with free access to food and water. Efforts were made to minimize animal suffering and to reduce the number of animals used. Experiments procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behavior (SBNeC) recommendations for animal care.

Hypoxia-induced seizure model

Rats on PND 10 were subjected to HS as described by Jensen with minor modifications (Jensen, 1995). Although it is not possible to accurately equate ages in rats and humans, a 10-day-old rat would be roughly equivalent to a human age range falling between a full-term newborn to an infant (Haut et al., 2004). Rats were removed from the litter and placed in a custom-made airtight acrylic chamber partially immersed in a water bath at 37 °C. One chamber (control) was left uncovered and continuously exposed to room air (21% O_2), whereas the other (hypoxia) was covered. Following this, the covered chamber had its O_2 concentration reduced to 5–7% and maintained at this level by the constant, regulated infusion of N_2 gas. Intra-chamber O_2 concentration was measured with a commercially available oxygen meter (Model DG-400, Instrutherm, SP, Brazil). This model results in acute hypoxic seizures in all the pups with minimal, if any, mortality. In order to avoid movement artifacts in the EEG recording, rat pups were prevented from moving excessively by the use of a tube rodent holder (CONTCA model, Beiramar, Brazil). Therefore, it was not possible to verify convulsive behavior as myoclonic jerks followed by tonic-clonic head and limb movements. To reduce the possible stress generated by immobilization, each animal remained a 5-min period without any intervention (handling or hypoxia induction) after being put into the holder with the sensors placed on the scalp. Control rats (normoxic) went through the same experimental conditions except by the hypoxia induction. The total duration of hypoxia was 12 min. Only rats exhibiting epileptiform activity on the EEG, characterized by high frequency spikes and/or bursts of polyspikes followed by marked voltage-attenuation of the EEG background (burst-suppression pattern), associated with cerebral tissue oxygen saturation (StO_2) reduction in at least 20% during the hypoxia period were used in this study. All rats were returned to their dams just after HS induction.

Measurement of physiological variables

Heart rate (HRT), StO_2 and EEG activity were continuously monitored using the Morfeu-bio apparatus (Dolsch Ltda, RS, Brazil). The recordings for each rat were divided into 4 stages: phase 1 corresponds to a 5-min period with 21% O_2 for signal stabilization and to minimize the immobilization-induced stress; phase 2 comprehends the transition period from 21% to 7% O_2; phase 3 represents the 12-min time required for HS induction with 5–7% O_2; phase 4 is for StO_2 and HRT reestablishment at basal levels within 5 min of recovery. Since near-infrared spectroscopy (NIRS) technology uses the relative transparency of biological tissue to near-infrared light to detect the presence of chromophores, such as hemoglobin, the StO_2 was determined by measuring the attenuation of light at two wavelengths and distances between the emitter and detector (von Siebenthal et al., 1992). The Morfeu-bio sensor has one detector to measure the ratio (percentage) of oxyhemoglobin to total hemoglobin, which reflects StO_2 because oxygenated hemoglobin transmits light of infrared wavelength much more efficiently than deoxygenated hemoglobin. The NIRS system was a pulse wave, broadband (650–950 nm) unit composed of two laser diode (LD) sources, 5 mW power. A pair of photodiodes was attached 0.25 cm apart and placed extradermally over the frontoparietal region of the rat brain before HS induction. Near-infrared light was emitted by diodes, and a fraction of the transmitted light was collected by a high sensitivity photo detector fixed in a probe holder to ensure a stable interoptode distance. Quantification of StO_2 was calculated from the differential signal obtained by the sensor, expressed as a tissue oxygenation index (percentage of oxygenated hemoglobin = oxygenated hemoglobin / total hemoglobin [oxygenated hemoglobin + deoxygenated hemoglobin]) (Chien et al., 2007). For acquiring simultaneous HRT measurements, an optical pulse wave reference was used. EEG data were acquired with transmitters coupled on the NIRS sensors configured to record two-channel scalp EEG. Any measurements associated with dislodgement of the sensor from the skin were not considered. Baseline EEG recordings and physiological measurements were performed for 5 min before and after hypoxia, as well as during the hypoxic period, in order to characterize the animal model of HS. Since these recording are aimed at measuring cerebral hemodynamics and oxygenation changes occurring on the time scale of seconds, we averaged 30 consecutive data points (corresponding to 1.0 s of acquisition) to improve the signal-to-noise ratio of our measurements.

NAP administration and hippocampal processing

NAP (NAPVSIPQ), with purity (>95%) and identity assessed by high-performance liquid chromatography and mass spectrometry analysis, was purchased from Sigma Genosys (Woodlands, TX). NAP was dissolved in 10% DMSO in PBS (0.13 M NaCl, 0.003 M KCl, 0.01 M Na_2HPO_4, and 0.002 M KH_2PO_4) and stored as 1 mM aliquots. NAP was stable in solution and could be aliquoted and frozen for later use without loss of activity. Final peptide solution was freshly prepared by stock solution diluted in distilled sterile PBS (Kumral et al., 2006). For rat pups treated with NAP, an intraperitoneal injection of NAP solution at a dose of 0.03, 0.3 or 3 μg/g of body weight (0.1 mL) was administered just after HS insult. In this study, four experimental groups of rat pups on PND 10 were used: vehicle-treated HS animals (HS), NAP-treated HS

animals (NAP 0.03, NAP 0.3 and NAP 3, respectively), vehicle-treated normoxic animals and NAP-treated normoxic animals. Rats were either killed by decapitation immediately after HS induction (0 h) or allowed to recover in ambient air for 1, 3, 6, 24, 72 and 168 h prior to killing. The number of animals sacrificed in each experimental group was 4. Brains were quickly removed, kept in ice-cold saline and hippocampal structures were dissected onto Petri dishes placed on ice. One hippocampus was gently homogenized in ice cold 0.3 mL Hanks' solution for alkaline comet assay, while the contralateral one was stored at −80 °C for posterior biochemical analysis.

Determination of DNA damage and oxidized bases

The alkaline comet assay was performed as described by Singh with minor modifications (Collins, 2009; Singh et al., 1988). Aliquots of the hippocampus homogenate (20 μL) were mixed with 0.75% warm low-melting point agarose (Invitrogen, Carlsbad, CA) and immediately spread onto an ordinary microscope slide pre-coated with a layer of 1% normal melting point agarose (Invitrogen, Carlsbad, CA). This mixture was then evenly distributed across the microscope slide following the application of a coverslip and allowed to set at 4 °C for 5 min prior to removal of the coverslips. Slides were then incubated in ice cold lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 1% Triton X-100 and 10% DMSO, pH 10.0; Gibco BRL, Grand Island, NY), protected from light and stored at 4 °C for at least 4 days in order to remove cell proteins, leaving DNA as 'nucleoids.' In the modified version of comet assay, the slides were removed from the lysis solution and washed three times in enzyme buffer (40 mM HEPES, 100 mM KCl, 0.5 mM Na_2EDTA, 0.2 mg/mL BSA, pH 8.0), drained and incubated at 37 °C in this buffer with one of the following: 60 μL of Fpg (New England BioLabs, Beverly, MA) for 30 min (for the detection of oxidized purines; 1 μg/mL solution; 100 mU per gel) and Endo III (New England BioLabs, Beverly, MA) for 45 min (for the detection of oxidized pyrimidines; 1 μg/mL solution; 100 mU per gel). The slides were then transferred to a tank containing electrophoresis solution (300 mM NaOH, 1 mM EDTA, freshly prepared, pH>13.0), and stored for 20 min at 4 °C prior to being transferred to a horizontal electrophoresis tank in a chilled incubator and covered in fresh electrophoresis solution (pH>13.0). Electrophoresis was performed for 20 min at 25 V and 300 mA (0.90 V/cm). All of the steps above were conducted under yellow light or in the dark in order to prevent additional DNA damage. Slides were then neutralized (0.4 M Tris, pH 7.5), washed in bi-distilled water and stained using a silver nitrate staining protocol as described elsewhere (Nadin et al., 2001). After drying at room temperature, the gels were analyzed using an optical microscope. For the visual score analysis, the slides were coded and after scored blindly. In total, 100 cells (50 cells from each of the two replicate slides) were selected and analyzed for each post-HS time point. When selecting cells for quantification, the edges and cells around air bubbles were avoided. Cells were visually scored according to tail length into five classes: (i) class 0: undamaged, without a tail; (ii) class 1: with a tail shorter than the diameter of the head (nucleus); (iii) class 2: with a tail length 1-2× the diameter of the head; (iv) class 3: with a tail longer than 2 × the diameter of the head and (v) class 4: comets with no heads. A value of damage index (DI) was assigned to each comet according to its class. The DI is based on the length of migration and on the amount of DNA in the tail, and it is considered a sensitive DNA measurement. DI ranged from 0 (completely undamaged: 100 cells×0) to 400 (with maximum damage: 100 cells×4), represented as arbitrary units (AU). The levels of Endo III and Fpg-sensitive sites were calculated from the DI score obtained with the enzymes treatment minus the score without enzyme (buffered). International guidelines and recommendations for the comet assay consider that visual scoring of comets is a well-validated evaluation method (Burlinson et al., 2007). The vehicle was used as a negative control while exposure of hippocampal cells to 150 μM H_2O_2 (Sigma-Aldrich, St. Louis, MO) for 5 min at 4 °C was used as a positive control.

Determination of lipid peroxidation

The extent of lipid peroxidation was determined by the reaction of TBA (thiobarbituric acid) with malondialdehyde (MDA), a product formed by lipid peroxidation, during an acid-heating reaction. The assays were performed according to Salgo and Pryor with minor modifications (Salgo and Pryor, 1996). Briefly, the samples were mixed after lysis with Tris–HCl (15 mM for 1 h). Next, 2 mL of 0.4 mg/mL TCA (Sigma-Aldrich, St. Louis, MO), 0.25 M HCl was added to the lysate, which was then incubated with 6.7 mg/mL TBA (Sigma-Aldrich, St. Louis, MO) for 15 min at 100 °C. The mixture was centrifuged at 750×*g* for 10 min. As TBA reacts with other products of lipid peroxidation in addition to MDA, TBARS (thiobarbituric acid reactive species) levels were expressed as MDA equivalents (nmol/mg protein), which were determined by absorbance at 532 nm. TMP (Sigma-Aldrich, St. Louis, MO) was used as the standard. The results were normalized by protein content (Lowry et al., 1951).

Determination of GSH levels

Intracellular GSH levels were determined by photometric determination of 5-thio-2-nitrobenzoate (TNB), which was produced from DTNB (Sigma-Aldrich, St. Louis, MO) in a kinetic assay, according to Akerboom and Sies with minor modifications (Akerboom and Sies, 1981). Samples were mixed in 0.1 M sodium phosphate with 5 mM EDTA pH 8.0, and sonicated to obtain the cell homogenate. An equal volume of 2 M $HClO_4$ with 4 mM EDTA was added to the cell extract, and the precipitated proteins were sedimented by centrifugation at 8000×*g* for 10 min at 4 °C. The supernatant was neutralized with 2 M KOH and 0.3 M MOPS, and the insoluble residue was removed by centrifugation under the same conditions. For the spectrophotometric determination, 910 μL of the cell extract supernatant or of the standard glutathione solution, in the same phosphate–EDTA buffer, were mixed with 50 μL of 4 mg/mL NADPH in 0.5% (w/v) $NaHCO_3$, 20 μL of 6 U/mL glutathione reductase (Sigma-Aldrich, St. Louis, MO) in phosphate–EDTA buffer, and 20 μL of 1.5 mg/mL DTNB in 0.5% $NaHCO_3$. The increase in absorbance was measured at 412 nm. Total glutathione content was normalized by protein content (Lowry et al., 1951). For GSSG determination, 4-vinylpyridine (Sigma-Aldrich, St. Louis, MO) was added to a final concentration of 0.1% (v/v), and then incubated for 1 h at room temperature. At this concentration, 4-vinylpyridine is able to react with all GSH without interfering with the GSSG determination. GSH was determined based on the total glutathione and GSSG concentration results.

Table 1
Induction of physiological changes during HS.

Physiologic parameter	Phase 1	Phase 2	Phase 3	Phase 4
StO_2 (%)	73.93 ± 11.75	67.82 ± 11.08	49.34 ± 15.21***	80.69 ± 12.13
HRT (beats/min)	179.90 ± 41.16	186.83 ± 43.68	85.54 ± 25.03***	153.76 ± 42.56

StO_2, cerebral tissue oxygen saturation; HRT, heart rate. Phase 1 corresponds to a 5-min period of signal stabilization with 21% O_2; phase 2 comprehends the transition period from 21% to 7% O_2; phase 3 represents the 12-min time required for HS induction with 5–7% O_2; phase 4 is for StO_2 and HRT reestablishment at basal levels within 5 min of recovery. Data significant in relation to phases 1, 2 and 4 at ***$p<0.001$ as tested by repeated ANOVA followed by Dunnett's multiple comparison post hoc test.

Statistical analysis

Statistical analysis was performed using PrismGraph 5.0 software (Graph-Pad Software, San Diego, CA). Data were expressed as mean ± standard deviation (S.D.). For physiological variables changes, repeated analysis of variance (ANOVA) followed by Dunnett's multiple comparison post hoc test was performed. The statistical analysis of the temporal patterns of hippocampal DNA damage, oxidized bases, TBARS levels, GSH levels and the effect of NAP treatment on DNA damage were performed using two-way ANOVA followed by Bonferroni post hoc test. The effect of NAP treatment on oxidative DNA damage, TBARS and GSH levels were analyzed by one-way ANOVA followed by Dunnett's multiple comparison post hoc test. A statistical significance level of $\alpha = 0.05$ and $p < 0.05$ was applied to all tests.

