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INVESTIGAÇÃO *IN VITRO* DE UM POSSÍVEL EFEITO ADVERSO DAS CÉLULAS TRONCO MESENQUIMAIS NO SISTEMA NERVOSO CENTRAL

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“A teoria sempre acaba,

mais cedo ou mais tarde,

assassinada pela experiência.”

Albert Einstein

DEDICO

Aos meus pais, aos meus amigos e ao Ivan
que tanto me ensinaram e
apoiaram ao longo desses anos...

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RESUMO

A terapia celular utilizando células tronco mesenquimais (MSC) derivadas da medula óssea surge como uma alternativa para o tratamento das doenças neurodegenerativas. Apesar dos resultados positivos com o uso dessas células nos ensaios pré-clínicos e clínicos após a isquemia cerebral, seus efeitos colaterais e seu mecanismo de ação permanecem desconhecidos. Os benefícios alcançados após a terapia celular para o tratamento da isquemia não são atribuídos à diferenciação dessas células em novos neurônios, mas sim aos fatores que elas podem secretar. Na tentativa de compreender como os fatores secretados pelas MSC podem influenciar o tecido hipocampal que sofreu ou não privação de oxigênio e glicose (POG) e como os fatores liberados pelo tecido lesionado podem atuar sobre as MSC, nós utilizamos culturas organotípicas de hipocampo expostas ao meio condicionado pelas MSC e MSC expostas ao meio condicionado pelas culturas organotípicas de hipocampo expostas à POG. Os resultados obtidos nesse trabalho mostram que o meio condicionado pelas MSC é tóxico para as culturas organotípicas de hipocampo, induzindo morte celular especificamente nas regiões do Corno de Ammon (CA) e agravando a lesão causada pela POG. Essa toxicidade parece ser específica das MSC, uma vez que o meio condicionado por outros tipos celulares não tem o mesmo efeito. As MSC isoladas tanto de rato como de camundongo e tanto de medula óssea como de pulmão induzem a morte celular de uma maneira semelhante, sugerindo que o efeito não é espécie ou órgão específico. Ainda, nós observamos que os fatores secretados pelas MSC ativam a microglia e os astrócitos, induzindo a produção de espécies reativas de oxigênio (ROS), o aumento da iNOS e um aumento de IL-6 e TNFα nas culturas organotípicas. O efeito tóxico do meio condicionado pelas MSC pode ser atenuado por antioxidantes, anti-inflamatórios, antagonistas NMDA e AMPA, bloqueadores de canal de cálcio dependentes de voltagem e por agonista GABA. Quando as MSC foram analisadas após a sua exposição ao meio condicionado pelo tecido hipocampal lesionado, nós não observamos morte celular ou mudanças morfológicas aparentes nessas células após 24 h de exposição ao meio. Surpreendentemente, as MSC aumentaram a proliferação quando expostas ao meio condicionado pelo hipocampo lesionado, não apresentando nenhum marcador neural após 72 h em contato com esse meio condicionado. Em conjunto, nossos resultados mostram um possível efeito adverso de fatores secretados pelas MSC, introduzindo uma nota de cautela na utilização dessas células.

ABSTRACT

Cell therapy using bone marrow-derived mesenchymal stem cells (MSC) seems to be a new alternative for the treatment of neurodegenerative diseases. In spite of several good and promising results with the use of these cells in preclinical and clinical studies after stroke, their side effects and their mechanism of action are still unknown. The benefits reached after cell therapy to treat stroke are not attributed to the differentiation of the cells in new neurons, but to the factors that these cells can secrete. In an attempt to understand how MSC secreted factors can influence the hippocampal tissue that suffer or not from oxygen and glucose deprivation (OGD) and how the factors secreted from the injured hippocampus can influence MSC behavior, we used organotypic hippocampal slice cultures exposed to MSC conditioned medium and MSC exposed to organotypic hippocampal cultures conditioned medium. The results obtained in this work show that MSC conditioned medium is toxic to organotypic hippocampal slice cultures, inducing cell death specifically in the CA (Cornus Ammonis) region of hippocampus and aggravating the lesion induced by OGD. This toxicity seems to be specific to MSC, once the medium conditioned by other cell types do not induce cell death. Also, MSC isolated from rat or mice and from bone marrow and lungs induce cell death in a similar manner, suggesting that the effect is not organ- or specie- specific. In addition, we have observed that MSC secreted factors activate microglia and astrocytes, inducing reative oxygem species (ROS) generation and iNOS, TNFα and IL-6 increase in organotypic cultures. The MSC conditioned medium-induced toxic effect can be attenuated by antioxidants, anti-inflammatory drugs, NMDA and AMPA antagonists, Ca^{2+} voltage-dependent channel blockers and GABA agonist. When MSC behavior was investigated after these cells were exposed to the conditioned medium from the lesioned hippocampus, we observed that these medium is not able to induce cell death or any apparent change in MSC morphology after a 24 h exposure period. Surprinsingly, MSC increase proliferation in response to the conditioned medium from the injured hippocampal tissue, do not presenting any neural marker after 72 h of contact with this medium. Taken together, our results show a possible side effect of MSC secreted factors, introducing a note of caution in the use of these cells.

APRESENTAÇÃO

Esta tese está organizada em seções dispostas da seguinte maneira: Introdução, Objetivos, Artigos Científicos publicados e/ou submetidos, Discussão, Conclusões, Perspectivas e Referências Bibliográficas. Em anexo está uma lista dos artigos realizados em colaboração durante o período do doutorado.

A **Introdução** mostra o embasamento teórico que nos levou a formular a proposta de trabalho. Os materiais, métodos e resultados, assim como as referências bibliográficas específicas, encontram-se no corpo de cada trabalho, os quais estão apresentados na forma de **artigos científicos**, denominados Capítulos 1, 2 e 3. Esses trabalhos foram realizados no Laboratório de Neuroproteção e Sinalização Celular do Departamento de Bioquímica da UFRGS, em colaboração com os laboratórios coordenados pelos professores Dr. Guido Lenz, Dra. Nance Nardi, Dra. Ângela Terezinha Wyse, Dra. Ana Maria Battasttini e Dra. Elena Bernard, da mesma Instituição, bem como em colaboração com a Dra. Maria Martha Campos, da Faculdade de Odontologia da PUC-RS.

A seção **Discussão** contém uma interpretação geral dos resultados obtidos nos diferentes trabalhos.

A seção **Conclusões** aborda as conclusões gerais obtidas na tese.

A seção **Perspectivas** discute as possibilidades de desenvolvimento de projetos a partir dos resultados obtidos, dando continuidade a essa linha de pesquisa.

A seção **Referências Bibliográficas** lista as referências utilizadas na Introdução e Discussão da tese.

A seção **Anexo** contém uma lista dos trabalhos realizados em co-autoria durante o período de doutorado.

LISTA DE ABREVIATURAS

ACM: **a**rtéria **c**erebral **m**édia

AMPA: ácido alfa amino-3-hidróxi-5-metil-4-isoxazolepropiônico

AVE: **a**cidente **v**ascular **e**ncefálico

CA: corno de Ammon (***C****ornus* ***A****mmonis*)

CAT: catalase

CCL5: quimiocina ligante do ligante 5 (***c****hemokine (***C***-C motif)* ***l****igand* ***5***)

COX-2: ciclooxigenase 2

CNQX: 6-**c**iano-7-**n**itro**q**uino**x**alina-2,3-dieno

CNS: sistema nervoso central (***C****entral* ***N****ervous* ***S****ystem*)

DCF-DA - **D**ihidrodi**c**loro**f**luoresceína **d**i**a**cetato

DG: giro denteado (***D****entate* ***G****yrus*)

DMSO: dimetil sulfóxido

GABA: ácido γ-aminobutírico (*γ-****A****mino****b****utyric* ***a****cid*)

GDNF: fator neurotrófico derivado da glia (***G****lial* ***d****erived* ***N****eurotrophic* ***F****actor*)

GFAP: proteína glial fibrilar ácida (***G****lial* ***F****ibrillary* ***A****cid* ***P****rotein*)

GLAST: transportador de glutamato-aspartato (***Gl****utamate-****As****partate* ***T****ransporter*)

GLT-1: transportador de glutamato-1 (***Gl****utamate* ***T****ransporter*)

HBSS: solução salina balanceada de Hanks (***H****anks* ***B****alanced* ***S****alt* ***S****olution*)

IFNγ: interferon gama

IL-4: interleucina 4

IL-6: interleucina 6

IL-10: interleucina 10

IL-1β: interleucina 1 beta

INDO: indometacina

iNOS: óxido nítrico sintase induzível (***i**nducible **N**itric **O**xide **S**ynthase*)

L-NAME: hidrocloreto de Nω-Nitro-L-arginina metil ester

LPS: lipopolissacarídeo

MEM: meio essencial mínimo (***M**inimal **E**ssential **M**edium*)

MHC: complexo de histocompatibilidade principal (***M**ajor **H**istocompatibility **C**omplex*)

MSC: célula tronco mesenquimal (***M**esenchymal **S**tem **C**ell*)

NFκB: fator nuclear kappa B (***N**uclear **F**actor **k**appa **B***)

NMDA: **N**-**M**etil-**D**-**A**spartato

NO: óxido nítrico (***N**itric **O**xide*)