Results

Monitoring of physiological parameters

EEG activity, HRT and StO_2 were simultaneously recorded at four different stages of the HS induction protocol ($n = 64$ rat pups). Table 1 depicts the physiological changes that occurred during the rodent model procedure. Both HRT and StO_2 measured at phase 3 were significantly lower than the corresponding values prior to ($p < 0.001$, phase 1; $p < 0.001$, phase 2) and after ($p < 0.001$, phase 4) HS induction. The basal StO_2 values were in agreement with other reports assessing this biological parameter through NIRS methodology in both adult rats and human infant brain (Chen et al., 2003; Franceschini et al., 2007). For rats subjected to hypoxia, bursts of polyspikes followed by background voltage-attenuation were detected in the EEG recordings (Fig. 1A). In Fig. 1B, a representative physiological profile during the

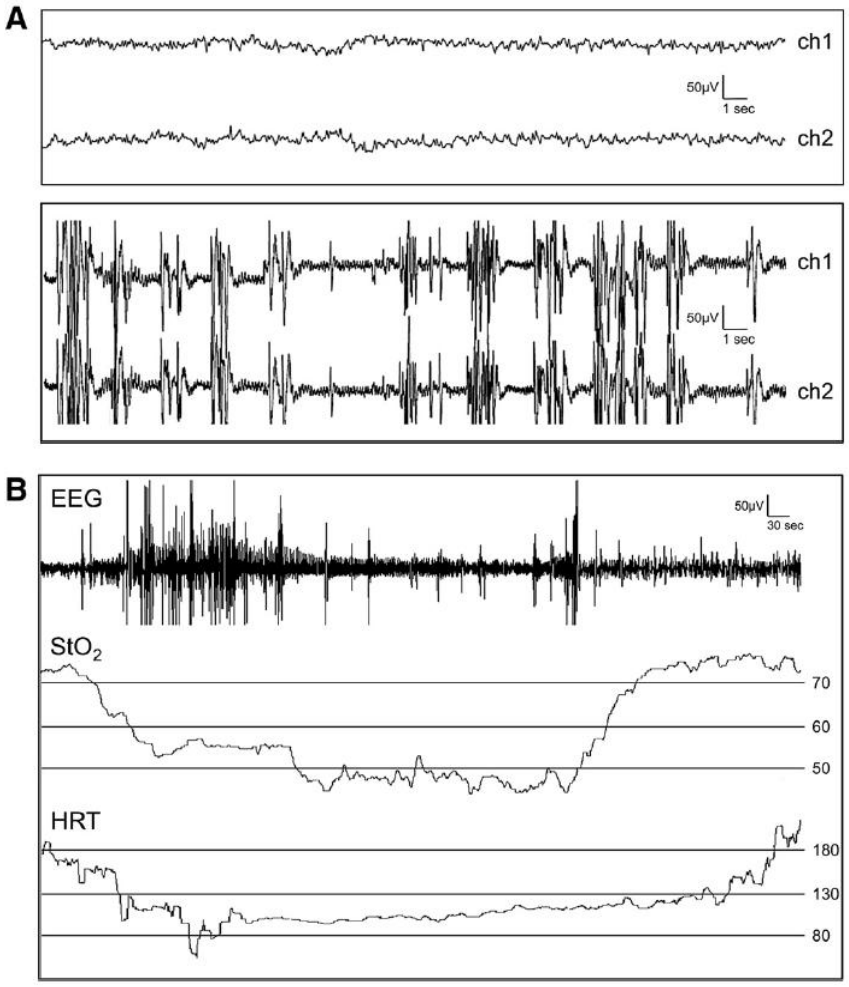

Fig. 1. Physiological measurements in neonatal rats subjected to HS insult. (A) Electroencephalographic recordings from immature rats at normoxic and hypoxic conditions were acquired with transmitters coupled to the NIRS sensors configured to record two-channel scalp EEG (ch1, channel 1; ch2, channel 2). At normoxic condition (21% O_2), it was possible to verify EEG waves at a normal physiological pattern (upper panel). However, when rats on PND 10 were subjected to global hypoxia (12-min period of 5–7% O_2), bursts of polyspikes followed by background voltage-attenuation were detected (lower panel). (B) Physiological changes induced by global hypoxia in immature rats. Rats on PND 10 were subjected to global hypoxia (12-min period of 5–7% O_2), and EEG activity, StO_2 and HRT were simultaneously measured during the HS induction protocol. In the beginning at 21% O_2, StO_2 and HRT data were at basal levels with normal EEG activity. As the intra-chamber O_2 concentration decreased, StO_2 and HRT started to diminish associated with concomitant epileptiform discharges (high frequency spikes and spikes-and-wave complexes). When the O_2 concentration returned to normoxic conditions at the end of HS protocol, all of the physiological parameters become normalized. EEG, electroencephalographic recording; StO_2, cerebral tissue oxygen saturation (%); HRT, heart rate (beats/min).

overall HS protocol is presented, showing epileptiform discharges (high frequency spikes and spike-and-wave complexes) in the EEG associated with the reduction of HRT and StO_2 parameters during the period of global hypoxia.

Temporal patterns of DNA damage and oxidative adducts formation in the hippocampus of immature rat subjected to HS induction

To determine the temporal pattern of DNA damage, hippocampi from normoxic and HS rats were isolated at distinct time points after HS insult and processed using the comet assay. For all samples, cell viability was evaluated by the trypan blue exclusion method and was found to be more than 90% in every experiment. The internal controls of the comet assay, using human blood cells, showed low damage in the negative control (DI = 0–10) and high damage in the positive control (DI = 180–300), thus validating the test conditions. As expected, exposure of hippocampal cells isolated from normal animals to H_2O_2 resulted in a significant increase in DNA damage (data not shown). In Fig. 2A, it is possible to observe the temporal pattern of DNA damage occurrence as detected by the alkaline comet assay in the hippocampus of normoxic and HS rats. Two-way ANOVA comparing the groups of animals and different reperfusion times revealed a significant difference [$F_{(1,42)} = 44.71$, $p<0.0001$]. Bonferroni post hoc test demonstrated an increase in hippocampal DNA damage immediately after HS insult (0 h, $p<0.001$), as well as at all other post-HS time points ($p<0.001$) except 168 hours. Considering these findings, it is possible to suggest that the reperfusion period is a more intense genotoxic condition than the HS event.

In order to verify the oxidative nature of the DNA damage detected in hippocampal cells from HS rats, we carried out a modified comet assay. While the alkaline test normally detects primary repairable DNA single and double-strand breaks and alkali-labile sites, the modified version is more specific to oxidative damage than the standard method. To this end, there is an incubation step with lesion-specific endonucleases that recognize resultant abasic sites and convert them into single-strand breaks. Here we used Fpg, which is specific for oxidized purines – including 8-oxo-7,8-dihydroguanine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine, and 4,6-diamino-5-formamidopyrimidine, as well as other ring-opened purines – and Endo III, which targets oxidized pyrimidines, including thymine glycol and uracyl glycol (Dizdaroglu, 2005). In this manner, increases in DI after enzymes incubation represent specifically the extent of oxidative DNA damage. Oxidative DNA damage identified by Endo III from HS rats was significantly higher than that found in normoxic animals, indicating the presence of oxidized pyrimidines [$F_{(1,42)} = 12.48$, $p<0.0001$, Fig. 2B]. Post hoc analysis demonstrates that while there was a significant increase of Endo III sensitive sites at 3 h ($p<0.001$), 6 h ($p<0.001$) and 24 h ($p<0.001$) post-HS induction, such an increase was not observed before and after this period. Similarly, oxidative DNA damage identified by Fpg could be observed [$F_{(1,42)} = 9.49$,

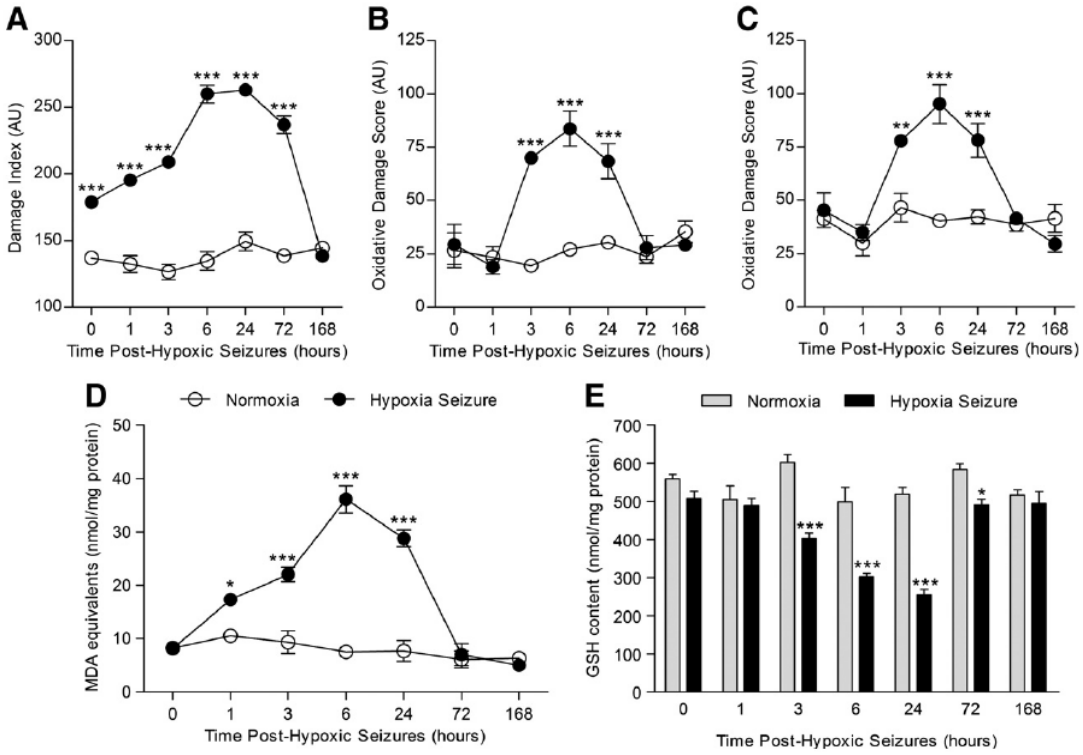

Fig. 2. HS insult promotes DNA damage, bases oxidation, lipid peroxidation and GSH depletion in the immature rat hippocampus. (A) Temporal pattern of DNA damage in immature rat hippocampus after HS. DNA damage was assessed with an alkaline comet assay of hippocampal tissue obtained from rat pups exposed to HS or room air at different time points. (B, C) Temporal pattern of oxidative DNA damage in immature rat hippocampus after HS. Oxidative DNA damage was assessed with a modified alkaline comet assay, using Endo III (B) and Fpg (C) enzymes, of hippocampal tissue obtained from rat pups exposed to HS or room air at different time points. (D) Temporal pattern of TBARS products in immature rat hippocampus after HS. Lipid peroxidation was assessed by the reaction of thiobarbituric acid with MDA during an acid-heating reaction of hippocampal tissue obtained from rat pups exposed to HS or room air at different time points. The results are expressed as MDA equivalents (nmol/mg protein). (E) Temporal pattern of reduced GSH content in immature rat hippocampus after HS. Intracellular total glutathione was determined by photometric determination of hippocampal tissue obtained from rat pups exposed to HS or room air at different time points. The results are expressed as GSH content (nmol/mg protein). All values represent the mean ± S.D.; $n = 4$ rats/group. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ vs. normoxic rats in Bonferroni post hoc test after two-way ANOVA.

$p<0.0001$, Fig. 2C], along with augmentation of oxidized purines shown by post hoc analysis, at 3 h ($p<0.01$), 6 h ($p<0.001$) and 24 h ($p<0.001$) post-HS induction. However, in the temporal profile for both DNA-modifying enzymes, oxidized DNA bases were not observed at 72 and 168 h after HS insult. Correlating results from Fig. 1, it is notable that DNA damage is still present at this time point, suggesting that these lesions are not mainly due to oxidative insult. The frequencies of ordinary and oxidatively generated DNA adducts were insignificant in the latest time point of recovery after HS. Moreover, the same is true immediately (0 h) and 1 h post-HS, indicating that during the initial time necessary to induce seizures through hypoxia, only non oxidative DNA damage is occurring.

Temporal patterns of lipid peroxidation and reduced GSH levels in the hippocampus of immature rat subjected to HS induction

We examined the time-dependent production of TBARS in the hippocampus of normoxic and hypoxic neonatal rats in order to determine whether lipid peroxidation is triggered by HS insult. Two-way ANOVA revealed significant differences in these groups [$F_{(1,42)}=21.77$, $p<0.0001$, Fig. 2D]. Within 3 h post-HS, hippocampal TBARS production is increasing ($p<0.001$), while it is sharply elevated at 6 h ($p<0.001$) and declines after 24 h ($p<0.001$). Although a slight increase in TBARS could be detected 1 h after HS ($p<0.05$), there was no difference in TBARS levels between normoxic and HS rats at 0 h, 72 h and 168 h.

In an attempt to further elucidate the possible mechanism of oxidative damage involved in HS insult, we also assessed whether GSH levels were reduced in the hippocampus of HS rats. GSH is a ubiquitous tripeptide and an important non-enzymatic constituent of the cellular defense mechanisms against oxidative stress. The reduced form is the most important free radical scavenging compound in the mammalian nervous system and prevents membrane lipid peroxidation via conversion into GSSG. The latter is rapidly recycled back into GSH by a specific NAD(P)H-dependent glutathione reductase. Since the conversion of GSH to its oxidized form generates an electron that acts to stabilize free radicals, it is generally believed that increases in free radical production occur in conjunction with decreases in GSH levels (Halliwell, 2006). It was visualized evident changes in the GSH level in this time-course study [$F_{(1,42)}=11.73$, $p<0.0001$, Fig. 2E]. There was no significant difference in GSH depletion between normoxic and HS rats at 0 and 1 h post-HS. However, there was a reduction in GSH content at 3 h post-HS ($p<0.001$), and further decreases from 6 h ($p<0.001$), until 24 h ($p<0.001$). The significant GSH depletion observed at 72 h ($p<0.05$) may represent the replenishment of glutathione content to homeostatic levels, as it was similar to the levels observed at 168 h post-HS.

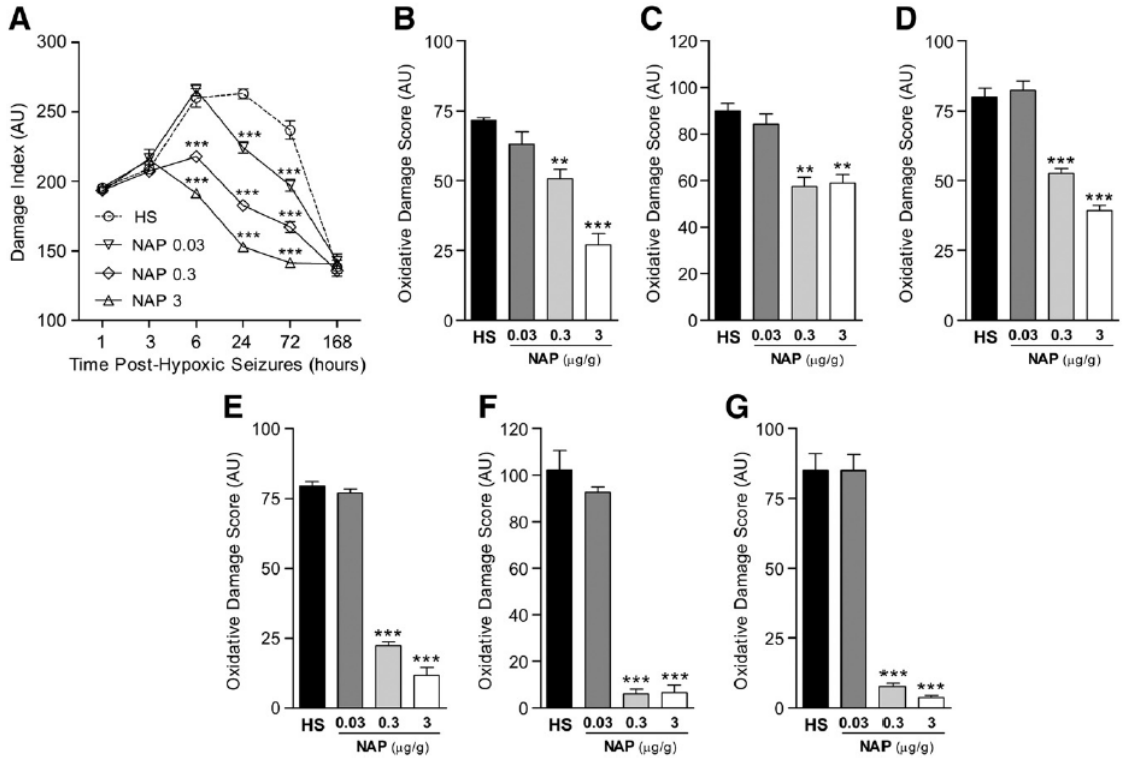

Fig. 3. NAP treatment protects hippocampal DNA integrity after HS insult. The rat pups received an intraperitoneal injection of NAP solution at a dose of 0.03, 0.3 or 3 μg/g just after HS insult (groups NAP 0.03, NAP 0.3, NAP 3, respectively). Some pups were exposed to HS but they received a vehicle injection instead of NAP (group HS). DNA damage and oxidized bases were assessed by an alkaline comet assay of hippocampal tissue at specific time points after the insult. Values represent the mean ± S.D.; $n=4$ rats per group. (A) The dose-dependent effect of systemic NAP treatment on DNA damage in immature rat hippocampus at different time points after HS. ***$p<0.001$ vs. HS vehicle-treated rats in Bonferroni post hoc test after two-way ANOVA. (B–D) The dose-dependent effect of systemic NAP treatment on Endo III-sensitive sites in rat hippocampus at 3 h (B), 6 h (C) and 24 h (D) post-HS. **$p<0.01$ and ***$p<0.001$ vs. HS vehicle-treated rats in Dunnett's multiple comparison post hoc test after one-way ANOVA. (E–G) The dose-dependent effect of systemic NAP treatment on Fpg-sensitive sites in rat hippocampus at 3 h (E), 6 h (F) and 24 h (G) post-HS. ***$p<0.001$ vs. HS vehicle-treated rats in Dunnett's multiple comparison post hoc test after one-way ANOVA.

NAP dose-dependently prevents HS-induced DNA damage and oxidative adducts formation in the immature rat hippocampus

To analyze whether NAP can hinder hippocampal DNA damage, rats were given intraperitoneal injections of three different NAP doses (0.03, 0.3 or 3 μg/g) immediately after HS insult. The assessment at 0 h was not included in this experimental design because the peptide could not reach central nervous system immediately after intraperitoneal access and thus would not have an effect (Gozes et al., 2005; Spong et al., 2001). Our results demonstrate a dose-dependent effect of systemic NAP administration on DNA damage in the immature rat hippocampus after HS insult (Fig. 3A). Two-way ANOVA comparing the experimental groups and different reperfusion times revealed a significant difference [$F_{(3,72)} = 38.09$, $p < 0.0001$]. Bonferroni post hoc test demonstrated that most doses of NAP had a similar effect on preventing DNA damage formation 6 h ($p < 0.001$), 24 h ($p < 0.001$) and 72 h ($p < 0.001$) after HS induction, with the exception of the dose of 0.03 μg/g at 6 h. No differences were observed at earlier 1 and 3 h post-HS time points, even with the highest NAP concentration. Although a two-way ANOVA comparing vehicle or three NAP doses administered to normoxic rats and different reperfusion times revealed a significant difference [$F_{(3,72)} = 1.95$, $p = 0.0311$], Bonferroni post hoc test demonstrated that any NAP concentration was not able to cause statistical differences in DI values at the entire time studied in comparison with vehicle-treated rats ($p > 0.05$). This is in agreement with previous report showing that NAP has no damaging effects upon DNA structure (Visochek et al., 2005).

On the basis of the positive outcome of NAP administration in reducing hippocampal DNA damage, further experiments were performed in an attempt to understand the molecular mechanisms associated with the protective capacity of this compound on oxidative lesions formation. First, we tested the dose-dependent effect of NAP by administering the same doses as above after HS insult. Then, we again performed the comet assay with the DNA-modifying enzymes Endo III and Fpg at three time points post-HS (3, 6 and 24 h) that had been identified as critical in oxidative adduct formation by our previous experiments. One-way ANOVA comparing DI among NAP-treated HS groups and vehicle-treated HS animals presented statistically significant differences in Endo III at 3 h ($p < 0.0001$, Fig. 3B), 6 h ($p = 0.0005$, Fig. 3C) and 24 h ($p < 0.0001$, Fig. 3D). In the same manner, Fpg assessment also demonstrated statistically significant differences at 3 h ($p < 0.0001$, Fig. 3E), 6 h ($p < 0.0001$, Fig. 3F) and 24 h ($p < 0.0001$, Fig. 3G). Further post hoc analysis revealed a dose–response effect of NAP treatment upon the formation of Endo III and Fpg-sensitive sites at 3, 6 and 24 h post-HS. Different but not contrasting results observed in Fig. 3B–D and E–G are exclusively inherent to the kind of oxidative damage naturally generated in the intracellular environment. It is known that among the great variety of oxidized bases resulted by pro-oxidant conditions into the cellular environment, there is a preference for guanine attack. Therefore, once the purine oxidation is more frequently observed, NAP efficacy becomes enhanced in comet assay applying Fpg enzyme (Dizdaroglu, 2005; Fortini et al., 2003). For all conditions, a NAP dose of 0.03 μg/g was not capable of preventing the oxidation of purines and

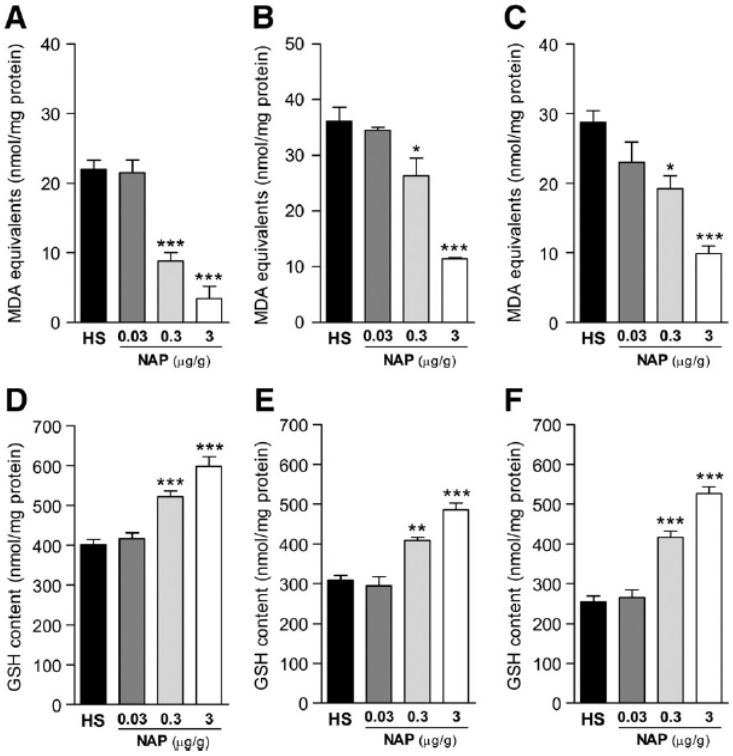

Fig. 4. NAP treatment prevents hippocampal lipid peroxidation and GSH depletion after HS insult. The rat pups with HS exposure received an intraperitoneal injection of NAP solution at a dose of 0.03, 0.3 or 3 μg/g just after HS insult (groups NAP 0.03, NAP 0.3, NAP 3, respectively). Some pups were exposed to HS but they received a vehicle injection instead of NAP (group HS). (A) The dose-dependent effect of systemic NAP treatment on TBARS levels in rat hippocampus subjected to HS. The time points analyzed (3, 6 and 24 h corresponds to (A), (B) and (C), respectively) were chosen based on previous evidence of TBARS production. The results are expressed as MDA equivalents (nmol/mg protein). (B) The dose-dependent effect of systemic NAP treatment on reduced GSH content in rat hippocampus subjected to HS. The time points analyzed (3, 6 and 24 h corresponds to (D), (E) and (F), respectively) were chosen based on previous evidence of GSH depletion. The results are expressed as GSH content (nmol/mg protein). All values represent the mean ± S.D.; $n = 4$ rats/group. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs. HS vehicle-treated rats in Dunnett's multiple comparison post hoc test after one-way ANOVA.

pyrimidines. Although NAP could prevent DNA damage stemming specifically from an oxidative origin at 3 h after HS insult (Fig. 3B and E), all NAP doses were insufficient to reduce ordinary DNA breakage at this same time point (Fig. 3A). These different NAP outcomes at 3 h post-HS are likely due to the specificity of the alkaline comet assay in detecting common DNA breakage without gathering together DI values from oxidative adducts. Therefore, the quantity of oxidized bases is only detected by endonuclease-mediated conversion into single-strand breaks.

NAP dose-dependently prevents lipid peroxidation and GSH homeostasis disturbance in the hippocampus of immature rat subjected to HS induction

Based on the positive effects of NAP activity in mitigating DNA damage and oxidized base formation, we also analyzed whether NAP could prevent the lipid peroxidation reactions induced by HS insult in neonatal rats. One-way ANOVA demonstrated statistically difference at 3 h ($p=0.0001$, Fig. 4A), 6 h ($p=0.0006$, Fig. 4B) and 24 h ($p=0.0005$, Fig. 4C). Additional post hoc test comparisons showed that doses of 0.3 and 3 μg/g NAP were able to protect developing hippocampus from HS-mediated TBARS production at each of the time points analyzed. As with oxidized lesion formation, it was not possible to detect any difference in MDA equivalents between the vehicle- and NAP-treated HS animals in NAP dose of 0.03 μg/g.

Reduced GSH levels were assessed in order to investigate a potential mechanism underlying the NAP treatment effects in HS rats, focusing on the possibility of an intracellular GSH modulation. We observed a beneficial impact of NAP administration on hippocampal GSH content at 3 h ($p<0.0001$, Fig. 4D), 6 h ($p<0.0001$, Fig. 4E) and 24 h ($p<0.0001$, Fig. 4F) after HS insult. Once again, Dunnett's multiple comparison post hoc test demonstrated that a NAP dose of 0.03 μg/g was not sufficient to augment the hippocampal GSH levels in the studied time frames. Interestingly, a single intraperitoneal injection of NAP on HS rat pups was effective in diminishing hippocampal DNA breakage, reducing oxidative base damage and lipid peroxidation, and increasing GSH levels in parallel.

Discussion

Here we demonstrated that HS decreases hippocampal GSH content in parallel with an increase in oxidative damage in neonatal rats. Establishing the temporal course of oxidative stress formation provides information on a possible therapeutic window for protecting the developing brain against HS insult. We observed that NAP administration protects against hippocampal oxidative DNA damage, lipid peroxidation and GSH depletion in a dose-dependent fashion. Here we show for the first time the *in vivo* ablative capacity of NAP on oxidative lesions in hippocampal cells from neonatal rats subjected to HS.

Previous studies documented DNA fragmentation following seizure induction by kainic acid (KA) and pilocarpine in adult rat brain (Fujikawa et al., 2000; Lan et al., 2000). However, conflicting outcomes were verified on PND 7 rats after pilocarpine and KA-induced status epilepticus (SE) (Mikati et al., 2003; Santos et al., 2000). Hypoxia promotes DNA damage in adult and immature brains (David and Grongnet, 2000; Englander et al., 1999), and in addition post-hypoxic reoxygenation contributes to DNA fragmentation in newborn piglet brains (Mishra et al., 2006; Parker et al., 2007). In our study, we detected DNA damage until 72 h post-HS in the hippocampus of immature rats. Two other studies have revealed a lack of hippocampal DNA fragmentation labeled by in situ end-labeling (ISEL) nick translation histochemistry at any point within 168 h after HS insult on PND 10 rats (Koh et al., 2004; Sanchez and Jensen, 2001). The contrasting results of HS impact on hippocampal DNA integrity are attributable to different methodological approaches. While the comet assay is able to detect repairable DNA damage that may or may not lead to cell death (Collins, 2009), ISEL-positive cells represent injured or dying neurons with irreversible apoptotic DNA breakage (Wijsman et al., 1993). Supporting evidence indicates that HS cannot induce long-term cell loss in the hippocampal CA1 subfield, as verified by Neu-N immunostaining (Chen et al., 2006). Hence, we conclude that HS is capable of inducing DNA breakage in the hippocampus of neonatal rats, but that damage is not sufficient to promote cell death.

It has been demonstrated that systemic KA administration produces 8-hydroxy-2-deoxyguanosine (8-OHdG), an oxidative lesion of DNA, in the hippocampus of adult rats (Lan et al., 2000; Liang et al., 2000). In maturing rats, KA-induced seizures did not result in increased mitochondrial aconitase inactivation, an index of steady-state mitochondrial O_2^- formation, or 8-OHdG levels, but they did so in older animals (Patel and Li, 2003). Besides, respiratory hypoxia gives rise to the formation of the same kind of oxidative DNA lesion in adult rat brain (Lee et al., 2002). Here we demonstrated that HS insult can induce oxidation of pyrimidines and purines in the immature hippocampus, as verified by Endo III and Fpg detection respectively. Changes in hippocampal lipid composition after pilocarpine and KA-induced SE have been reported in adult rats (Dal-Pizzol et al., 2000; Freitas et al., 2005). KA seizures in the developed brain produce an increase in isofurans formation that coincides with mitochondrial oxidative stress (Patel et al., 2008). While in the immature brain there was no increase in lipid peroxidation after KA-induced SE (Bruce and Baudry, 1995; Shin et al., 2008), sustained hypoxia could induce augmentation of TBARS levels in the brain of neonatal rats (Lee et al., 2008). In the present work, we showed that HS promotes an increase in hippocampal TBARS products. After a wide variety of hypoxic insults, the glutathione redox system is altered in newborn rat brain (Kretzschmar et al., 1990; Reuter and Klinger, 1992; Wallin et al., 2000). Moreover, studies have shown GSH depletion in adult rat hippocampus following pilocarpine and KA-induced SE (Freitas et al., 2005; Liang and Patel, 2006), and GSH depletion appears to be present in chronic human epilepsy (Mueller et al., 2001). Recently, it was again demonstrated that hippocampal oxidative stress is more pronounced in adult than in immature genetically epilepsy-prone rats (GEPR-9) (Shin et al., 2008). Here we verified depletion of GSH in the hippocampus of HS neonatal rats that coincides with oxidative DNA damage and lipid peroxidation. To analyze these data in a context that comprehends hypoxic and reoxygenation insults together with seizures events, the age dependency of seizure-induced oxidative stress must be taken into consideration.

Developing neurons are less vulnerable, in terms of neuronal damage and cell loss, than adult neurons to a wide variety of pathological insults (Holmes et al., 2002). Although the threshold for seizure generation is lower in immature than in developed brains, the degree of SE-induced morphological damage and oxidative stress is highly age-dependent (Ben-Ari and Holmes, 2006; Bruce and Baudry, 1995; Haut et al., 2004; Patel and Li, 2003; Shin et al., 2008). It was reported that KA seizures do not increase the formation of reactive species or mitochondrial dysfunction in neonatal brain (Sullivan et al., 2003). However, it is possible to observe an opposing outcome after hypoxic-ischemic insult, since the adult mouse brain accumulates less H_2O_2 than the developing brain (Lafemina et al., 2006). Similarly, high free radicals generation and protein oxidation products have been detected in the plasma of preterm hypoxic newborns at birth and on the seventh day of life (Buonocore et al., 2000). Our experiments demonstrate oxidative damage in the DNA and membrane lipids together with an impairment of the glutathione-mediated antioxidant system in developing hippocampus of HS rats. Thus, we suggest that the oxidative stress detected in HS is more likely to be contributed to global hypoxia and reoxygenation than would be the case in non-hypoxia-induced neonatal seizures.

Based on the evidence that GSH depletion is involved in redox homeostasis imbalance triggered by HS in the immature rat hippocampus, stimulation of antioxidative mechanisms may reduce or even abolish this consequence. The rationale for choosing NAP as a potential protective agent against HS insult is based on previous evidences of its *in vitro* and *in vivo* neuroprotective properties (Beni-Adani et al., 2001; Gozes et al., 2005; Leker et al., 2002; Rotstein et al., 2006; Shiryaev et al., 2009). NAP can cross membranes and facilitate microtubule assembly by binding tubulin in neuronal cells (Divinski et al., 2006; Gozes, 2007). Furthermore, its mechanism of protection also involves cGMP formation, control of nitric oxide production (Ashur-Fabian et al., 2001), modulation of inflammatory mechanisms (tumor necrosis factor-α) (Beni-Adani et al., 2001), interference of apoptotic-related processes as regulation of protein p53 expression (Gozes et al., 2004), mitochondrial cytochrome C release (Zemlyak et al., 2009), caspase-3 activation and DNA fragmentation (Leker et al., 2002). Studies also indicate potential protective activity against oxidative stress. Indeed, NAP at femtomolar concentrations protects cells against H_2O_2, dopamine and 6-OHDA toxicity (Gozes et al., 2004; Offen et al., 2000; Steingart et al., 2000). Moreover, NAP showed significant protection against depletion of cellular GSH by buthionine sulfoximine treatment (Offen et al., 2000). In a mouse model of fetal alcohol syndrome, NAP + ADNP-9 treatment hindered fetal death and growth abnormalities, as well as prevented the alcohol-induced changes in reduced to oxidized glutathione levels in the fetuses and resulted in GSH/GSSG ratio not different from that of control (Spong et al., 2001). Additionally, recombinant ADNP, which includes the active site NAP, was shown to protect against H_2O_2 and β amyloid toxicity, reducing the elevation of p53 protein levels that is associated with oxidative stress (Steingart and Gozes, 2006). Recently, NAP was reported to be neuroprotective at very low concentrations in H_2O_2-treated human cortical neurons and Down's syndrome cortical neurons (Busciglio et al., 2007). These studies indicate an involvement of the glutathione system in the NAP-associated neuroprotective mechanism across a wide variety of oxidative stress models.