PGE2: prostaglandina E 2

PI: iodeto de propídio (***P**ropidium **i**odide*)

POG: privação de oxigênio e glicose

RNS: espécies reativas de nitrogênio (***R**eactive **N**itrogen **S**pecies*)

ROS: espécies reativas de oxigênio (***R**eactive **O**xygen **S**pecies*)

SNC: sistema nervoso central

SOD: superóxido dismutase

TGFβ: fator de crescimento tumoral beta (***T**umor **G**rowth **F**actor beta*)

TH1: linfócito T auxiliar tipo 1 (***T h**elper **1***)

TNFα: fator de necrose tumoral alfa (***T**umor **N**ecrosis **F**actor alpha*)

TROLOX: ácido 6-hidroxi-2,5,7,8-tetrametilcroman-2-carboxílico

INTRODUÇÃO

As doenças neurodegenerativas

As doenças neurodegenerativas afetam diretamente as características que fazem a vida dos seres humanos tão especial, como a memória, a fala, a personalidade e os movimentos especializados. Dentre as doenças do Sistema Nervoso Central (SNC), podemos destacar, dentre outras, as desordens cerebrovasculares, a epilepsia, a doença de Alzheimer, a doença de Parkinson e a esclerose múltipla. Em conjunto, essas doenças afetam um grande segmento da população e, na maioria dos casos, levam à incapacidade física e/ou mental (Price, 1999). Para cada uma dessas doenças, na maioria das vezes, apenas tratamentos sintomáticos estão disponíveis. No caso da isquemia cerebral, por exemplo, apenas a terapia trombolítica está aprovada e os critérios de exclusão dos pacientes são tantos que poucos podem receber esse tratamento (Chopp e Li, 2002; Gilman 2006).

Cada uma das doenças neurodegenerativas tem suas próprias características, afetando diferentes populações de neurônios em diferentes locais do SNC. Um ponto em comum nessas doenças parece ser a presença do componente inflamatório, que na maioria das vezes contribui para acelerar a degeneração (Floden et al., 2005; Mosley et al., 2006; Sriran e O'Callaghan, 2007; Wang et al., 2007a).

Muitas substâncias neuroprotetoras já foram caracterizadas e mostraram-se eficazes em pesquisas pré-clínicas, não apresentando infelizmente o mesmo

efeito quando utilizadas em pacientes (De Keyser et al., 1999; Ginsberg 2008). Novas estratégias terapêuticas precisam ser desenvolvidas e, nesse contexto, a terapia celular surge como uma esperança no tratamento das doenças que acometem o SNC (Kan et al., 2007, Dharmasaroja, 2009).

A isquemia cerebral

A isquemia cerebral, que pode ser a causa do popularmente conhecido derrame cerebral ou acidente vascular encefálico (AVE), é uma das principais causas de morbidade e mortalidade entre adultos e idosos. A doença está intimamente ligada a uma variedade de fatores de risco como hipertensão, hipercolesterolemia e diabete (Price, 1999). Dados do Ministério da Saúde mostram que em 2005 as doenças cerebrovasculares foram a maior causa de morte no Brasil, com um número de óbitos ultrapassando os 90.000, correspondendo a 10% da mortalidade no país. Outro dado importante é que as doenças circulatórias (aqui incluídas a isquemia cerebral e a isquemia cardíaca) são a principal causa de morte em todas as regiões brasileiras, tanto para homens quanto para mulheres acima dos 40 anos (Ministério da Saúde, 2008). São números altos que justificam o estudo de estratégias para reversão desse quadro.

O tipo mais freqüente de isquemia cerebral em humanos é a isquemia focal, definida como a interrupção do fluxo sanguíneo para uma parte do cérebro, afetando apenas as regiões circundantes à área não irrigada, em geral a artéria cerebral média (ACM). O segundo tipo é a isquemia global, que resulta da interrupção transitória do fluxo sangüíneo para todo o cérebro, o que ocorre

durante uma parada cardíaca, por exemplo. Além dos danos causados pela falta de oxigênio e metabólitos durante a isquemia, a volta da circulação sangüínea pode aumentar a morte neuronal, especificamente nas áreas mais vulneráveis do cérebro, como a região CA1 do hipocampo (Schmidt-Kastner e Freund, 1991). Estratégias de proteção das células das áreas afetadas ou de resgate das populações de células que estão morrendo tornam-se necessárias na tentativa de diminuir a morbidade e as seqüelas dos pacientes vítimas de isquemia cerebral. Como citado anteriormente, até o momento não existe um protocolo efetivo que melhore as condições de vida desses pacientes, instigando-nos ainda mais na busca de novas alternativas terapêuticas.

O mecanismo da excitotoxicidade

Estudos experimentais identificaram quatro processos dominantes que, sozinhos ou combinados, levam ao dano neuronal pós-isquêmico: aumento de cálcio intracelular, neurotoxicidade mediada por receptores glutamatérgicos, formação de radicais livres e inflamação (Dirnagl *et al.*, 1999; White et al, 2000).

Os eventos relacionados ao mecanismo da excitotoxicidade estão exemplificados na figura 1, onde os itens circulados em azul merecem atenção especial por terem sido diretamente estudados nesse trabalho. Cabe lembrar aqui que a excitotoxicidade não é uma característica exclusiva da morte induzida pela isquemia, podendo ser disparada por outros estímulos, como veremos adiante.

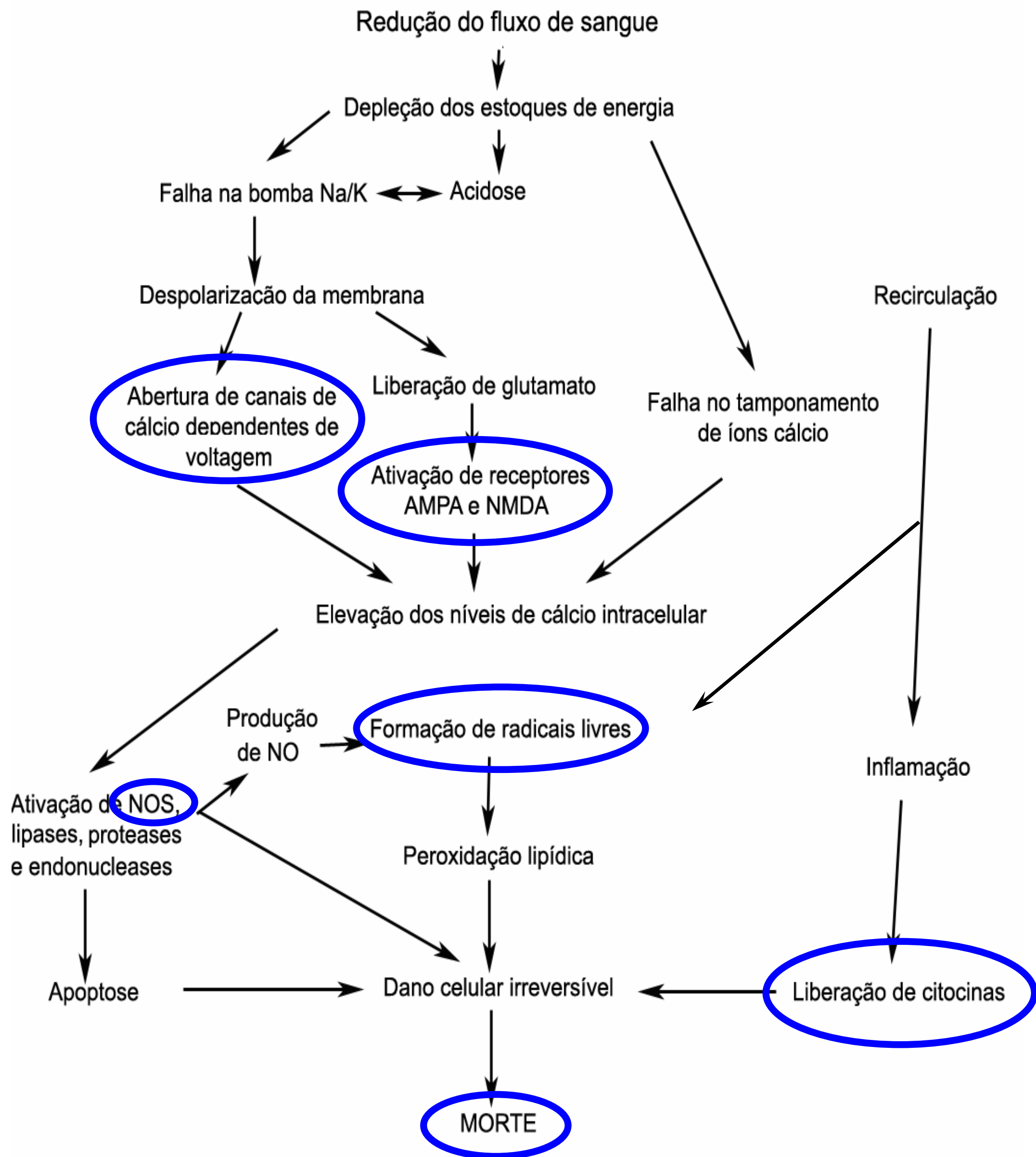

Figura 1: Cascata neurotóxica na isquemia e na recirculação. Alguns eventos principais são: despolarização descontrolada, excitação por glutamato, aumento de cálcio intracelular, geração de radicais livres, ativação de enzimas como proteases e lipases e indução de inflamação. Adaptada de De Keyser *et al.*, 1999.