Since it has been reported that there is a narrow therapeutic window for effective clinical treatment of HS (Jensen, 1995; Koh and Jensen, 2001; Sanchez and Jensen, 2001), we administered an injection of NAP solution immediately after HS insult. A previous study detected NAP in the adult rat hippocampus and midbrain 30 min after intravenous application (Leker et al., 2002). Besides, after either intranasal or intravenous administration, NAP rapidly appeared in the cerebrospinal fluid earlier than 30 min into the rat brain (Morimoto et al., 2009). NAP subcutaneous administration provided neuroprotection in head-injured mice (Beni-Adani et al., 2001). Besides, a therapeutic concentration of NAP was identified in embryos 30 min after intraperitoneal injection of pregnant mice (Spong et al., 2001). Then NAP delivery via intraperitoneal administration was considered appropriate based on brain bioavailability evidence and also suitable for neonatal rats. Here we demonstrated a dose-dependent effect of NAP upon the reestablishment of GSH levels after HS insult in the neonatal rat hippocampus. Together with this finding, we also proved that NAP provides the same dose-response beneficial impact on mitigating DNA damage and lipid peroxidation. Recently, it was reported that there is also a NAP neuroprotective effect in β amyloid-treated neurons mediated by polyADP-ribosylation of histone H1 and chromatin relaxation. Poly(ADPribose) polymerase-1 activation by NAP could render DNA accessible to repair and transcription factors, thereby facilitating DNA repair and enabling rapid gene expression (Visochek et al., 2005). Perhaps this additional mechanism of action, together with the enhancement of glutathione system, converged and influenced hippocampal DNA integrity and lipid membranes recovery. Until now, no work has reported the effects of NAP treatment on seizure-related models. In a hypoxic context, NAP treatment ameliorates cognitive and motor abilities of newborn apolipoprotein E-deficient mice subjected to hypoxia (Rotstein et al., 2006). Moreover, NAP prevents neuronal apoptosis and nitric oxide production in a newborn rat model of hypoxic-ischemic brain injury (Kumral et al., 2006).

To our knowledge, this is the first study to demonstrate a protective effect of NAP on hippocampal oxidative stress in a rodent model of HS. Recently, it was reported that major antiepileptic drugs that block voltage-gated sodium channels, enhance GABAergic inhibition or block glutamate-mediated excitation, may trigger apoptotic neurodegeneration in the developing rat brain (Bittigau et al., 2002). While not highly efficacious, phenobarbital and the benzodiazepines continue to be used to treat neonatal seizures (Sankar and Painter, 2005). Thus the potential toxicity of existing therapies renders the immature brain vulnerable to a critical condition. The results and observations presented here raise the possibility that NAP may expand upon and improve the therapeutical approaches to neonatal seizures. NAP could maintain the integrity of brain structures at a molecular level by stimulating glutathione biosynthesis, while the convulsive activity may not be completely suppressed by drugs. However, in order to become a an acceptable strategy for the treatment of neonatal HS, additional studies must be undertaken to determine whether NAP has the ability to control seizure activity and prevent long-term behavioral deficits, without interfering in the processes of neurodevelopment.

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APÊNDICE D

ARTIGO ORIGINAL

Hemispheric brain injury and behavioral deficits induced by severe neonatal hypoxia-ischemia in rats are not attenuated by intravenous administration of human umbilical cord blood cells

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Hemispheric Brain Injury and Behavioral Deficits Induced by Severe Neonatal Hypoxia-Ischemia in Rats Are Not Attenuated by Intravenous Administration of Human Umbilical Cord Blood Cells

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ABSTRACT: Neonatal hypoxia-ischemia (HI) is an important cause of mortality and morbidity in infants. Human umbilical cord blood (HUCB) is a potential source of cellular therapy in perinatology. We investigated the effects of HUCB cells on spatial memory, motor performance, and brain morphologic changes in neonate rats submitted to HI. Seven-day-old rats underwent right carotid artery occlusion followed by exposure to 8% O_2 inhalation for 2 h. Twenty-four hours after HI, rats received either saline solution or HUCB cells i.v. After 3 wk, rats were assessed using a Morris Water Maze and four motor tests. Subsequently, rats were killed for histologic, immunohistochemical, and polymerase chain reaction (PCR) analyses. HI rats showed significant spatial memory deficits and a volumetric decrease in the hemisphere ipsilateral to arterial occlusion. These deficits and decreases were not significantly attenuated by the injection of HUCB cells. Moreover, immunofluorescence and PCR analysis revealed few HUCB cells located in rat brain. Intravenous administration of HUCB cells requires optimization to achieve improved therapeutic outcomes in neonatal hypoxic-ischemic injury. (***Pediatr Res* 65: 631–635, 2009**)

Neonatal hypoxia-ischemia (HI) is a major cause of mortality and morbidity in infants and occurs in approximately 2–4 per 1000 full-term births. Between 20 and 50% of asphyxiated newborns with hypoxic-ischemic encephalopathy die within the neonatal period, and up to 25% of the survivors will exhibit neurodevelopment morbidity, such as cerebral palsy, mental retardation, and epilepsy. The most widely used and accepted animal model of neonatal HI is the Levine method as modified by Rice *et al.* (1), which represents a useful tool to study long-term effects of neuroprotective strategies in behavioral changes, especially in learning and memory tasks (2). Although promising neuroprotective strategies have been studied in animal models and clinical trials, current management techniques have reached only limited success (3).

Human umbilical cord blood (HUCB), is rich in adult stem cells and seems to be a potential source for transplantation, especially for perinatal neuronal repair. Studies have shown behavioral and neurologic recovery in stroke (4–7) and HI-insulted animals (8,9) that received i.v. injection of HUCB, indicating that cells migrate toward ischemic regions and cross the blood brain barrier (BBB), especially in acute periods postischemia (10). The i.v. route is less invasive and a safer access to clinical applications when compared with intracerebral delivery. However, very few transplanted cells are found in the brain when delivered intravascularly. Therefore, evidence suggests that these cells increase endogenous mechanisms of brain repair by trophic factor secretion rather than by replacing the damaged tissue (11,12).

The aim of this study was to assess the effects of HUCB cells on spatial memory, motor performance, and brain morphologic changes in 30-d-old rats after neonatal HI on postnatal d 7. In addition, we tested whether the injected HUCB cells migrate to the injured brain 24 h, and 1 and 3 wk after HI insult.

METHODS

Experimental groups. The animals were randomly divided into three experimental groups: A) sham-operated animals ($n = 9$); B) rats infused with saline solution 24 h after HI injury (HI + saline; $n = 10$); and C) rats infused with HUCB cells 24 h after HI injury (HI + HUCB; $n = 15$). After 3 wk, rats were assessed in Morris Water Maze (MWM) and four motor tests. All evaluations were performed by blinded investigators.

Neonatal hypoxia-ischemia model. We used the Levine rat model, modified by Rice *et al.* (1) for neonatal rats. On postnatal d 7, Wistar rats were briefly anesthetized with halothane, and the right common carotid artery was identified, isolated from the vagus nerve, and permanently occluded with 7.0 surgical silk sutures. The entire surgical procedure was completed within 15 min.

After surgery, animals were put back into their cages and allowed to recover for 2–4 h in the company of their mothers. Rats were then placed for 2 h in a hypoxia chamber, with constant flow of humidified 8% oxygen balanced with nitrogen. The environmental temperature was maintained at 37–38°C.

The sham-operated animals were anesthetized with halothane and exposure of the right common carotid artery without ligation and hypoxia. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the National Institutes of Health (USA).

Human umbilical cord blood cell separation procedure and administration. Cord blood collections were obtained *ex utero* using sterile syringes containing anticoagulant from full-term births of healthy donors. All samples ($n = 5$) were collected after obtaining written informed consent forms.

Blood samples were processed within a range of 24 h after collection. HUCB was diluted with RPMI medium 1640 (GIBCO, Langley, OK) and this suspension was fractionated on Histopaque density (Sigma Chemical-Aldrich) at 400 g for 30 min at room temperature. The mononuclear fraction was collected and rinsed twice with phosphate-buffered saline (PBS) contain-

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Abbreviations: BBB, blood brain barrier; **HI,** hypoxia-ischemia; **HUCB,** human umbilical cord blood; **MWM,** Morris Water Maze

ing 1% of Liquemine (Roche, Swiss). The cell viability was evaluated using the Trypan Blue Stain 0.4% (GIBCO, Langley, OK) exclusion method. CD 34+ cells represented, on average, 0.05% of cord blood mononuclear cells after flow cytometric analysis.

Twenty-four hours after HI, randomly selected rats received i.v. either HUCB cells or saline solution. Using a micropipette with ultrafine tip (diameter <5 μm) connected to an insulin syringe, we injected 1×10^7 mononuclear cells (6,8) suspended in 100 μL total fluid volume into the external jugular vein.

Morris water maze. The spatial memory performance was evaluated 3 wk after HI lesion using an MWM (13,14). The water maze consists of a black circular pool conceptually divided in four equal imaginary quadrants. Two centimeters beneath the surface of the water and hidden from the rat's view was a black circular platform. The water maze was located in a well-lit white room with several spatial cues.

Training on spatial version of the MWM was carried out over 5 consecutive days. On each day, rats received four training trials in which the hidden platform was kept in a constant location. The mean latency to find the platform was measured for individual animals on each day.

A different starting location was used in each trial, which consisted of a swim followed by a 30 s platform sit. Rats that did not find the platform within 60 s were guided to it by the experimenter. To assess long-term memory, 24 h after the final trial, the platform was removed from the maze and the parameter measured was time spent in the target quadrant.

Open-field activity. Rats were placed in a 40×30 cm wood box and left to explore the arena freely for a 5-min period. The floor was divided into 12 equal squares by black lines. The number of line crossings, number of rearings, and locomotion time were measured (15).

Cylinder test. Forelimb use was analyzed by videotaping movements of each rat during vertical exploration in a transparent cylinder (15×30 cm). Each animal was placed in the cylinder and observed for 5 min. The forelimb use asymmetry score was calculated as the percentage of nonimpaired forelimb use subtracted from the percentage of impaired forelimb use (16).

Grid walking test. The rats were placed on a stainless steel grid floor elevated 1 m above the floor. For 1-min observation period, the total number of steps was counted as well as the number of foot fault errors (15).

Tapered/ledged beam walking test. The beam-walking apparatus consisted of a tapered wooden beam with ledges on each side to permit foot faults without falling. The end of the beam was connected to a black box. A bright light was placed above the start point to motivate the rats to traverse the beam. The score for each trial was calculated as follows: [(vertical slips $\times$ 0.5 + horizontal slips)/(steps + vertical slips + horizontal slips)] $\times$ 100 (17).

Morphologic study. The animals were deeply anesthetized with thiopental sodium (0.1 mL/100 g, i.p.) and perfused transcardially with saline followed with 4% paraformaldehyde. The brains were removed from the skull and stored in the same solution for 24 h. Coronal sections of the brain (50 μm) were cut using a cryostat (Shandon, United Kingdom), with 250 μm intervals, and stained with cresyl violet using the Nissl method. Digitized images of cross-sectional areas were obtained with a video camera installed in an Olympus BX40 microscope, interfaced by a software (Image Pro-Plus 6.1, Media Cybernetics) run on a personal computer. Images of the hemispheres were displayed onto a high-resolution video monitor and its boundaries were outlined for area measurements in accordance with the Paxinos and Watson atlas (18).

The Cavalieri method was used to estimate the hemispheric volumes (mm^3) by summation of areas multiplied by the distance between sections. The cross-sectional area of the hemisphere was obtained outlining edges of each hemisphere. Volume estimation was performed in about 10 sections for each rat (19,20).

Immunofluorescent staining. Rats from the HUCB cell group were killed 24 h ($n = 2$), and 1 ($n = 2$) and 3 ($n = 2$) wk after neonatal HI injury. Brains were removed from the skulls and frozen in liquid nitrogen (-70°C). Coronal sections (15 μm) were obtained using a cryostat at -20°C and postfixed in acetone. To identify grafted human cells, sections were incubated with mouse antihuman nuclear monoclonal antibody (MAb) (MAb-1281; dilution 1:100; Chemicon International) overnight at 4°C. After washing in 0.1 M PBS, FITC (dilution 1:100; Novocastra Laboratories) was added and incubated at room temperature in the absence of light for 1 h. To specifically stain the nuclei with blue fluorescence, 4′,6-diamino-2-phenylindole (DAPI, Santa Cruz Biotechnology) was used. To detect human cells in rat brain, double-stained sections were examined to identify those MAb-1281 positive cells that colabeled with DAPI, as previously described (6).

Reactivity of these antibodies with human cells had been confirmed in positive samples and negative control sections were processed as per the experimental tissue, but the primary or secondary antibodies were omitted. Slides were examined qualitatively in a Nikon Eclipse E800 fluorescence microscope coupled to a Pro-Series High Performance CCD camera and Image Pro Plus Software 6.1 (Media Cybernetics, Bethesda, MD).

Polymerase chain reaction analysis. DNA was obtained from the brain and other organs 24 h, and 1 and 3 wk after HUCB cell injection using Trizol Reagent (Carlsbad, CA). To evaluate HUCB cells migration, polymerase chain reaction (PCR) analysis for human β-actin gene was performed using primers (direct 5′-CCTCATGAAGATCCTCAC-3′, and reverse 5′-TGGAGAAGAGCTACAAGC-3′), which results in a 161 bp amplicon. Amplicons were analyzed after electrophoresis on 2% agarose gel and visualized with ethidium bromide staining.

Statistical analysis. The sample size (nine animals per group) was calculated to provide more than 80% power to detect a reduction in behavioral outcomes of 40% between the groups.

Variables with normal distribution were presented as means $\pm$ SEM. Comparisons between the groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test, and behavioral performance on the training day was analyzed using a two-way ANOVA followed by the Bonferroni test. Variables with abnormal distribution were presented as median (minimum and maximum), and the Kruskal-Wallis test was used after Dunn's comparison. Data were considered significantly different if $p < 0.05$.

RESULTS

Eight rats died during different steps of the experiment (19%). These animals were not considered in the final sample size.

To determine whether i.v. administration of HUCB cells can improve cognitive deficits, 30-d-old rats were trained in the spatial version of the MWM. Except on d 1, two-way ANOVA revealed significant differences between the experimental groups during the training period. As shown in Figure 1*A*, the mean latency of the second to fifth day was shorter in sham-operated rats when compared with the saline and HUCB cell groups, indicating that HI impaired memory performance in the injured animals. During this test, rats that had received i.v. HUCB cells 24 h after HI did not present a statistically significant shorter latency to find the platform on the d 2 to d 4.

Analysis of the probe trials (Fig. 1*B*) using one-way ANOVA followed by the Tukey test also showed that the sham-operated group spent significantly more time in the target quadrant when compared with HI animals treated with HUCB cells ($p = 0.008$) and saline ($p < 0.001$). No statistically significant difference was found between the transplanted HI rats and the saline group in terms of the latency to swim over the previous location of the escape platform ($p = 0.25$).

To analyze the motor performance of the experimental groups, we performed four motor tests. Table 1 shows that open-field activity, cylinder, grid walking, and ledged beam walking tests did not reveal significant differences between the HUCB, saline, and sham-operated groups.

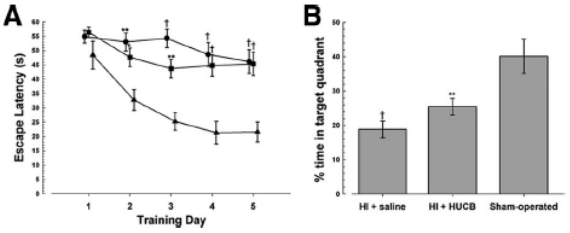

Figure 1. MWM performed 3 wk after HI injury. (*A*) Mean escape latency during the 5 d of training for sham-operated (▲; $n = 9$), HI + saline (●; $n = 10$) and HI + HUCB (■; $n = 15$) rats. (*B*) Mean percentage of time spent in the target quadrant during a 60-s probe test in the absence of the escape platform carried out 24 h after the fifth training day for rats that received saline or HUCB cells as in *A*. *Post hoc* test shows HI + saline *vs.* sham: $p < 0.001$; HI + HUCB *vs.* sham: $p = 0.008$; HI + saline *vs.* HI + HUCB: $p = 0.25$. $^*p < 0.05$; $^{**}p < 0.01$; †$p < 0.001$ *vs.* sham-operated rats.

Table 1. *Motor performance in three groups within the study*

Test/group	HI + HUCB	HI + saline	Sham-operated	*p*
Locomotion time (s): mean ± SEM	107.09 ± 5.61	99.75 ± 5.39	109.13 ± 4.76	0.44
No. rearing: mean ± SEM	31.64 ± 3.08	35.75 ± 2.01	29.38 ± 2.01	0.21
No. crossings: mean ± SEM	115.55 ± 6.55	113.17 ± 5.50	124.63 ± 4.47	0.40
Cylinder test (%): median (minimum–maximum)	12.90 (−2.7 to 69.23)	13.17 (−7.14 to 53.85)	2.94 (−1.37 to 11.32)	0.06
Grid walking (%): median (minimum–maximum)	16.67 (4 to 21.43)	11.24 (7.14 to 21.43)	5.72 (0 to 33.33)	0.07
Ledged beam walking (%): means ± SEM	10.94 ± 1.01	8.62 ± 0.59	10.98 ± 1.47	0.15

Variables with normal distribution are presented as means ± SEM and are compared using one-way ANOVA. Otherwise, variables are presented as median (minimum and maximum) and are compared using the Kruskal-Wallis test.

We also examined whether HI resulted in morphologic deficits and if i.v. administration of HUCB cells attenuated neuronal loss. The results of the morphologic assessment in animals treated only with saline solution showed that neonatal HI lesion caused a significant decrease in the volume of the hemisphere ipsilateral to carotid occlusion (right) when compared with the contralateral hemisphere ($p < 0.001$) 3 wk after injury (Fig. 2*A*). The group that received HUCB cells also presented significantly reduced volume in the right hemisphere when compared with the left hemisphere ($p < 0.01$). Figure 2*B* shows that there is no statistically significant difference between the HI + saline and HI animals treated with HUCB cells in terms of the relative difference between left and right hemispheric volume (hemisphere volume ratio = left hemisphere volume/right hemisphere volume) ($p = 0.095$).

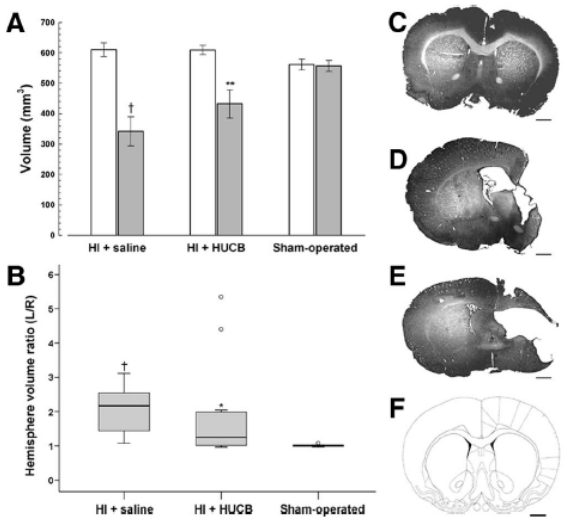

Figure 2. (*A*) The estimated volumes of brain hemispheres, using the Cavalieri method in sham-operated ($n = 9$), HI + saline ($n = 10$) and HI + HUCB ($n = 15$) rats 3 wk after HI injury. There is a significant decrease in the right hemisphere volume (*gray columns*) in HI + saline ($p < 0.001$) and HI + HUCB groups ($p < 0.01$), when compared with the left side (*white columns*). Data expressed as means ± SEM. ANOVA was used followed by a Tukey test (*$p < 0.05$; **$p < 0.01$; †$p < 0.001$) (*B*) Hemispheric volume ratio determined by division of the left hemisphere by the right hemisphere, showing a tendency to reduction in HUCB cell group when compared with saline animals. Data are expressed as median (minimum and maximum). Circles represent outliers. Post hoc analysis shows HI + saline *vs.* sham: $p < 0.001$; HI + HUCB *vs.* sham: $p = 0.02$; HI + saline *vs.* HI + HUCB: $p = 0.095$. (*C–F*) We can observe digitized images of coronal sections of the rat brains stained using the Nissl procedure. (*C*) Sham-operated. (*D*) HI + saline. (*E*) HI + HUCB. (*F*) Schematic drawings obtained from Paxinos and Watson's atlas (interaural 9.70 mm; bregma 0.70 mm). Calibration bars = 1 mm.

Qualitative analysis of HUCB cell migration detected few MAb-1281 immunoreactive cells in either the ipsilateral or contralateral hemispheres 24 h, and 1 and 3 wk after HI injury (Fig. 3*A–I*). To confirm the distribution of HUCB cells after i.v. administration, PCR analysis using human β-actin was performed (Fig. 3*J*). The expression of human β-actin was detected in the ipsilateral and contralateral hemispheres 24 h, and 1 and 3-wk postinjury.

DISCUSSION

This study showed that neonatal HI brain injury induced significant long-term spatial memory deficits and extensive brain hemisphere atrophy. In those animals that received i.v. administration of HUCB cells 24 h after HI induction, the degree of behavioral and morphologic impairment was not significantly reduced. Importantly, the administration of human cells did not worsen the outcomes in HI rats.

As with other investigations, our study showed that, in spite of severe brain damage, motor function tests were insufficiently sensitive to detect neuromotor alterations in all HI rats (2). In contrast to human neonates, pups that underwent HI injury did not show obvious postural or locomotor abnormalities due to a higher degree of plasticity in immature rat brain (21). However, studies have demonstrated that injured brain regions required for memory and learning processing result in significantly decreased spatial memory (2). Our results showed that the MWM test was sensitive to brain damage in neonatal HI rats.

Several groups have reported that HUCB cells delivered either intracerebrally or i.v. dramatically enhance functional recovery after ischemic injury in adult rats. The mechanisms behind such reported neuroprotection are not known, but may include cytokines and trophic factors produced by HUCB cells (6,7,22–24). However, some authors do not confirm these results (25).

Although there are few reported studies on the use of cellular therapy in neonatal brain damage models, there is a wide range of methods used (8,9,26,27). Ma *et al.* used a HI mouse model similar to ours when studying the effect of stem cell transplantation. They showed that the transplanted cells significantly improved the learning and memory deficits 8 mo posttransplantation. However, this study used mice embryonic stem cells injected directly into the lesion site (26).

The route of delivery may contribute to contrasting data. To date, no study had used the acute transplant (24 h after insult)

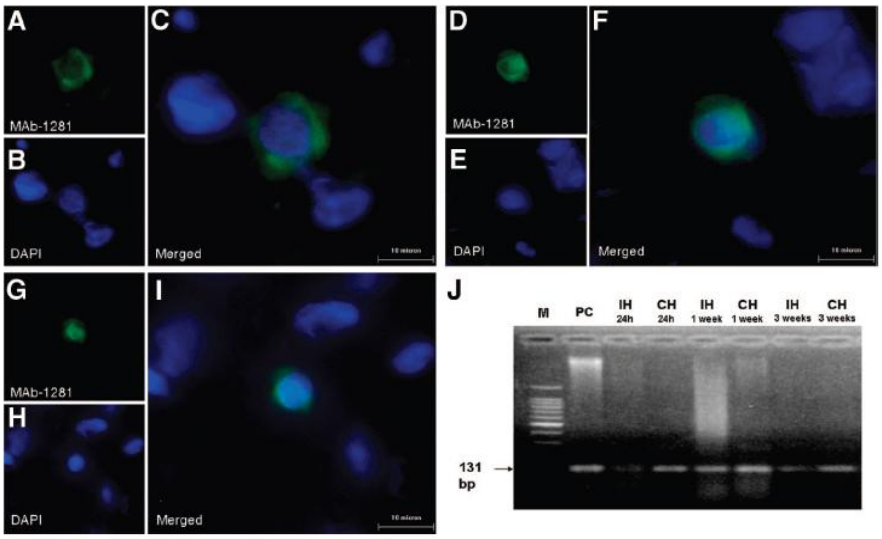

Figure 3. (*A–I*) Digitized images of rat brain showing the immunoreaction to human nuclei antibody (MAb-1281). Use of immunofluorescence FITC (*green*) shows few MAb-1281 immunoreactive cells in rat brain at (*A*) 24 h, and (*D*) 1 and (*G*) 3 wk after HI injury. (*B*, *E*, *H*) Nuclei visualized by DAPI staining (*blue*). (*C*, *F*, *I*) Colocalization of immunofluorescent labels DAPI and MAb-1281 was observed. (*J*) Representative PCR analysis results for human β-actin of rats after 24-h, and 1- and 3-wk postinjury. Positive control (PC) = human DNA extracted from human hippocampus. IH, ipsilateral hemisphere; CH, contralateral hemisphere.

of adult stem cells into neonatal rat by i.v. delivery. Intravenous administration has the advantage of being less invasive but raises the problem of cell homing to organs and target site (11). Yasuhara *et al.* (9) demonstrated that both intracerebral and i.v. injection of multipotent adult progenitor cells resulted in behavioral improvement in HI rats although the percentage of graft survival was small. However, Borlongan *et al.* (28) showed that in contrast with intracerebral transplantation, i.v. delivery of bone marrow stem cells produced only limited functional effects in stroke rats. In our study, the nonsignificant functional recovery could also be explained by the small number of MAb1281-positive cells found in rat brain.

We chose to administer the cells 24 h after injury to target acute ischemic processes, such as the inflammation and BBB opening. The optimal time for transplantation after injury is also a critical factor in initiating the repair process. Acute delivery of cells will be critical once the ischemic brain elicits a strong inflammatory response about 24 h postinsult (10,22,28). Also, the BBB may be more accessible at 24–72 h postischemia and combined opening of the BBB and the high expression of chemokines could have facilitated the present mononuclear cell graft migration toward and survival within the ischemic area (29). However, Borlongan *et al.* reported that BBB opening produced by middle cerebral artery occlusion was not permissive enough to allow CNS entry of graft-derived trophic factors. In that study, improved behavioral functions were observed only in rats treated with cord blood stem cells associated with mannitol (4). Previously, we evaluated the BBB permeability in five rats 24 h after HI. Mild leakage was observed in these animals after trypan blue injection (data not shown).

Our morphologic analyses showed that neonatal HI resulted in an extensive infarcted area and that i.v. injection of HUCB cells was unable to significantly reduce the severity of the morphologic damage. We used a dose of 1×10^7 HUCB cells in accordance with previous studies (8). However, few reports about stem cell therapy in brain damage have studied specific dose range (6).

Our study has some limitations. Seven-day-old rats are considered to be similar to human newborns with regard to brain maturation, and the animal is highly suitable for long-term behavioral evaluations. However, Levine rat model results in variable degrees of damage due to individual differences in brain susceptibility among animals (2,30). We hypothesized that the large extension of the hemispheric brain lesion obtained in our study could have contributed to the fact that we did not observe repair after the infusion of 1×10^7 HUCB cells. It is possible that rats with smaller brain injury will have a different response to i.v. injection of HUCB cells.

Only a low number of HUCB cells were detected in rat brain 24 h, and 1 and 3 wk after neonatal HI, as reported in other investigations (4,6,8,22). Despite controversies, immunosuppressive treatment may be a key determinant for homing of HUCB cells i.v. infused into rats (31). Several investigations suggest that T cell-mediated immune reaction plays a significant role in graft rejection and that interspecies incompatibility contributes significantly to phagocytosis of xenogeneic cells (32). Although xenoreactivity to human mesenchymal stem cells transplanted into infarcted rat myocardium has been demonstrated (33), these findings contrast with the results of Saito *et al.* (34). Indeed, xenotransplantation of human bone marrow stem cells ameliorates neurologic deficits after grafting into ischemic brain of rats (35). Also, the results obtained by Chen *et al.* (22) with HUCB cells in rats with stroke suggest an immunologic tolerance to human cells. Although we do not have evidence of host immune response to the transplanted cells, the administration of human cells could have played a role in the negative results obtained in this study.

In addition, the unilateral carotid artery permanent occlusion results in reduction of cerebral blood flow within the various structures of the hemisphere ipsilateral to the vascular occlusion (30). We speculated that low perfusion in distal field

regions may hamper migration of HUCB cells to injured structures, which could also explain our immunohistochemical, behavioral, and morphologic results.

In conclusion, this study suggests that, according to our experimental design, HI neonatal rats with severe brain damage that received i.v. administration of HUCB cells showed nonsignificant improvement to functional and morphologic outcomes. Aspects such as dose, timing, peripheral route of HUCB cell delivery, immunosuppression, and use of associated therapies need to be investigated in depth before cellular therapy is clinically applied to optimize neuroprotection and, consequently, neurobehavioral outcomes.

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APÊNDICE E

ARTIGO ORIGINAL

The dose-response effect of acute intravenous transplantation of human umbilical cord blood cells on brain damage and spatial memory deficits in neonatal hypoxia-ischemia

de Paula S, Greggio S, Marinowik DR, Machado DC, DaCosta JC.