A cultura organotípica

As técnicas *in vitro* são ferramentas importantes para o estudo de diversas doenças neurodegenerativas, uma vez que são um modelo mais simples e controlado para os estudos moleculares e celulares. As culturas organotípicas tornaram-se uma importante alternativa para a investigação da morte neuronal induzida por excitotoxinas (Abdel-Hamid e Tymianski, 1997), privação de oxigênio e glicose (Valentim et al., 2003; Horn et al., 2005), toxicidade induzida pelo peptídeo β-amilóide (Nassif et al., 2007; Frozza et al., 2009), dentre outras aplicações. Além disso, essas culturas vêm sendo utilizadas em nosso grupo para o estudo de compostos com possível atividade neuroprotetora frente a lesões (Cimarosti et al., 2005; Zamin et al., 2006; Simão et al., *in press*).

Esse modelo de culturas foi desenvolvido em 1981 por Gähwiller e modificado por Stoppini e colaboradores em 1991. Basicamente, trata-se de um método que mantém fatias de um determinado tecido em cultivo, sobre uma interface entre o ar e o meio de cultivo, podendo assim permanecer por diversas semanas. Uma das principais características da cultura organotípica é a de manter a organização do tecido como ela ocorre *in vivo*. Isso significa que todos os tipos celulares estão presentes e organizados da mesma forma que estariam *in vivo*, sendo essa uma grande vantagem dessa cultura sobre a cultura primária de tipos celulares específicos (Stoppini *et al.*, 1991; Gähwiler *et al.*, 1997).

Para a simulação de uma "isquemia *in vitro*" a cultura organotípica de hipocampo pode ser submetida a um modelo experimental que simula as condições de isquemia cerebral *in vivo,* o qual chamamos de privação de oxigênio

e glicose (POG). Esse método consiste em expor as culturas a um meio sem glicose e a uma atmosfera saturada de nitrogênio. Essas condições simulam a falta de fluxo sangüíneo durante a isquemia, sendo uma ótima alternativa para o estudo da isquemia cerebral e para uma triagem de potenciais agentes terapêuticos (Sundstrom et al., 2005), uma vez que essas culturas mantêm muitos aspectos da isquemia *in vivo*, como a morte neuronal tardia e a vulnerabilidade seletiva (Strasser e Fischer, 1995; Noraberg et al., 1999; Noraberg et al., 2005).

Ativação glial e Neuroinflamação

Já foi demonstrado por vários autores que a inflamação contribui para a patogênese de doenças neurodegenerativas como doença de Alzheimer, doença de Parkinson e esclerose múltipla. Além disso, ela participa na degeneração neuronal observada após uma isquemia ou um trauma cerebral (Lucas et al, 2006; Brown, 2007), somando-se aos efeitos deletérios já presentes nessas patologias. Drogas com propriedades antiinflamatórias, imunossupressoras e imunomoduladoras já vem sendo utilizadas na clínica com relativo sucesso para doenças como esclerose múltipla e adrenoleucodistrofias (Zipp e Aktas, 2006).

A inflamação, porém, possui duas faces. Pode ser benéfica em algumas situações, onde as citocinas e quimiocinas secretadas possuem propriedades neuroprotetoras, mas também pode ser muito prejudicial em outras, onde essas mesmas moléculas induzem a morte das células. Os efeitos que as moléculas inflamatórias terão sobre o tecido nervoso dependerão da intensidade do estímulo, das suas características e dos diferentes níveis e estados de ativação dos

receptores dessas moléculas em cada um dos tipos celulares afetados (Sriram e O'Callaghan, 2007).

É importante salientar que a resposta imunológica dentro do encéfalo é limitada e peculiar, condição essa que o levou a ser descrito como "órgão imunologicamente privilegiado", um privilégio garantido pela permeabilidade seletiva da barreira hemato-encefálica. Sabe-se que essa é uma verdade parcial, uma vez que em determinadas situações pode-se observar a infiltração de outras células, principalmente linfócitos (Engelhardt e Ransohoff, 2005). Na imensa maioria dos casos, porém, apenas a microglia e os astrócitos são ativados, sendo esses dois tipos celulares, em especial o primeiro, os responsáveis pela resposta imunológica no SNC (Minghetti e Levi, 1998; Liberto et al., 2004; Rock et al., 2004; Sriram e O'Callaghan, 2007). A figura 2 exemplifica como a ativação da microglia e dos astrócitos pode atuar na morte neuronal em resposta a um estímulo que a dispare (adaptada de Block et al., 2007).

A microglia

As células microgliais são de origem mesodérmica e são consideradas os macrófagos que residem no SNC. Estão localizadas no parênquima tecidual, próximas aos vasos, em todas as regiões do SNC, correspondendo a 10-20% das células gliais. Podem ser encontradas no cérebro adulto na forma ramificada, quando estão no estado não ativado ou quiescente, ou na forma amebóide, quando em resposta a algum estímulo adquirem a forma arredondada e passam a secretar uma gama de fatores relacionados com a resposta imunológica. Devido a sua plasticidade e reatividade a um amplo espectro de estímulos, parecem possuir

um papel importante na defesa do SNC, na neuroproteção e no reparo do tecido pós lesões, uma vez que podem migrar para os locais de dano tecidual (Heppner et al, 1998; Minghetti e Levi, 1998; Rock et al, 2004). Essas células podem ser facilmente reconhecidas no tecido pela marcação com anticorpos específicos como anti-CD11b (OX42) (Strassburger et al., 2008) e anti-lipocortina-1 (LC1) (Skibo et al., 2000) ou por sua reatividade com a lectina isolada de *Griffonia simplicifolia* Isolectina IB_4, também conhecida como GSA, que marca resíduos de galactose presentes apenas na membrana dessas células e que é amplamente utilizada na literatura para identificação da microglia (Streit e Kreutzberg, 1987; Heppner et al., 1998; Hailer et al., 2005; Buffo et al., 2008).

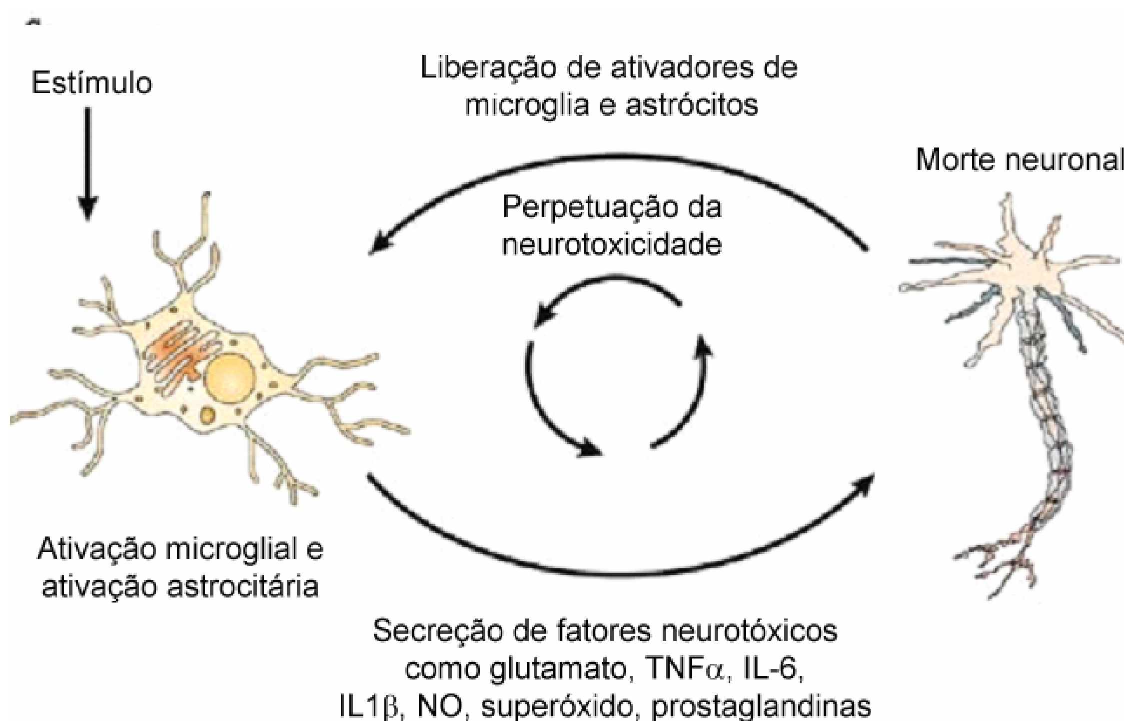

Figura 2: Participação da ativação glial na morte neuronal. Adaptada de Block et al., 2007.

Dentre as substâncias secretadas pela microglia ativada podemos citar o glutamato, importante na excitotoxicidade (Takeuchi et al., 2006), fatores de crescimento como NGF e TGFβ, citocinas como interleucina 1β (IL-1β), interleucina 6 (IL-6) e TNFα, espécies reativas como óxido nítrico e ânions superóxido (Koutsilieri et al., 2002), além de quimiocinas, mediadores lipídicos, fatores de coagulação e componentes de matriz extracelular. Além disso, na forma ativada aumentam a produção de COX-2, participando ativamente na síntese de prostanóides como prostaglandinas, leucotrienos e tromboxamos, importantes mediadores da resposta inflamatória (Minghetti e Levi, 1998; Rock et al, 2004; Sriram e O' Callaghan, 2007). O potencial protetor *versus* destrutivo dessas células é ditado pelo tipo de estímulo, sua intensidade e sua duração. Os fatores secretados podem ser tóxicos ou protetores dependendo da sua concentração e da disponibilidade de receptores para ligarem.