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THE DOSE-RESPONSE EFFECT OF ACUTE INTRAVENOUS TRANSPLANTATION OF HUMAN UMBILICAL CORD BLOOD CELLS ON BRAIN DAMAGE AND SPATIAL MEMORY DEFICITS IN NEONATAL HYPOXIA-ISCHEMIA

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Abstract—Despite the beneficial effects of cell-based therapies on brain repair shown in most studies, there has not been a consensus regarding the optimal dose of human umbilical cord blood cells (HUCBC) for neonatal hypoxia-ischemia (HI). In this study, we compared the long-term effects of intravenous administration of HUCBC at three different doses on spatial memory and brain morphological changes after HI in newborn Wistar rats. In addition, we tested whether the transplanted HUCBC migrate to the injured brain after transplantation. Seven-day-old animals underwent right carotid artery occlusion and were exposed to 8% O_2 inhalation for 2 h. After 24 h, randomly selected animals were assigned to four different experimental groups: HI rats administered with vehicle (HI+vehicle), HI rats treated with 1×10^6 (HI+low-dose), 1×10^7 (HI+medium-dose), and 1×10^8 (HI+high-dose) HUCBC into the jugular vein. A control group (sham-operated) was also included in this study. After 8 weeks of transplantation, spatial memory performance was assessed using the Morris water maze (MWM), and subsequently, the animals were euthanized for brain morphological analysis using stereological methods. In addition, we performed immunofluorescence and polymerase chain reaction (PCR) analyses to identify HUCBC in the rat brain 7 days after transplantation. The MWM test showed a significant spatial memory recovery at the highest HUCBC dose compared with HI+vehicle rats ($P<0.05$). Furthermore, the brain atrophy was also significantly lower in the HI+medium- and high-dose groups compared with the HI+vehicle animals ($P<0.01$; 0.001, respectively). In addition, HUCBC were demonstrated to be localized in host brains by immunohistochemistry and PCR analyses 7 days after intravenous administration. These results revealed that HUCBC transplantation has the dose-dependent potential to promote robust tissue repair and stable cognitive improvement after HI brain injury. © **2012 IBRO. Published by Elsevier Ltd. All rights reserved.**

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Abbreviations: ANOVA, analysis of variance; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; HI, hypoxia-ischemia; HUCBC, human umbilical cord blood cells; HuNu, human nuclei-positive cells; MWM, Morris water maze; PCR, polymerase chain reaction; PND, postnatal day; SEM, standard error of the mean.

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Key words: stem cells, hypoxia-ischemia, asphyxia, cord blood stem cell transplantation, dose-response relationship.

The hypoxia-ischemia (HI) that occurs during the neonatal period is an important cause of mortality and severe neurologic morbidity in children, affecting approximately 1 to 3 cases per 1000 full-term live births in developed countries (Wyatt et al., 2007). Approximately 50% of infants with severe HI die, and up to 25% of survivors have long-term disabilities, such as epilepsy, cerebral palsy, and cognitive impairments (Ferriero, 2004). Despite technological and scientific advances in the perinatal care of at-risk newborns, until recently, the management of newborn infants with HI has been limited to supportive care in the neonatal intensive care unit. Consequently, new neuroprotective strategies have been investigated in experimental studies and clinical trials because of the clinical relevance and socioeconomic impact generated by neonatal brain damage. However, with the exception of hypothermia, which shows satisfactory outcomes in infants with mild or moderate HI injury, these therapies have limited results (Sahni and Sanocka, 2008; Johnston et al., 2011). As a result, cell based-therapy has been proposed as a novel treatment approach for severe neurological diseases, including HI. In the neonatal context, obtaining stem cells from umbilical cord blood offers low risk or discomfort to the newborn, and the cells can be transplanted after autologous collection. In addition, umbilical cord blood can be used therapeutically during the perinatal period or can be cryopreserved for later use (Santner-Nanan et al., 2005; Harris, 2008; Liao et al., 2011).

Current investigations using different cell dosages, delivery routes, and types of human umbilical cord blood cells (HUCBC) have reported that this therapy is neuroprotective in most animal models of neonatal brain injury (Meier et al., 2006; Pimentel-Coelho et al., 2009; Yasuhara et al., 2010; Rosenkranz et al., 2010; Xia et al., 2010). Although these preclinical studies have demonstrated promising results for brain damage, we have recently shown in a rat model of severe neonatal HI that a single dose of 1×10^7 transplanted HUCBC can migrate to the brain after intravenous injection but does not improve the cognitive and morphological outcomes 3 weeks post-transplantation (de Paula et al., 2009). These previous data demonstrated that several variables will need to be explored to optimize the use of cell therapy in children with HI brain damage, including aspects such as timing, route of transplantation,

cellular type, and dosage (Bliss et al., 2007; Janowski et al., 2010).

One of the most important questions in terms of efficacy and tolerance for the clinical approach of stem cell treatment is the number of administered cells (Wechsler et al., 2009). Some reports have assessed the dose-response association between cell concentration and the functional effects of the treatment on animal models of heart damage (Iwasaki et al., 2006; Wolf et al., 2009). However, there are insufficient data showing the relationship between cell dose and long-term neurological outcomes (Vendrame et al., 2004; Garbuzova-Davis et al., 2008; Omori et al., 2008; Stroemer et al., 2009; Yang et al., 2011), and there are no dose-ranging studies in neonatal HI. Hence, we conducted a pioneer investigation to compare the effect of intravenous administration of HUCBC at three different doses on spatial memory and brain morphological changes in 60-day-old rats previously subjected to neonatal HI. In addition, we tested whether the transplanted HUCBC migrate to the injured brain 7 days after intravenous administration.

EXPERIMENTAL PROCEDURES

Animals

All experimental procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Ethics Committee of Pontifícia Universidade Católica do Rio Grande do Sul, RS, Brazil (CEP 09/04761). A total of 50 male Wistar rats were kept under a constant 12:12-h light–dark cycle at room temperature (23±1 °C), with free access to food and water. After each normal delivery, the litter sizes were adjusted to eight pups per litter. Pups were kept with their dams until weaning at postnatal day (PND) 21. Efforts were made to minimize animal suffering and to reduce the number of animals used.

Experimental groups

The animals were randomly assigned to five experimental groups (n=10 each): sham-operated rats, rats administered with vehicle (HI+vehicle), and rats with 1×10^6 (HI+low-dose), 1×10^7 (HI+medium-dose), or 1×10^8 (HI+high-dose) HUCBC transplanted into the jugular vein 24 h post-HI. The rat pups from each litter were randomly divided among the five experimental groups to avoid "litter effects" on the results of brain injury and cognitive performance. Only male rats were used in our study. An additional cohort of HI animals was injected with HUCBC as described earlier in the text but was euthanized 7 days after transplantation. In these animals, brain samples were collected for immunofluorescence staining and PCR analyses. All experiments were performed by blinded investigators.

Hypoxic-ischemic model

In this study, we used the Levine rat model, modified by Rice et al (Rice et al., 1981) for neonatal rats. On PND 7 (weights ranging from 12 to 15 g), each animal was briefly anesthetized with halothane delivered by a face mask. The right common carotid artery was identified through a midline longitudinal neck incision, isolated from the vagus nerve, and permanently double-ligated with a 7.0 surgical silk suture. The entire surgical procedure was completed within 15 min. After the wounds were sutured, the animals were put back into their cages and allowed to recover for 2–4 h in the company of their dams. The rats were then placed in a hypoxia chamber for 2 h, with a constant flow of humidified 8% oxygen balanced with nitrogen. The hypoxia chamber was kept in a water bath to maintain the ambient temperature inside the chamber at a normal range (37–38 °C). After hypoxic exposure, the pups were returned to their dams for recovery. The sham-operated animals underwent anesthesia and incision only.

HUCBC preparation and intravenous administration

After obtaining informed consent, HUCBC were collected *ex-utero* from healthy volunteers using sterile syringes containing 5.000 UI of heparin, immediately after full-term delivery. We have used a proportion of one donated umbilical cord to 4–7 transplanted rats, depending on the amount of available mononuclear cells. Blood samples were kept at a temperature of 4 °C during the transport and storage procedures, and all units were processed within 24 h after collection. For the separation of mononuclear cells, the obtained material was diluted in RPMI-1640 medium (1:1) (Gibco, Grand Island, NY, USA). The cells were resuspended and fractionated on a density gradient generated by centrifugation, over Ficoll-Paque solution with a density of 1.077 g/L (Histopaque 1077, Sigma Aldrich, St. Louis, MO, USA), at $400\times g$ for 30 min at 25 °C. The mononuclear fraction over the Ficoll-Paque layer was collected and washed twice with Dulbecco's Phosphate Buffered Saline (DPBS) (Gibco, Grand Island, NY, USA). The cell density was determined with a Neubauer-counting chamber, and the number of viable cells was determined using the Trypan Blue 0.4% exclusion method. For the detection of surface antigens, HUCBC were incubated with fluorescein isothiocyanate- (FITC) or phycoerythrin- (PE) conjugated monoclonal antibody against CD45 (hematopoietic precursor cells), CD105 (bone marrow precursor cells), CD34 (hematopoietic and endothelial precursor cells), and CD117 (hematopoietic precursor cells) (Becton Dickinson Biosciences, San Jose, CA, USA). Labeled cells were collected and analyzed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Twenty-four hours after HI, animals weighting approximately 20 g received HUCBC (1×10^6, 1×10^7, or 1×10^8 cells resuspended in saline) or vehicle delivered into the left jugular vein using an ultrafine insulin syringe with a 31-gauge needle in a volume of 100 μl. For that procedure, animals were anesthetized with halothane, the previous neck suture was carefully opened, and the left external jugular vein was isolated from adjacent tissue to facilitate the intravenous injection. Thereafter, the skin was once again closed with suture, and the animals were returned to their dams for recovery.

Spatial version of the Morris water maze task

Spatial memory performance was evaluated 8 weeks after HI exposure or sham operation using the Morris water maze (MWM) as previously described (Greggio et al., 2011; Venturin et al., 2011). The water maze consisted of a black circular pool (200 cm in diameter) conceptually divided into four equal imaginary quadrants. The water temperature was maintained at 21–24 °C. Two centimeters beneath the surface of the water and hidden from the rat's view was a black circular platform (15 cm in diameter). The water maze was located in a well-lit white room, and cues were placed on the walls around the pool, which could be used by the rats for spatial orientation. Training on the spatial version of the MWM was performed over five consecutive days. On each day, the rats received eight training trials during which the hidden platform was kept at a constant location. The movements of the animals were monitored during the sessions with a video camera fixed to the ceiling over the center of the maze. A different starting location was used in each trial, which consisted of a swim followed by a 30-s platform sit. The rats that did not find the platform within 60 s were guided to it by the experimenter. To assess long-term memory, 24 h after the final trial, the platform was removed from the maze and the parameters measured were (1) the percentage

of time spent in the target quadrant and (2) the latency to reach the original platform position.

Brain morphological study

After completing behavioral tests, the animals were deeply anesthetized with a ketamine and xylazine mixture (90:10 mg/ml; i.p.) and perfused transcardially with saline followed by 4% paraformaldehyde, pH 7.4. The brains were removed from the skulls, post-fixed in the same solution at room temperature for 24 h, and cryoprotected by immersion in a 30% sucrose solution in phosphate buffer at 4 °C until they sank. The brains were then quickly frozen in isopentane that was cooled in liquid nitrogen (−70 °C). Coronal sections of the brains (50 μm) were cut using a cryostat (Shandon, UK), collected at equidistant intervals, and stained with Cresyl Violet using the Nissl method. The cerebellum was excluded from the study. Digitized images of the coronal sections overlaid on a point counting grid were obtained with a high-performance CCD camera installed on a stereoscopic light microscope (DF Vasconcellos MU-M19, Brazil), interfaced with Image Pro Plus 6.1. (Media Cybernetics, Silver Spring, MD, USA), and run on a personal computer. Images of the hemispheres were displayed on a high-resolution video monitor, and the boundaries were defined in accordance with the Paxinos and Watson atlas (Paxinos and Watson, 1986). The Cavalieri method was used to estimate the hemispheric volume by the summation of points multiplied by the distance between sections. With the coronal sections displayed on the point counting grid, we counted the number of points hitting the hemisphere. Volume estimation was performed in 10 equally spaced sections for each rat brain in the affected and control hemispheres. The number of points counted was used for the estimation of hemispheric volume (mm^3) using the following equation: $V=T.a/p.\Sigma P$, where V=volume estimation; T=distance between the analyzed sections (1200 μm); a/p=point area (1 mm^2); and ΣP=the sum of points overlaid in the image (Galvin and Oorschot, 2003; Alles et al., 2010). Besides, to evaluate the extent of brain injury, we calculated the percentage of brain tissue loss in the ipsilateral hemisphere (left hemisphere—residual ipsilateral hemisphere divided by left hemisphere× 100%), as previously described (You et al., 2007).

Immunofluorescent staining and confocal laser scanning

Migration of HUCBC was performed in low-, medium-, and high-dose groups using the indirect immunofluorescence method. Seven days after intravenous HUCBC transplantation, a subgroup of HI animals (n=6) was anesthetized with a ketamine and xylazine mixture (90:10 mg/ml; i.p.) and perfused transcardially first with a saline solution containing heparin, followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4. The brains were removed, post-fixed in 4% paraformaldehyde, and then processed for paraffin sectioning. A series of four 15-μm thick coronal sections containing the cortex and hippocampus were cut with a microtome. After being deparaffinized, the sections were placed in boiling Target Unmasking Fluid solution (TUF; PanPath, Amsterdam, NL) in a microwave oven for 10 min. After cooling at room temperature, nonspecific protein binding was blocked with 2.5% albumin serum bovine (Sigma Aldrich, St. Louis, MO, USA) for 1 h. For the detection of the grafted human stem cells, the coronal slices were incubated overnight at 4 °C with a primary mouse anti-human nuclear antigen monoclonal antibody (HuNu) (1:100; Chemicon, Temecula, CA, USA). The sections were then washed with 0.1 M PBS and incubated with Alexa Fluor 488 goat anti-mouse IgG (H+L) secondary antibody (1:1.000; Invitrogen, Carlsbad, CA, USA) at 37 °C in the absence of light for 1 h. Glass coverslips were mounted using ProLong Gold antifade mounting medium with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) Invitrogen (Carlsbad, CA, USA) to visualize cell nuclei. Reactivity of the antibody was confirmed in positive samples and negative control sections. Colocalization of DAPI with the human-specific marker HuNu was detected, analyzed, and photographed qualitatively using a confocal laser scanning microscope (LSM 5 Exciter, Carl Zeiss, Germany) coupled to a Pro-Series High Performance CCD camera and Zen 5.0 software (Carl Zeiss, Inc.). Blue (DAPI) and green (Alexa 488 for HuNu) fluorochromes in the slices were excited by a laser beam at 405 nm (Diode) and 488 nm (argon), and the emissions were sequentially acquired with two separate photomultiplier tubes through LP 420- and BP 505–530-nm emission filters, respectively. The areas of interest were scanned with a pinhole under each laser, set to a value of 1.0 Airy unit for the×63 oil immersion objective lens (Plan-Neofluar, NA=1.4). The scanning dimensions were 1024×1024 pixels, and the specimens were scanned an average of four times with a 12-bit pixel depth. The Z-stacks (±15 optical slices) were obtained at a thickness of 1 μm, and colocalization was evaluated in single optical planes taken through the entire z-axis of each cell. Only $HuNu^+$ cells contained entirely within the three dimensions of a stack were included in the analysis.

Polymerase Chain Reaction (PCR analysis

An additional group of HI rats (n=7) was euthanized, and samples were collected 7 days after HUCBC intravenous injection. DNA was obtained from the rat brains using the phenol/chloroform method described by Isola et al (Isola et al., 1994). PCR analysis was performed to identify the presence of administrated HUCBC in the brains of transplanted animals using complementary primers to the human β-actin gene sequence. We used the forward primer 5′-TCCCTGTACGCCTCTGGCCATA-3′ and the reverse primer 5′-CCTTCTGCATCCTGTTGGTGATGCTA-3′ complimentary to the human β-globin DNA sequence and reamplified with the forward primer 5′-TGACTGGCCGGAACCTGACT-3′ and the reverse primer 5′-GGTGATGACCTGGCCATTGGG-3′ using the nested PCR technique, resulting in fragments of 535 and 209 bp, respectively. The positive control (DNA from human peripheral blood) and negative control (without any DNA) samples were assayed along with experimental samples in every reaction. Amplified products were detected by gel electrophoresis (2% agarose containing ethidium bromide) for 30 min, at a voltage of 100 V and an amperage of 400 mA. The gels were visualized under an ultraviolet transilluminator (3UV™), and the images were captured using photodocumentation equipment connected to Quantity One software (Bio-Rad, CA, USA).