Os astrócitos

São as células mais abundantes no SNC. De origem neural, estão associados à manutenção da homeostase no SNC adulto. Entre as suas principais funções estão a participação na estrutura da barreira hemato-encefálica, a produção de fatores tróficos, o metabolismo de neurotransmissores e a garantia de suporte energético para os neurônios (He e Sun, 2007; Seth e Koul, 2008). Expressam numerosos receptores, o que os capacita a responder a praticamente todos os compostos neuroativos, como neurotransmissores, neuropeptídeos, fatores de crescimento, citocinas e toxinas (Liberto et al., 2004). Atualmente seu

papel na geração de novos neurônios está sendo estudado, sugerindo que essas células, além de todas as outras funções já descritas, participem na neurogênese (Buffo et al., 2008). Além disso, já foi constatado o envolvimento dos astrócitos em doenças neurodegenerativas como doença de Alzheimer, doença de Huntington, esclerose múltipla, isquemia, demência associada à infecção pelo vírus da AIDS, dentre outras (Seth e Koul, 2008).

Em situações de isquemia, traumas ou patologias diversas os astrócitos são ativados e ocorre um fenômeno chamado de astrogliose reativa. Esse processo é caracterizado por hipertrofia, proliferação celular, extensão dos processos celulares, aumento na produção das proteínas GFAP, vimentina e nestina (Liberto et al., 2004; Sofroniew, 2005; Buffo et al., 2008) e secreção de citocinas como IL-6 (Gao et al., 2008). A gliose reativa resulta na formação da cicatriz glial, que se acredita ser responsável pela inibição do crescimento dos neuritos, dificultando a regeneração no SNC após lesões. Além disso, essa cicatriz inibe a comunicação entre os processos neuronais já existentes (Silver e Miller, 2004).

Acompanhando as mudanças morfológicas acima há uma série de mudanças fisiológicas associadas à gliose reativa. Assim como a microglia, os astrócitos passam a secretar várias citocinas quando ativados em resposta a estímulos, dentre elas TNFα, IL-6 e IL-1β, além de passar a produzir e liberar prostanóides e óxido nítrico, o que pode agravar o dano causado por uma lesão (Liberto et al., 2004). Cabe salientar que, apesar da microglia produzir quantidades maiores dessas moléculas que os astrócitos, a contribuição deles no agravamento das lesões não pode ser considerada desprezível.

O estresse oxidativo

É definido como um desequilíbrio entre os processos bioquímicos que levam à geração de espécies reativas de oxigênio (ROS) e de nitrogênio (RNS) e os processos responsáveis pela sua remoção. O SNC é particularmente sensível ao insulto oxidativo, uma vez que utiliza grandes quantidades de O_2, possui concentrações baixas de antioxidantes e enzimas relacionadas, possui um grande conteúdo de lipídios poliinsaturados e um alto conteúdo de metais de transição, muito importantes na catálise das reações geradoras de espécies reativas (Sayre et al., 2008).

O estresse oxidativo, assim como a inflamação, pode possuir um papel benéfico ou deletério, dependendo da sua intensidade (Valko et al., 2007). Ele está descrito como participando em várias doenças neurodegenerativas e como sendo crucial na neurotoxicidade e morte celular nessas patologias (Bal-Price e Brown, 2001; Koutsilieri et al., 2002; Block et al., 2007; Valko et al., 2007; Sayre et al., 2008). Como já citado anteriormente, tanto a microglia quanto os astrócitos são importantes fontes de espécies reativas como NO e superóxido, participando ativamente no dano oxidativo (Block et al., 2007). Além disso, citocinas como TNFα e IL-6 aumentam a produção de ROS na mitocôndria das células, alimentando o sistema positivamente (Chapple, 1997; Koutsilieri et al., 2002).

A figura 3 mostra de uma maneira simples algumas das conseqüências do estresse oxidativo nas células, que geralmente culminam na morte celular, principalmente dos neurônios. Novamente os círculos em azul merecem especial atenção por terem sido alvo deste trabalho.

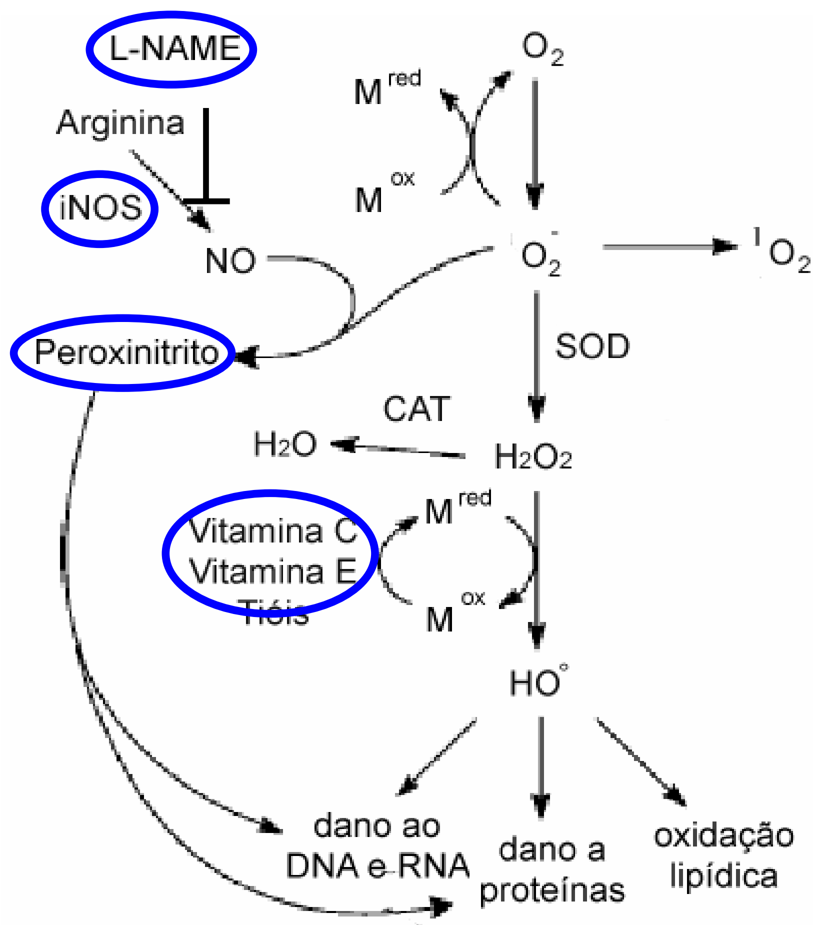

Figura 3: Estresse oxidativo e suas conseqüências. A figura mostra de maneira simplificada as principais enzimas e produtos gerados durante a cascata oxidativa, culminando com dano ao DNA/RNA, dano a proteínas e peroxidação lipídica, muitas vezes letais às células. Adaptada de Sayre et al., 2008. SOD: superóxido dismutase, CAT: catalase, M^{red}: metal reduzido, M^{ox}: metal oxidado, iNOS: óxido nítrico sintase induzível.

As células tronco mesenquimais

As células tronco podem ser definidas como células com capacidade de auto renovação e capazes de originar outras células. São classificadas em embrionárias, localizadas na massa interna do blastocisto, e adultas ou somáticas, encontradas nos tecidos já desenvolvidos desde o feto até o organismo adulto (Krabbe et al., 2005). Ambas são muito estudadas atualmente e nesse trabalho focaremos apenas nas células tronco adultas, especificamente nas mesenquimais (MSC), aqui obtidas da medula óssea e do pulmão de ratos e camundongos.

Dentre as células tronco adultas encontramos as MSC, que estão ganhando atenção devido ao seu possível uso para terapias celulares. São definidas como células multipotentes, capazes de originar tecidos de origem mesodérmica como o cartilaginoso, o ósseo, o adiposo e o muscular (Baksh et al., 2004). As principais vantagens da utilização dessas células sobre as células embrionárias são a fácil obtenção e manutenção, a possibilidade de transplante autólogo (sem a rejeição imunológica decorrente de transplantes), a baixa imunogenicidade e a inexistência de conflitos éticos. Elas podem ser facilmente obtidas de várias fontes como medula óssea, tecido adiposo e cordão umbilical. Acredita-se que estejam presentes em todos os órgãos e tecidos do organismo (da Silva Meirelles et al., 2006) e estejam situadas no corpo como pericitos, com a função de estabilizar os vasos sanguíneos e contribuir para a homeostase dos tecidos (da Silva Meirelles et al., 2008). O método de obtenção dessas células é bastante simples e baseia-se na adesão ao plástico, podendo ser facilmente mantidas e expandidas em cultura (da Silva Meirelles, 2003; Baksh et al., 2004; Jori et al., 2005).