Statistical analysis

Statistical analysis was performed using the PrismGraph 5.0 program (GraphPad Software, San Diego, CA, USA). The variables are presented as the means±standard error of the mean (SEM). Behavioral and morphological outcomes were compared between the groups using one-way analysis of variance (ANOVA) followed by post-hoc Dunnett's or Bonferroni's tests, as appropriate. Pearson's correlation coefficient and linear regression analysis between the variables were calculated. Data were considered significantly different if $P<0.05$.

RESULTS

HUCBC characterization

To determine the phenotypic characteristics of mononuclear cells derived from human umbilical cord blood, four fluorescence-activated cell sorting (FACS) analyses were used to examine the expression of cell-surface marker (Fig. 1A–D). In brief, 2.4% of the cells expressed CD34,

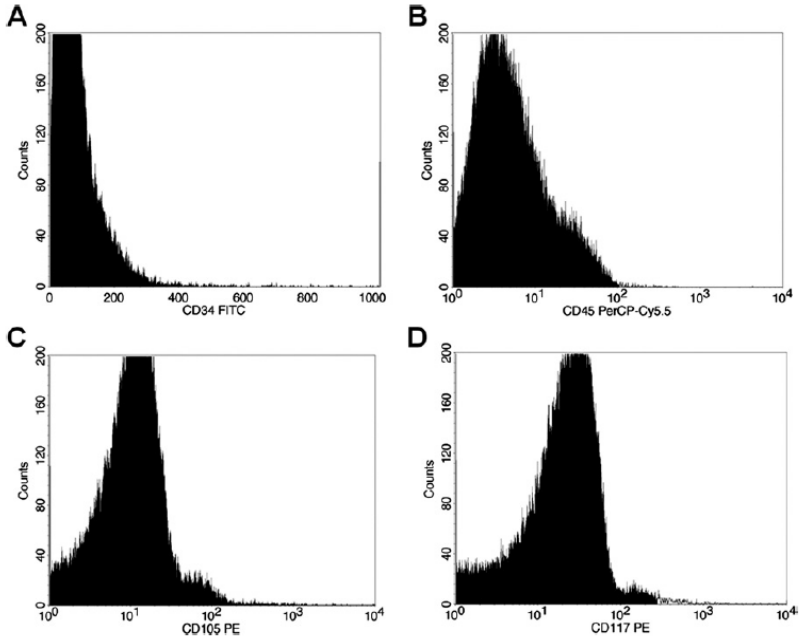

Fig. 1. Immunophenotypic analysis of mononuclear fraction derived from human umbilical cord blood. The histograms show the fluorescence intensity of HUCBC reacting with (A) CD34, (B) CD45, (C) CD105, and (D) CD117 during flow cytometry. A total of 5000 events were considered for each analysis.

23.14% expressed CD45, 50.41% expressed CD105, and 71.25% expressed CD117. Taken together, the data showed that the isolated cord blood cells exhibited a mixture of different cellular types, consistent with the literature (Mayani and Lansdorp, 1998; Park et al., 2009; Ruhil et al., 2009).

A high dose of HUCBC transplanted intravenously rescues long-term spatial memory impairments in hypoxic-ischemic rats

To assess the effects of three different doses of HUCBC on long-term spatial memory deficits, we employed the MWM task. The mean escape latencies to the hidden platform were shortened as training progressed, and the groups showed a different rate of change over time [$F_{(4,\ 220)}$=1.10, P=0.34, Fig. 2A]. As shown in Table 1, Bonferroni post hoc test showed a significant difference (P<0.001) between the HI+vehicle and sham groups throughout the entire 5-day training session. Only the group of HI animals that were administered with the highest HUCBC dose presented learning similarity to sham rats and a significant difference to HI+vehicle group. Besides, there were no statistical differences in the training performance among the transplanted groups. The probe test was performed 24 h after the last training session in the absence of the escape platform, and HI+vehicle rats exhibited significant spatial memory deficits. One-way ANOVA followed by Dunnett's test indicated that the escape latency to swim over the previous position of the escape platform was longer in the HI+vehicle group (37.66±6.99 s) than in the sham group (7.89±1.19 s) (P<0.01) (Fig. 2B). Among treatment groups, only the HI+high dose of HUCBC had a markedly shorter time required to reach the platform location (16.86±3.48 s) (P<0.05 vs. HI+vehicle). For the second analyzed variable, the HI+vehicle group spent less time swimming in the target quadrant that previously contained the escape platform (32.97±3.91%) compared with either the sham-operated (62.52±4.17%) (P<0.001) or HI+high-dose groups (48.17±2.93%) (P<0.05) (Fig. 2C). No significant differences were observed for either variable when HI+low- and medium-dose animals were compared with HI+vehicle animals, suggesting that only a high dose of HUCBC rescues the learning and memory impairments.

Intravenously transplanted HUCBC dose-dependently prevent brain lesions after neonatal hypoxia-ischemia in rats

Absolute hemispheric volume analysis showed significant atrophy of the hemisphere ipsilateral to the carotid occlusion (right side) in the HI+vehicle group when compared with the contralateral hemisphere (296.76±74.23 vs. 605.28±47.39 mm^3; P<0.001) (Fig. 3A). Similarly, the HI+low-dose group also had a significantly reduced volume of the right hemisphere compared with the left one (451.92±64.82 vs. 639.12±17.14 mm^3; P<0.05). No difference was observed between the right and left hemispheres in sham animals (641.40±7.49 vs. 658.92±6.23 mm^3) or in the HI groups that received only medium (560.16±63.65 vs. 664.56±16.74 mm^3) or high doses (658.20±22.32 vs. 658.56±18.75 mm^3) of HUCBC. As shown in Fig. 3B, we also examined cerebral atrophy in terms of the percentage of brain tissue loss, which was calculated using the contralateral (left, non-ischemic)

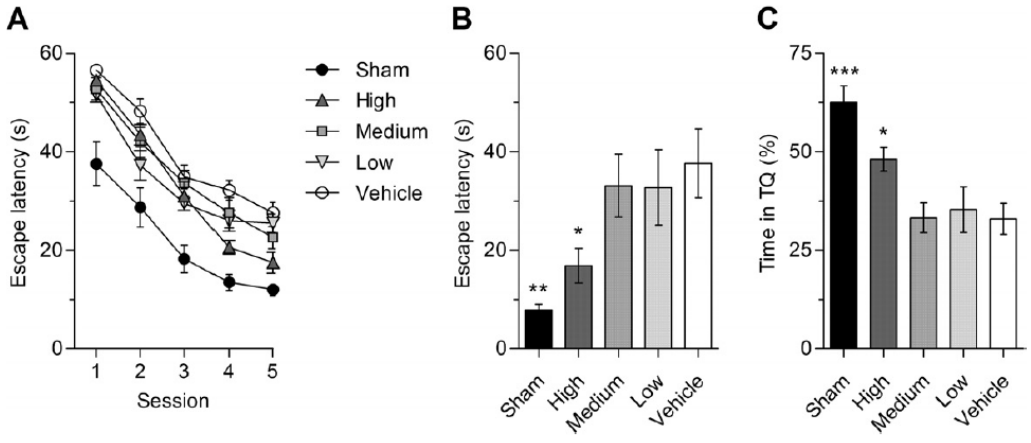

Fig. 2. A high dose of HUCBC transplantation resulted in a significant attenuation of injury-induced spatial memory impairment in hypoxic-ischemic rats. (A) The mean escape latencies to the hidden platform were obtained from a 5-d training session. Data are presented in blocks of eight trials as mean±SEM. (B) The sham-operated and HI+high-dose (1×10^8 cells) groups showed a significantly faster latency of swimming over the previous platform location when compared with HI+vehicle animals. (C) The sham-operated and HI+high-dose groups spent a greater percentage of time searching the quadrant in which the platform had been submerged during training compared with HI+vehicle rats. No significant differences were observed when low- or medium-dose groups were compared with the HI+vehicle group for either variable. The values were presented as the mean±SEM; $n=10$ per group. Differences between groups were analyzed by one-way ANOVA followed by the Dunnett post hoc test; * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ vs. HI+vehicle.

hemisphere as a control. One-way ANOVA followed by Dunnett's test indicated that the percentage of damage in the HI+vehicle group (54.90±9.16%) was markedly higher than in the sham-operated group (3.09±0.60%; $P<0.001$). However, the right hemispheric volume loss caused by HI was significantly lower in rats of the medium-dose (18.29±8.79%; $P<0.01$) and high-dose (3.21±0.84%; $P<0.001$) groups when compared with vehicle-treated animals, suggesting a protective dose-dependent effect. There was no statistically significant difference between the HI+vehicle and low-dose groups (29.70±9.89%). Representative samples of Nissl staining from the brain hemispheres of rats after HI insult are shown in Fig. 3C. Examination of brain tissues showed vast ischemic damage with extensive atrophy and the formation of porencephalic cysts in the ipsilateral hemisphere of the HI+vehicle rats. Additionally, we observed a positive linear relation between the degree of hemispheric tissue volume loss and the escape latency of the MWM probe trial when all groups were pooled for correlation analysis ($R=0.71$, $P<0.0001$, Fig. 4A). Besides, a negative correlation between the degree of hemispheric tissue volume loss and the time spent in the target quadrant of the MWM probe trial was detected ($R=-0.57$, $P<0.0001$, Fig. 4B). Taken together, these findings suggest that neonatal HI-induced brain injury contributes to spatial learning and memory deficits in rats, and that HUCBC dose-dependently hinders brain lesion and cognitive impairments due to neonatal HI.

HUCBC are detected in the rat brain 7 days post-transplantation as determined by PCR analysis and immunofluorescence staining

Additional groups of HI rats were euthanized 7 days after HUCBC transplantation for human cell detection in host rat brains using an anti-human nuclear antibody. Double-stained sections were examined using a confocal laser scanning microscope to identify human nuclei-positive cells (HuNu) that colabeled with DAPI. Most of the surviving human cells were located in the cortex and the hip-

Table 1. Morris water maze acquisition performance

Groups	Training session				
	1	2	3	4	5
Sham	37.6±4.4***	28.7±4.0***	18.3±2.8***	13.5±1.6***	12.0±1.2***
Vehicle	56.6±0.9†††	48.3±2.5†††	34.9±2.4†††	32.2±2.0†††	27.6±2.1†††
Low	51.6±1.5†††	37.3±3.0**	29.5±1.0††	26.0±2.1††	25.5±1.9†††
Medium	52.8±2.3†††	41.9±3.1†††	33.7±2.6†††	27.6±3.1†††	22.6±2.3†
High	54.5±1.0†††	43.5±2.1†††	31.0±2.9††	20.6±1.4**	17.5±2.1*

Values represent the mean±SEM ($n=10$ rats/group).
* $P<0.05$, ** $P<0.01$, and *** $P<0.001$ vs. vehicle group; † $P<0.05$, †† $P<0.01$, and ††† $P<0.001$ vs. sham group in Bonferroni post hoc test after two-way ANOVA.

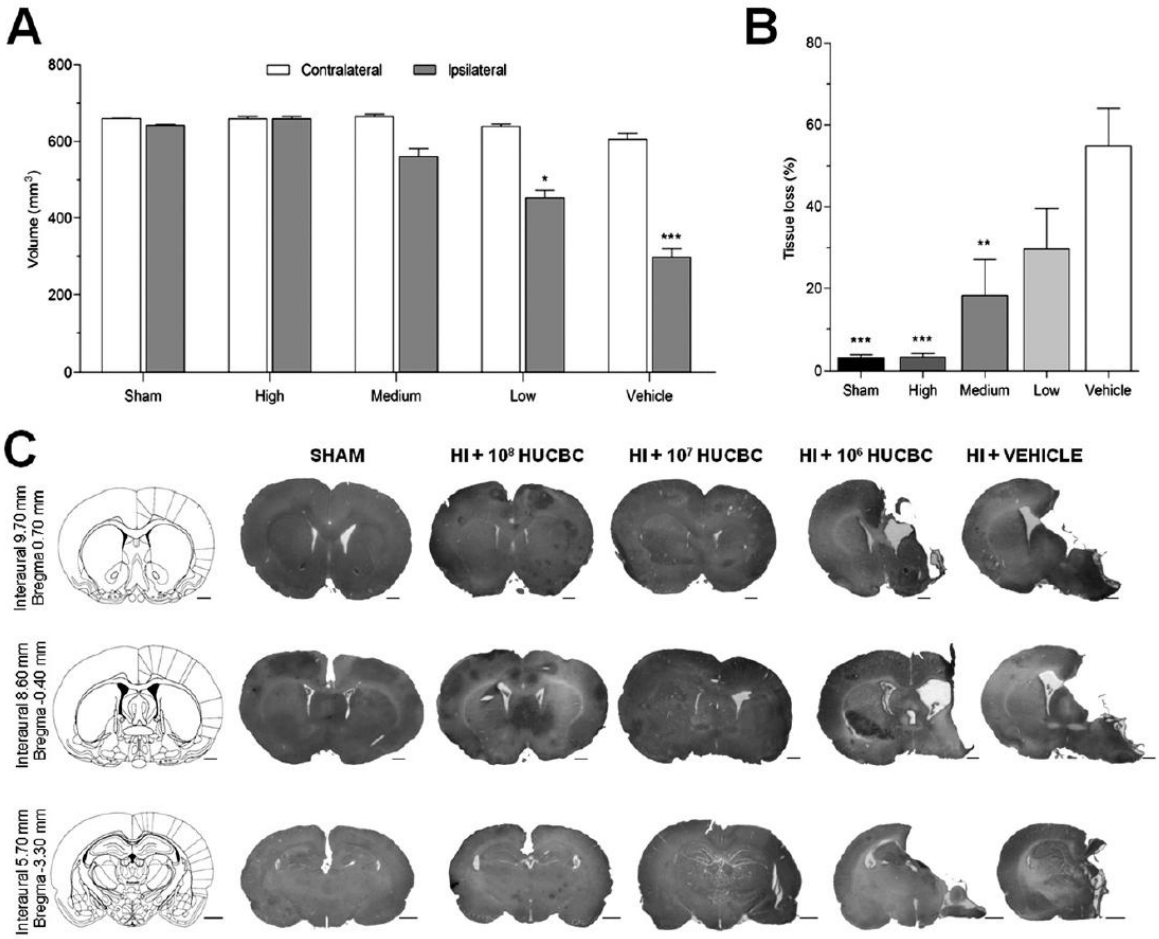

Fig. 3. HUCBC dose-dependently protect against brain atrophy in rats subjected to neonatal hypoxia-ischemia. (A) Absolute volumes of brain hemispheres are shown. The rats in the HI+vehicle and HI+low dose of HUCBC groups showed a significant decrease in the right hemispheric volume compared with the left. There was no difference between the hemispheric volumes in the medium- and high-dose HI animals. The values were presented as the mean±SEM; $n=10$ per group. Differences were analyzed by one-way ANOVA followed by the Bonferroni post hoc test; * $P<0.05$; *** $P<0.001$ vs. the left hemisphere. (B) Percentage of brain tissue loss is shown in a graph. When compared with sham-operated rats, the HI+vehicle group showed marked brain atrophy that was significantly lower in the medium- and high-dose HUCBC groups, suggesting a relationship between cell dosage and brain damage rescue. There was no statistically significant difference between the HI+vehicle and the HI+low-dose group. All values represent the mean±SEM; $n=10$ per group. ** $P<0.01$, and *** $P<0.001$ vs. the HI+vehicle group using Dunnett's multiple comparison post hoc test after one-way ANOVA. (C) Digitized images of coronal sections of the rat brains were stained using the Nissl procedure. The first column shows schematic drawings obtained from Paxinos and Watson's atlas. The slices show visible ipsilateral cortical atrophy (right hemisphere) in the HI+vehicle group that was attenuated at the highest doses of HUCBC. Calibration bars=1 mm.

pocampus of both hemispheres (Fig. 5A, B). Confocal photomicrographs with orthogonal reconstruction of grafted cells (Fig. 5C) revealed merged images of $HuNu^+$ cells and DAPI in brain parenchyma of all analyzed 7 days after transplantation. To confirm the migration of the delivered cells, we performed nested PCR analysis in seven animals using complementary primers to the human β-globin sequence 7 days after HUCBC transplantation. The expression of the band corresponding to the human gene was detected in the ipsilateral and contralateral hemispheres of six rats that received HUCBC via the jugular vein (Fig. 5D). The observations made from immunofluorescence staining and PCR analysis were consistent for all doses.