As MSC são capazes de secretar fatores como GDNF (fator neurotrófico derivado de linhagem glial), BDNF (fator neurotrófico derivado do cérebro), NGF (fator de crescimento de nervos), VEGF (fator de crescimento de endotélio vascular) e HGF (fator de crescimento de hepatócitos), além de outros (Chen et al., 2002; Pan et al., 2007). A gama de fatores secretados classifica as MSC como "fábricas tróficas", uma esperança para o tratamento de várias patologias.

Além da produção de fatores tróficos, as MSC também mostraram-se capazes de reduzir a resposta inflamatória (Uccelli et al., 2006; Chamberlain et al., 2007), atuando principalmente na modulação da função dos linfócitos T (Bartholomew et al. 2002), inibindo a proliferação de linfócitos B (Corcione et al., 2006) e dificultando a maturação e funcionamento das células dendríticas (Jiang et al., 2005). Além disso, sabe-se que são pouco imunogênicas, uma vez que expressam baixos níveis de MHC classe I e não possuem MHC classe II, não expressando também as moléculas de superfície CD40, CD80 e CD86, o que permite seu transplante em tecidos alogênicos com um risco menor de rejeição (Uccelli et al., 2006). Ainda nas propriedades de influenciar a resposta inflamatória foi demonstrado que as MSC podem secretar citocinas como IFN-γ, TNFα, TGFβ, IL-4 e IL-6 (Karnoub et al., 2007).

Transdiferenciação das células tronco mesenquimais em neurônios

O termo transdiferenciação é comumente utilizado para descrever a habilidade das células tronco adultas em originarem células de tecidos onde elas não residem ou células de outros folhetos embrionários (Krabbe et al., 2005). É o

caso, por exemplo, da geração de neurônios e células gliais a partir de MSC, já descrito por vários autores (Sanchez-Ramos et al., 2000; Woodbury et al., 2000; Abouelfetouh et al., 2004; Suzuki et al., 2004; Bossolasco et al., 2005; Jori et al., 2005; Rivera et al., 2006; Lei et al., 2007). A figura 4 mostra alguns dos tipos celulares já gerados a partir das MSC, onde a interrogação em azul questiona se realmente a transdiferenciação das MSC em neurônios e células gliais existe.

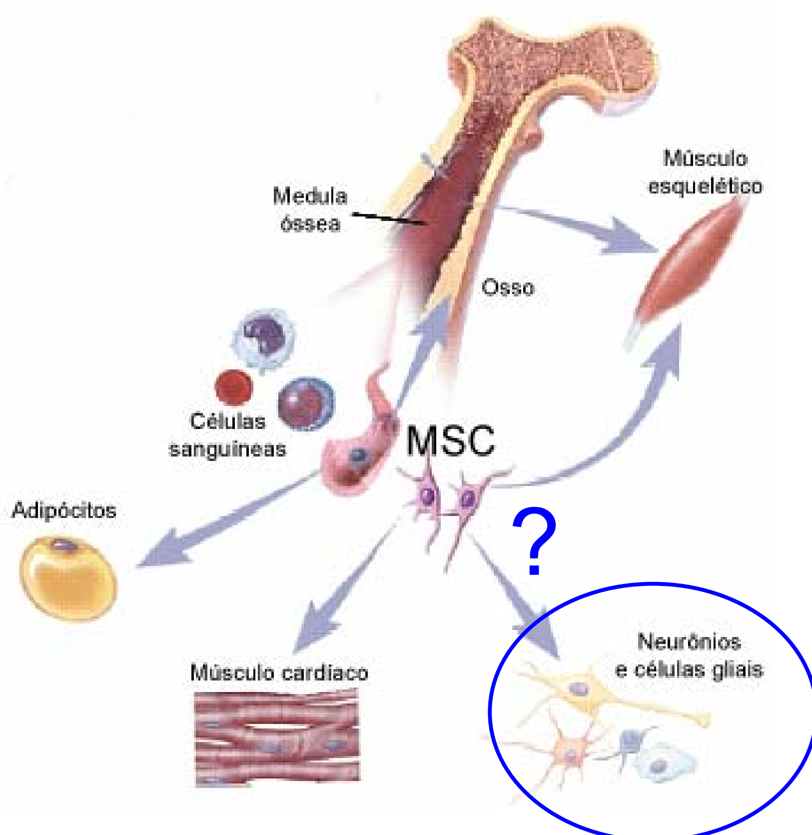

Figura 4: Tecidos originados a partir das células tronco mesenquimais. Por sua origem mesodérmica sabe-se que são capazes de gerar tecido adiposo, cartilaginoso, ósseo e muscular. A transdiferenciação em células neuronais e gliais é muito questionada e acredita-se ser apenas um artefato dos protocolos utilizados. Adaptada do livro “*Stem Cells: Scientific Progress and Future Research Directions*”, publicado e fornecido pelo National Institute of Health, EUA.

Os protocolos utilizados para a diferenciação *in vitro* das MSC em células da linhagem neural incluem a utilização de compostos como β-mercaptoetanol (Lei et al., 2007), DMSO (Suzuki et al., 2004), ácido retinóico (Abouelfetouh et al., 2004) e fatores de crescimento como FGF e EGF (Bossolasco et al., 2005). A formação de neuroesferas já foi evidenciada, sendo essa capaz de originar tanto neurônios quanto astrócitos e oligodendrócitos, visualizados utilizando-se imunoistoquímica para marcadores específicos (Suzuki et al., 2004). Também *in vivo* vários autores já observaram que as MSC são capazes de expressar marcadores neurais após serem transplantadas em áreas cerebrais lesionadas (Azizi et al., 1998; Chen et al., 2001; Zhao et al., 2002; Lee et al., 2003; Deng et al., 2006).

Há muita discussão em torno da real transdiferenciação das MSC em neurônios e células gliais (Krabbe et al., 2005). Muitos autores acreditam que as MSC não são capazes de diferenciar em neurônios (Castro et al., 2002) e que os marcadores neurais observados, principalmente após a utilização de substâncias agressivas como β-mercaptoetanol e DMSO, sejam apenas o resultado de um grande estresse celular e, portanto, artefato da técnica (Lu et al., 2004; Neuhuber et al., 2004; Lu e Tymanski, 2005). Essa crítica ocorre tanto para os experimentos *in vitro* quanto para os *in vivo*, onde já foi mostrado que o análogo da timidina BrdU, utilizado para marcação das MSC a serem transplantadas, é capaz de ser incorporado pelas células em divisão no cérebro, levando a interpretações completamente equivocadas quando observada a sua co-localização com proteínas marcadoras neurais (Burns et al., 2006; Coyne et al., 2006).

A terapia celular como tratamento da isquemia cerebral

Como já citado anteriormente, a terapia celular mostra-se uma estratégia promissora para o tratamento de doenças neurodegenerativas. O uso de MSC isoladas ou da fração mononuclear de medula óssea (que além de MSC contém células tronco hematopoiéticas, macrófagos e outras células nucleadas) em outras patologias como a isquemia cardíaca, por exemplo, tem tido relativo sucesso, mostrando-se uma importante perspectiva no seu tratamento (Perin et al., 2004; Nagaya et al., 2005; Schuleri et al., 2007). Para as doenças neurodegenerativas muitos protocolos pré-clínicos já mostraram benefício da terapia celular em modelos animais de isquemia (Chen et al., 2001; Chopp e Li, 2002; Zhao et al., 2002; Borlongan et al., 2004; Mendez-Otero et al., 2007; Guzman et al., 2008; Dharmasaroja, 2009), doença de Parkinson (Wang et al., 2007b), lesão de medula espinhal (Lee et al., 2003), dentre outras.

Para a isquemia cerebral há apenas um estudo clínico publicado em 2005 mostrando a melhora dos pacientes que receberam MSC autólogas após isquemia focal (Bang et al., 2005). Vários experimentos clínicos estão sendo realizados pelo mundo e no Brasil há um protocolo clínico Fase I em andamento para avaliação da segurança e exeqüibilidade de um possível tratamento utilizando as células da fração mononuclear da medula óssea (de Mendonça et al., 2006). O critério de seleção para inclusão nesses protocolos restringe-se a pacientes que sofreram oclusão da artéria cerebral média (isquemia focal aguda). Porém, até o momento, não há na literatura nenhum estudo mostrando algum benefício da terapia celular após o modelo de isquemia cerebral global.

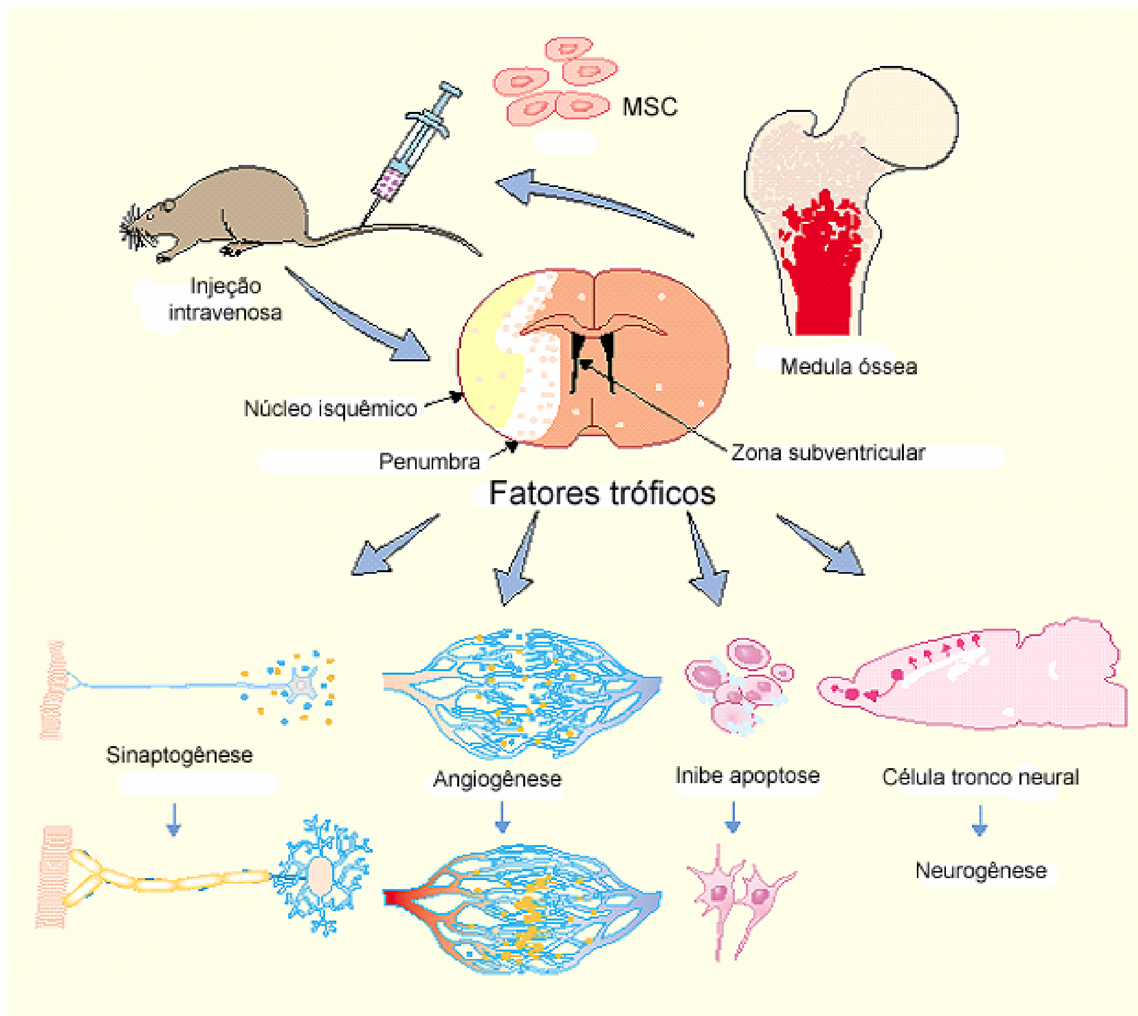

Figura 5: A terapia celular e seus possíveis benefícios após a isquemia. Acredita-se que as MSC possam migrar até o local da lesão e secretar fatores tróficos responsáveis pelo aumento da sinaptogênese, aumento da angiogênese e aumento da neurogênese no tecido danificado. Adaptada de Chopp e Li, 2002.

A melhora observada nos estudos pré-clínicos e clínicos baseia-se em testes neurológicos e comportamentais, sendo o mecanismo de ação das células ainda desconhecido. Acredita-se que o percentual de células tronco capazes de diferenciar em neurônios seja muito pequeno ou até inexistente, sendo a melhora

observada nos estudos pré-clínicos e clínicos atribuída aos fatores tróficos que essas células secretam, capazes de estimular mecanismos endógenos de reparo (Chopp e Li, 2002; Zhao et al., 2002; Kan et al., 2007; Guzman et al., 2008). A figura 5 mostra um esquema do possível mecanismo pelo qual as MSC possam exercer a melhora após a isquemia (Chopp e Li, 2002).

Questões como quantas células injetar, qual a melhor via de administração, até quanto tempo após a isquemia é possível iniciar o tratamento, qual o melhor tipo de célula a ser utilizada e quais as características do paciente a receber a terapia ainda precisam ser discutidas e padronizadas (Guzman et al., 2008; Dharmasaroja, 2009).

Levando em consideração que a terapia celular é uma estratégia promissora no tratamento de pacientes que sofreram uma isquemia cerebral e que já está sendo utilizada em testes clínicos, embora seu mecanismo de ação e possíveis efeitos adversos ainda não estejam totalmente elucidados, o objetivo principal desse trabalho foi investigar, em um modelo *in vitro,* como os fatores secretados pelas MSC podem influenciar o tecido nervoso com ou sem uma lesão e como os fatores secretados pelo tecido nervoso podem influenciar as MSC.

OBJETIVOS

Objetivo geral

Investigar como o meio condicionado pelas células tronco mesenquimais de medula óssea de ratos age sobre o tecido nervoso na presença ou ausência de uma lesão e como o meio condicionado pelo tecido nervoso age sobre as células tronco mesenquimais.

Objetivos específicos

- ✔ Expor culturas organotípicas de hipocampo de ratos ao meio condicionado por células tronco mesenquimais de ratos para investigação do potencial efeito neuroprotetor desse meio frente à lesão por privação de oxigênio e glicose;
- ✔ Investigar o efeito da exposição de culturas organotípicas de hipocampo ao meio condicionado por células tronco mesenquimais, estudando parâmetros de excitoxicidade, estresse oxidativo e indução de resposta inflamatória;
- ✔ Verificar se os fatores secretados pelo tecido nervoso com ou sem lesão são capazes de induzir mudanças significativas nas células tronco mesenquimais, avaliando para isso parâmetros de morte celular, proliferação e possível transdiferenciação dessas células em células neurais.

CAPÍTULO 1

CONDITIONED MEDIUM FROM MESENCHYMAL STEM CELLS INDUCES CELL DEATH IN ORGANOTYPIC CULTURES OF RAT HIPPOCAMPUS AND AGGRAVATES LESION IN A MODEL OF OXYGEN AND GLUCOSE DEPRIVATION

Ana Paula Horn, Rudimar Luiz Frozza, Patrícia Bencke Grudzinski, Daniéli Gerhardt, Juliana Bender Hoppe, Alessandra Nejar Bruno, Pedro Chagastelles, Nance Beyer Nardi, Guido Lenz e Christianne Salbego

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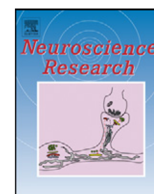

Conditioned medium from mesenchymal stem cells induces cell death in organotypic cultures of rat hippocampus and aggravates lesion in a model of oxygen and glucose deprivation

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ABSTRACT

Cell therapy using bone marrow-derived mesenchymal stem cells (MSC) seems to be a new alternative for the treatment of neurological diseases, including stroke. In order to investigate the response of hippocampal tissue to factors secreted by MSC and if these factors are neuroprotective in a model of oxygen and glucose deprivation (OGD), we used organotypic hippocampal cultures exposed to conditioned medium from bone marrow-derived MSC. Our results suggest that the conditioned medium obtained from these cells aggravates lesion caused by OGD. In addition, the presence of the conditioned medium alone was toxic mainly to cells in the CA1, CA2 and CA3 areas of the hippocampal organotypic culture even in basal conditions. GABA stimulation and NMDA and AMPA receptors antagonists were able to reduce propidium iodide staining, suggesting that the cell death induced by the toxic factors secreted by MSC could involve these receptors.
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1. Introduction

Neurological diseases affect the qualities that make the lives of humans so special, including memory, cognition, language, personality and skill movements (Price, 1999). Among these diseases we find stroke, the third cause of mortality and the leading cause of morbidity in the majority of industrialized countries (Dirnagl et al., 1999). While accumulating scientific evidence has increased our understanding of the biological and molecular events of stroke pathology, only intravenous tissue-plasminogen activator treatment within 3 h of the ischemic stroke onset has shown some efficacy in the clinic (Mohammad et al., 2004; Gilman, 2006). Currently, new approaches are under investigation, including clot disruption, neuroprotective treatments and cell therapy, being the latter the most promising of them.

Several in vivo and in vitro models have been used to study the mechanism underlying neuronal degeneration and to evaluate the potential neuroprotective effect of pharmacological treatments (Noraberg et al., 2005). Among the in vitro models used to study neurodegeneration and ischemia-induced injuries, organotypic hippocampal slice cultures, combined with oxygen and glucose deprivation (OGD), offer great advantages because they mimic closely the majority of the conditions found in vivo (Valentim et al., 2003; Horn et al., 2005; Zamin et al., 2006). Cultured slices maintain their cell architecture and interneuronal connections with a physiological maturation of its neurons, allowing an extended survival study (Gähwiler et al., 1997; Tavares et al., 2001).

Cell therapy with stem cells brings new hope for the long-term care of patients that suffered ischemic stroke, mainly due to the self-renewal and differentiation potentials of these cells (Savitz et al., 2004). Animal studies show that bone marrow stromal cells injection after focal cerebral ischemia in rats (MCA occlusion) improve their functional deficit with very rare differentiation of the stem cells (Chen et al., 2001; Chopp and Li, 2002; Zhao et al., 2002). These experimental studies suggest

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that stem cells would support cerebral plasticity via growth factor production and stimulation of endogenous mechanisms of local repair. Mesenchymal stem cells (MSC) have been shown to secrete trophic factors, including glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), vascular endothelial growth factors (VEGF) and hepatocyte growth factor (HGF) (Chen et al., 2002; Pan et al., 2007). Assessment of effectiveness and safety in the use of stem cells in cerebral ischemia still require thorough investigation before clinical use can be approved. Bang et al. (2005) showed for the first time that MSC improved the outcome of patients 1 year after they suffered a cerebral infarct within the middle cerebral artery territory and received autologous MSC therapy (Bang et al., 2005). Despite these positive results, little is known about the basic biology of the stem cells, their interaction with the nervous system and their potential side effects.