DISCUSSION

The neonatal HI rodent model produces long-term cognitive deficits and severe brain atrophy. We found that after a high dose of HUCBC (1×10^8 cells) transplanted intravenously, the rats demonstrated a significant attenuation of HI-induced spatial memory impairment 8 weeks after the treatment. Furthermore, medium (1×10^7 cells) and transplantation of high-doses of cells hindered the brain lesions caused by HI, which was not observed in low-dose-(1×10^6 cells) treated animals. In addition, HUCBC were identified in host rat brains 7 days after intravenous administration using immunofluorescence and PCR analysis.

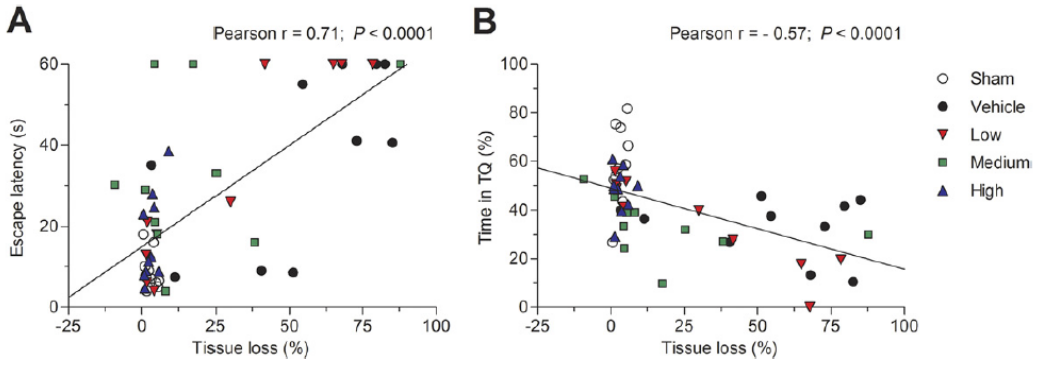

Fig. 4. Correlation of the degree of hemispheric tissue volume loss with spatial learning and memory in HI rats after HUCBC transplantation. (A) Correlation of the degree of hemispheric tissue volume loss and the escape latency of the MWM probe trial (R=0.71, P<0.0001). (B) Correlation of the degree of hemispheric tissue volume loss and the time spent in the target quadrant of the MWM probe trial (R=−0.57, P<0.0001). All experimental groups were pooled for both correlation analyses. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

To investigate cognitive function after HUCBC transplantation, we performed the MWM test, which is an important measure of hippocampal-dependent spatial learning and memory after brain injuries, such as neonatal HI (Golan and Huleihel, 2006). Using this tool, our study revealed that injured animals treated with low and medium doses of HUCBC did not have a reduction of behavioral deficits. These results support our previous investigation that failed to show significant benefits in behavior performance 3 weeks after administration of 1×10^7 HUCBC (medium dose) in HI rats (de Paula et al., 2009). However, in the current report, the spatial learning and memory of HI animals treated with a high dose of HUCBC was significantly rescued compared with HI+vehicle animals 8 weeks after treatment. Only a few studies have addressed the effects of cellular therapy on the cognitive consequences after neonatal brain damage (Katsuragi et al., 2005; Ma et al., 2007). Consistent with our data, Ma et al (2007) reported that stem cells were effective in reducing behavioral impairments caused by HI, 2 and 8 months after cell transplantation. In addition to functional restoration in the MWM test, the authors also observed a neuropathological recovery in stem cell-treated animals (Ma et al., 2007).

In the current investigation, we observed that both medium and high doses of HUCBC provided significant brain damage repair after HI, in contrast to our previous data (de Paula et al., 2009). This discrepancy might be owing to the length of time that was chosen to assess neuronal injury changes. As previously shown in adult rats with ischemic lesions, no beneficial tissue effect of mesenchymal stem cell treatment was observed 29 days after the treatment. However, the authors found a statistically significant decrease in the lesion size in treated animals when evaluated 60 days post-transplantation (Kranz et al., 2010). In addition, we emphasize that, despite the brain tissue regeneration presented here, 1×10^7 HUCBC (medium dose) were not enough to mediate functional recovery after neonatal HI injury.

Although basic research has shown promising results in the field of cell-based therapy for neonatal brain injury (de Paula et al., 2010), the optimal dose for intravenous administration of stem cells has not yet been determined. According to a recent meta-analysis on intravenous stem cell delivery, there is a dose-response association between the number of stem cells injected and the functional effects of the treatment in experimental neurological diseases (Janowski et al., 2010). A pioneer study demonstrated that neural stem cells dose-dependently improved functional outcomes when transplanted into the ischemia-damaged striatum of rats (Saporta et al., 1999). In addition, Stroemer et al. (2009) observed that neural stem cell transplantation in rats after stroke promoted significant sensorimotor recovery depending on cell dosage (Stroemer et al., 2009). In line with our study, Yang et al. (2011) demonstrated that the highest doses of bone marrow mononuclear cells led to reduced lesion size and better functional performance in an animal model of stroke (Yang et al., 2011). The authors of these investigations observed discrete cell survival in the majority of the high-dose-treated animals, proposing a neuroprotective paracrine trophic mechanism.

Multiple mechanisms have been proposed to explain the promising behavioral and morphological outcomes observed in cell transplantation reports. However, the specific mechanisms of action responsible for the successful stem cell transplantation in HI brain injury have not yet been fully elucidated. It has been suggested that HUCBC treatment decreases brain damage and consequently improves neurological deficits, mainly by enhancing paracrine repair processes (Luo, 2011). It is possible that stem cells might serve as vehicles for specific molecules, acting as vectors for the production and/or release of neurotrophic factors. Consistent with *in vitro* studies (Arien-Zakay et al., 2009) and investigations using adult rats in a stroke model (Li et al., 2002), Yasuhara et al. (2010) reported an increase in GDNF, NGF, and BDNF brain levels in HI rats 3 days post-HUCBC transplantation (Ya-

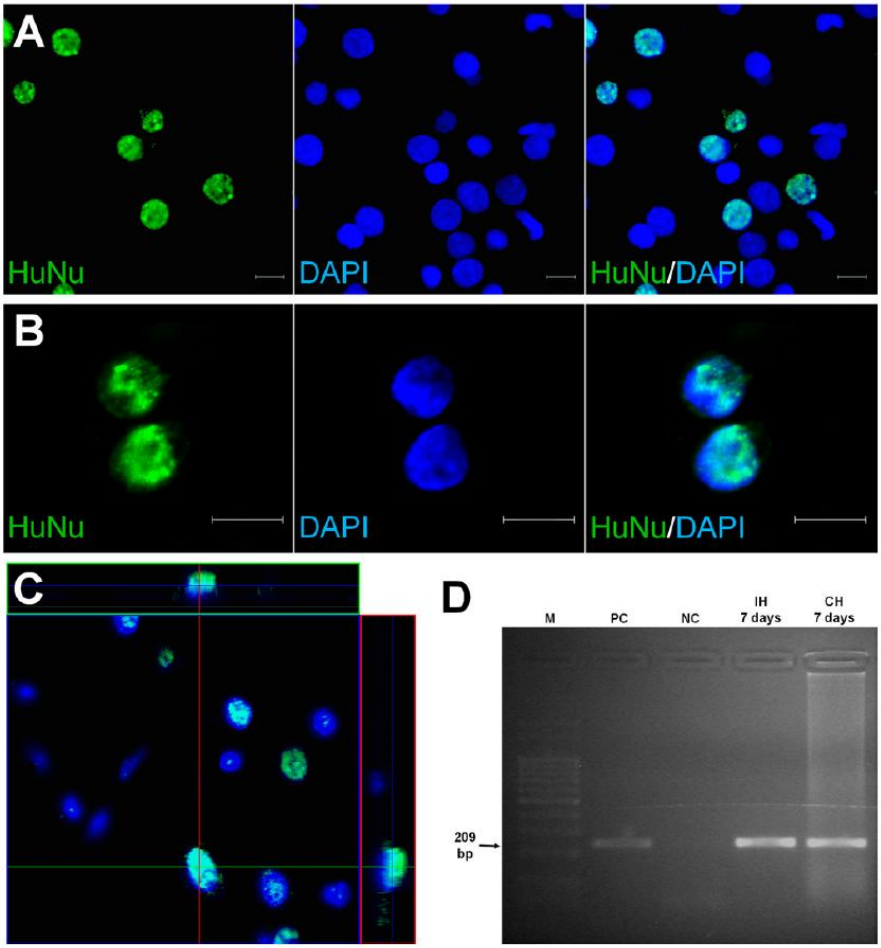

Fig. 5. Evidence of surviving HUCBC in a rat brain 7 d after a high dose of intravenous cell transplantation. Laser scanning fluorescent imaging of double-labeled cells and three-dimensional image reconstruction revealed that $HuNu^+$ cells (Alexa 488, green) were colocalized with DAPI (blue). (A) The individual channels and their merged image clearly demonstrate the colocalization of DAPI and HuNu in the HI rat cortex ipsilateral to the lesion. (B) $HuNu^+$ cells in the graft are shown at higher magnification in left hemisphere. The co-labeling of DAPI and $HuNu^+$ cells was highly cell-type specific. (C) Representative orthogonal images showing the colocalization of $HuNu^+$ cells and DAPI are presented. Red and green lines indicate corresponding points in the orthogonal planes, confirming the localization of the labeling within the cell after the summation of serial optical sections. (D) PCR analysis was performed to identify human cells in the rat brain. An agarose gel shows the presence of a band corresponding to the human β-globin gene sequence in the ipsilateral and contralateral hemispheres. Positive control (PC); negative control (NC); ipsilateral hemisphere (IH); contralateral hemisphere (CH). Calibration bars=10 μm. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

suhara et al., 2010). Neurotrophic support may also be responsible for axonal sprouting and consequently rescue behavior. Using neuroanatomical tracing, Daadi et al. (2010) demonstrated significant contralesional sprouting in HI rats after neural stem cell transplantation (Daadi et al., 2010). Additionally, some studies have reported that the functional benefits of mononuclear cord blood are related to an increase in endogenous neurogenesis. Bachstetter et al. (2008) demonstrated that intravenous administration of the mononuclear fraction of umbilical cord blood stimulates neurogenesis in the brains of aged rats (Bachstetter et al., 2008). Some authors have also suggested that transplanted cord mononuclear cells have the ability to modulate the post-injury inflammatory response thus facilitating the process of endogenous neurogenesis (Vendrame et al., 2005). Another important action mechanism that needed to be considered is that a high dose of HUCBC used in our study may have enhanced the blood vessel formation in ischemic areas. As demonstrated by Ramos et al. (2010), human cord blood is a source of endothelial, myeloid, and lymphoid precursors. The endothelial cells generated from common progenitors for the hematopoietic and vascular lineages in cord blood mononuclear cell fraction may be able to participate in new blood vessel formation (Ramos et al., 2010). However, more studies are needed to confirm these suggested mechanisms.

In the present study, we detected double-labeled $HuNu^+$ cells 7 days after cell transplantation. Most of the

HUCBC were widely dispersed throughout the cortical regions and other structures of both hemispheres. In addition, PCR analysis performed 7 days after HUCBC transplantation showed the presence of human β-globin in the rat brains. Recently, it has been shown that stem cells rapidly migrate to the lesion site within 4–10 days post-transplantation in a rat model of HI (Obenaus et al., 2011). Stromal cell-derived factor-1 (SDF-1) has been reported to be an important player in the recruitment and homing of transplanted HUCBC-derived mononuclear cells to the site of the HI brain lesion in newborn rats (Rosenkranz et al., 2010).

Translation of the experimental knowledge to human trials using cell-based therapy should consider important factors to bridge this gap. The optimization of the cell dose, route and devices for cellular delivery, selection of the optimal cell donor, timing of administration, and use of associated interventions are fundamental issues that should be addressed in the laboratory. As recommended by the Stem cell Therapeutics as an Emerging Paradigm in Stroke (STEPS) (Savitz et al., 2011) and Baby STEPS guidelines (Borlongan and Weiss, 2011), the experimental design for restorative therapies should include a cell dose-response study to reveal not only the optimal therapeutic dose, but also the maximum tolerable dose. With these considerations in mind, we demonstrated that a single intravenous transplantation of HUCBC dose-dependently hinders brain damage and spatial memory deficits in neonatal HI. Although only the high dose (1×10^8 HUCBC) promoted robust tissue neuroprotection and cognitive improvement, it may be interesting to test repeated dosing regimen. Some clinical studies have indicated that transplants using two cord blood units in hematological disease are feasible and may increase the applicability of grafts with a high cell dose. The authors also suggested that techniques such as *ex vivo* cell expansion may provide a good alternative to clinical use of high doses of HUCBC transplantation (Stanevsky et al., 2010; Ballen et al., in press; Kindwall-Keller et al., in press).

Today, four clinical trials promise relevant data on the therapeutic use of umbilical cord blood transplantations in pediatric patients with HI (phase I study at Duke University, NCT00593242) and cerebral palsy (phase II study at Duke University, NCT01147653; phases I and II at Georgia Health Sciences University, NCT01072370; phase I study at Sung Kwang Medical Foundation, NCT01193660). The cellular dosing regimen varies from 1 to 5×10^7 cells/kg, which is similar to human dose recommended for hematological diseases in infants but smaller than high-dose cell transplantation used in our study in rats weighting approximately 20 g. Although the use of 1×10^8 HUCBC dose may be difficult in the clinical setting because of the large number of cells needed, we suggest that our results may support clinical trials using high doses of cord blood cells taking into consideration three important factors presented here: (1) neonate rats tolerated well the high dose of human cells without specific side effects such as tumorigenicity or embolism; (2) a proper human umbilical cord blood collection provided a high dose of mononuclear cells ($\geq1\times10^8$) for transplantation in newborn rats; and (3) there was a strong relationship between cell dosage, brain damage regeneration, and impaired behavior recovery in HI. Finally, we emphasize that the outcomes of further clinical trials with a careful selection of homogeneous patients will determine whether high doses of HUCBC transplantation are clinically feasible and safe. Then, we could verify whether the benefits of HUCBC transplantation correspond to the expectations observed on experimental scenario.

CONCLUSIONS

To our knowledge, this is the first study to demonstrate that acute intravenous administration of HUCBC exerts a dose-dependent effect on long-term behavior and morphological outcomes in HI-injured rats. Thus, these results bring us closer to the clinical applications of cell-based therapy in neonatal HI. In summary, our current study reveals the therapeutic dose (1×10^8 mononuclear cells) necessary to promote robust tissue neuroprotection and stable functional improvement. We emphasize that dosage is an important factor in optimizing cellular transplantation. However, other questions as to the follow-up time required for neuroprotection and the additional action mechanisms demand further investigations, as these are essential tools for the translation of basic science to safe and effective clinical therapies after neonatal brain damage.

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