The aim of this study was to investigate whether the factors secreted by cultured MSC were neuroprotective in an in vitro model of cerebral ischemia. Surprisingly, when in contact with the hippocampal tissue, the conditioned medium from MSC was cytotoxic and increased cell death caused by oxygen and glucose deprivation.

2. Materials and methods

2.1. Materials

Propidium iodide (PI) and Hepes were obtained from Sigma (St. Louis, MO, USA). Millicell culture inserts were obtained from Millipore (Millicell®-CM, 0.4 μm, Millipore®, Bedford, MA, USA) and 6-well culture plates were from TPP (tissue culture test plates TPP®, Switzerland). Culture medium, HBSS, fungizone, Trypsin/EDTA solution, horse heat inactivated serum and fetal bovine serum (FBS) were obtained from GIBCO (Grand Island, NY, USA). Gentamicin was from Schering-Plough (Rio de Janeiro, Brazil). The cell lines U87, HEK293 and H647 were obtained from ATCC (American Type Culture Collection, USA). The NMDA glutamate receptor antagonist (+)-MK801 hydrogen maleate was obtained from RBI (Natick, MA, USA). CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) was from Tocris Bioscience (Elliswille, USA). Nimodipine, GABA (γ-aminobutyric acid) and bicuculline were obtained from Sigma (St. Louis, MO, USA).

2.2. Organotypic hippocampal slice cultures

All animal use procedures were approved by the local Animal Care Committee and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Organotypic hippocampal slice cultures were prepared according to the method of Stoppini et al. (1991) with some modifications (Valentim et al., 2003; Horn et al., 2005). Briefly, 400 μm thick hippocampal slices were prepared from 6–8-day-old male *Wistar* rats using a McIlwain tissue chopper and separated in ice-cold Hank's balanced salt solution (HBSS), pH 7.2. The slices were placed on Millicell culture membranes and the inserts were transferred to a 6-well culture plate. Each well contained 1 mL of tissue culture medium consisting of 50% minimum essential medium (MEM), 25% HBSS, 25% heat inactivated horse serum supplemented with (mM, final concentration): glucose 36, HEPES 25 and $NaHCO_3$ 4; fungizone 1% and gentamicin 0.1 mg/mL, pH 7.3. Organotypic cultures were maintained in a humidified incubator gasified with a 5% CO_2 atmosphere at 37 °C for 14 days. Culture medium was changed twice a week.

2.3. Organotypic cortical slice cultures

Organotypic cortical slice cultures were also prepared according to the method of Stoppini et al. (1991) with some modifications. Briefly, coronal 300 μm thick anterior cortical slices were prepared from 6-day-old male *Wistar* rats using a McIlwain tissue chopper and separated in ice-cold Hank's balanced salt solution (HBSS), pH 7.2. The slices were placed on Millicell culture membranes and the inserts were transferred to a 6-well culture plate, each containing two slices. Each well contained 1 mL of tissue culture medium consisting of 50% minimum essential medium (MEM), 25% HBSS, 25% heat inactivated horse serum supplemented with (mM, final concentration): glucose 36, HEPES 25 and $NaHCO_3$ 4; fungizone 1% and gentamicin 0.1 mg/mL, pH 7.3. Organotypic cultures were maintained in a humidified incubator gasified with a 5% CO_2 atmosphere at 37 °C for 10 days. Culture medium was changed every 2 days.

2.4. Mesenchymal stem cell culture

Mesenchymal stem cell cultures were prepared according to the method described by da Silva Meirelles and Nardi (2003) and Nardi and da Silva Meirelles (2006). Briefly, bone marrow and lungs were obtained from adult *Wistar* rats or C57Bl/6 mice. The animals were anesthetized and killed by cervical dislocation and bone marrow was flushed out of tibias and femurs. Cells from lungs were dissociated using collagenase type 1 (0.5 mg/mL in DMEM 10 mM HEPES). After washing by centrifugation at $400 \times g$ for 10 min and counting viable cells with trypan blue, the cells were resuspended in DMEM with 10% FBS and 10 mM HEPES in a final concentration of 5×10^6 viable cells per ml. To initiate the cultures, cells were plated in 6-well tissue culture dishes at 3.5 ml/well (1.94×10^6 cells/cm^2) and kept in a humidified 5% CO_2 incubator at 37 °C for 72 h, when non-adherent cells were removed by changing the medium. Confluent cultures from MSC derived from bone marrow and lungs were incubated with 0.25% trypsin solution containing 0.01% EDTA for detachment and maintained in culture by changing the medium every 3–4 days. For the experiments, cells between the 10th and 25th passages were seeded in 6-well plates in DMEM with 10% FBS and 10 mM HEPES in a density of 30,000 cells/well 2 days before the contact with the organotypic culture. Twenty-four hours before the contact, the medium was changed and conditioned during the next 24 h. The same protocol was used for conditioning the medium with U87 glioma, H647 carcinoma and HEK293 cell lines.

When indicated, conditioned medium was boiled for 10 min at 100 °C or diluted with unconditioned medium. These media stayed in contact with hippocampal cultures during the next 24 h.

2.5. Oxygen and glucose deprivation (OGD)

The induction of OGD was based on the method described by Strasser and Fischer (1995), with some modifications (Valentim et al., 2003). Cultures were carefully rinsed twice with OGD medium composed of $CaCl_2$ 1.26 mM, KCl 5.36 mM, NaCl 136.9 mM, H_2PO_4 0.34 mM, $MgCl_2$ 0.49 mM, $MgSO_4$ 0.44 mM, HEPES 25 mM, pH 7.2. Slices were left in 1 ml of this medium for 15 min, and then changed to the same medium previously bubbled with nitrogen for 30 min. The cultures were transferred to an anaerobic chamber at 37 °C in which the oxygen was replaced by nitrogen, and left in these conditions for 60 min (hippocampal slice cultures) or 90 min (cortical slice cultures). Slices were washed twice with HBSS and placed in a 6-well plate containing rat or mice MSC (bone marrow- or lung-derived) conditioned medium or only in DMEM/10% FBS medium (control slices). After that, they

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recovered for 24 h. It is important to note that only the factors secreted by MSC acted in hippocampal slices, once the organotypic slices were not in direct contact with the cells.

When indicated, MK801 (30 μM), CNQX (100 μM), nimodipine (10 μM), GABA (100 μM) or bicuculline (6 μM) were added simultaneously to the MSC conditioned media. GABA was dissolved in water and all the other drugs had DMSO as a vehicle. In these cases DMSO in a final concentration of 0.1% was used as a control.

2.6. Quantification of cell death

Cellular damage was assessed by fluorescent image analysis of propidium iodide (PI) uptake (Noraberg et al., 1999; Valentim et al., 2003; Horn et al., 2005). PI 5 μM was added 1 h before the end of the recovery period and cultures were observed with an inverted microscope (Nikon Eclipse TE 300) using a standard rhodamine filter. Images were captured and analyzed using Scion Image software (www.scioncorp.com). The area where PI fluorescence was detectable above background levels was determined using the "density slice" option of Scion Image software and compared to the total slice area to obtain the percentage of damage.

2.7. Statistical analysis

Data are expressed as mean ± S.E.M. One-way or two-way analysis of variance (ANOVA) was applied to the means to determine statistical differences between experimental groups. Post hoc comparisons were performed by Tukey's test. Differences between mean values were considered significant when $p < 0.05$.

3. Results

3.1. Effect of MSC conditioned medium in organotypic hippocampal cultures after OGD

Exposure of cultures to the medium conditioned with bone marrow-derived rat MSC caused a marked increase in cell death, as shown by the intense fluorescence due to incorporation of PI in hippocampus after 24 h of contact with this medium (Fig. 1A).

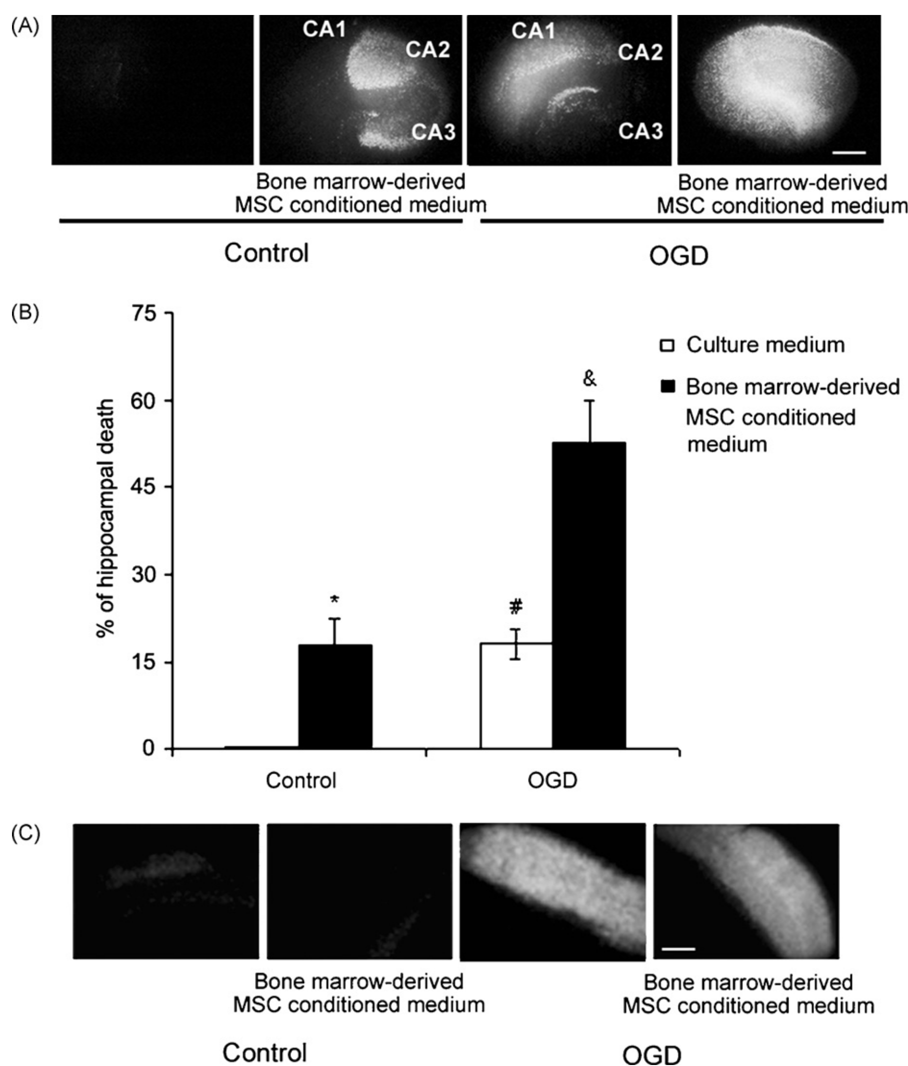

Fig. 1. Medium conditioned with bone marrow-derived rat MSC induces cell death in hippocampal slice cultures and increases OGD-induced damage. (A) Photomicrographs showing the damage in hippocampus after 24 h of exposure to rat bone marrow MSC conditioned medium (magnification: 40×; scale bar: 0.5 mm). (B) Quantification of PI incorporation from pictures showed in (A). (C) Representative pictures showing the damage in cortical slice cultures after OGD conditions and exposure to conditioned medium from rat bone marrow MSC for 24 h. Scale bar: 1 mm. Bars represent the mean ± S.E.M., $n = 9$. *Significantly different from control cultures and from cultures exposed to OGD in the presence of MSC conditioned medium during recovery. #Significantly different from control cultures; &significantly different from all other groups; $p < 0.05$ (two-way ANOVA followed by Tukey's test).

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Table 1
Rat and mice bone marrow- and lung-derived MSC conditioned media aggravate lesion caused by OGD in rat organotypic hippocampal cultures.

	Rat		Mice	
	Control	OGD	Control	OGD
Bone marrow				
Culture medium	02 ± 0.1	18.1 ± 2.5[#]	0.7 ± 0.5	19.2 ± 1.8[#]
MSC conditioned mudium	17.9 ± 4.5[*]	52.7 ± 7.2[&]	30.1 ± 4.2[*]	62.7 ± 3.0[&]
Lung				
Culture medium	04 ± 0.1	18.3 ± 1.6[#]	0.7 ± 0.5	19.2 ± 1.8[#]
MSC conditioned mudium	33.5 ± 3.2[*]	56.7 ± 3.5[&]	29.9 ± 3.1[*]	54.7 ± 5.3[&]

Data represent the mean ± S.E.M. from nine animals.
[*] Mean different from culture medium.
[#] Mean different from control.
[&] Mean different from all other groups.

Quantification of PI fluorescence showed that the exposure of cultures to conditioned medium from bone marrow-derived MSC caused around 18% of damage in hippocampus after 24 h of exposition, a significant increase when compared to control cultures (Fig. 1B). The damage induced by MSC conditioned medium was mainly observed in CA1, CA2 and CA3 areas, apparently sparing some parts of the CA1 and CA3 areas. Additionally, a small damage was sometimes observed in the inner blade of the DG area, as observed in Fig. 1A. When cultures were exposed to OGD, to mimic ischemic conditions, around 18% of increase in PI staining was observed, but in this lesion model the damage was mainly in CA1 area (Fig. 1A). Surprisingly, in the presence of rat bone marrow-derived MSC conditioned medium, the cell death increased to 53% in OGD exposed slices (Fig. 1B). Similar results were obtained with medium conditioned with MSC prepared from rat lungs and from bone marrow and lungs from C57BL6 mice as shown in Table 1.

Contrary to hippocampal cultures, in cortical slice cultures we did not observe any propidium iodide incorporation when these cultures were treated with conditioned medium. Also this treatment did not cause any protection or sensitization to OGD as well (Fig. 1C).

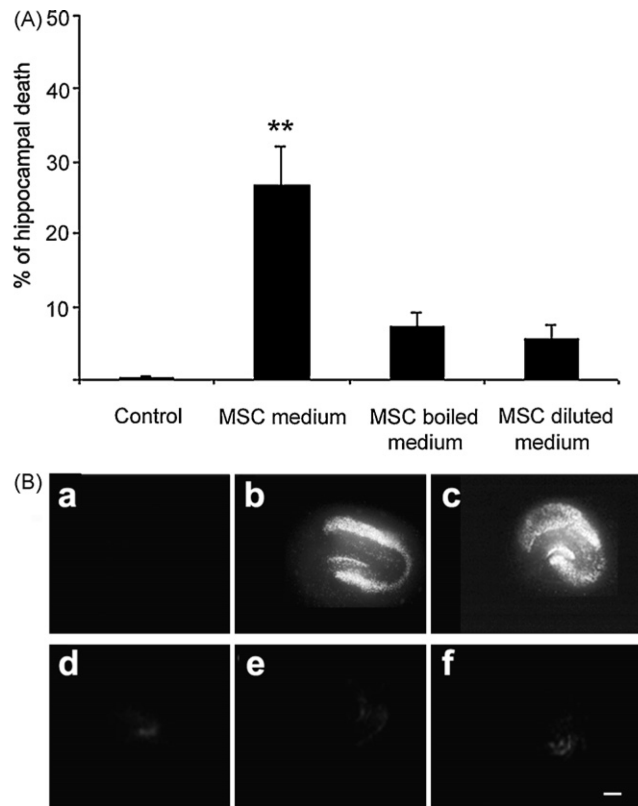

Fig. 2. Toxicity induced by MSC conditioned medium in organotypic hippocampal slice cultures can be attenuated by boiling or diluting this medium. (A) Quantification of PI incorporation after 24 h exposure to bone marrow MSC medium; (B) shows representative pictures of organotypic hippocampal cultures exposed to (a) control medium, (b) bone marrow-derived rat MSC conditioned medium, (c) lung-derived rat MSC conditioned medium, (d) U87 glioma cell line-derived conditioned medium, (e) H647-derived conditioned medium and (f) HEK293-derived conditioned medium. Bars represent the mean ± S.E.M., $n = 6$. [**]Significantly different from the other groups, $p < 0.01$ (one-way ANOVA followed by Tukey's test). The pictures in (B) are representative of six slices. Scale bar: 0.5 mm.

3.2. Toxicity induced by rat MSC conditioned medium in organotypic hippocampal slice cultures seems to be exclusive of MSC and is heat sensitive

In an attempt to understand the nature of the soluble factors responsible for the cell death, conditioned medium was boiled, to eliminate the majority of the proteins and other heat labile factors. This treatment significantly reduced toxicity. Dilution of the medium 1:1 with fresh medium also reduced the observed effect (Fig. 2A).

To test whether these toxic factors secreted by MSC were not just a product from the normal metabolism of cells in general, we also exposed the hippocampal cultures to conditioned medium from other cell types: glioma cell line (U87MG), carcinoma cell line (H647) and HEK293, in the same conditions and periods, and no toxic effect, i.e. cell death, was observed (Fig. 2B). Taken together, these results strongly suggest that specifically MSC secreted factors are toxic to hippocampal cultures.

3.3. Toxicity induced by rat MSC conditioned medium in organotypic hippocampal slice cultures can be attenuated by GABA treatment and by NMDA and AMPA receptors antagonists

Cell death induced by MSC conditioned medium shares regional similarities in the hippocampus with the cell death induced by bicuculline, i.e. GABA inhibition (Fig. 3A). Therefore the reduction in cell death observed with GABA suggests that this receptor may be reversibly blocked by the MSC-produced toxic factors (Fig. 3A and B).

When the NMDA antagonist MK801 (30 μM), the AMPA antagonist CNQX (100 μM) and the L-type calcium channel blocker nimodipine (10 μM) were added to MSC conditioned medium, PI incorporation in hippocampal tissue was also decreased (Fig. 4A), but differently from MK801 and nimodipine, CNQX reduced cell death only partially (Fig. 4B–D).

It is relevant to mention here that neither morphological changes nor massive cell death were observed on MSC which were exposed to organotypic cultures. After 72 h in presence of the organotypic tissue no alterations were observed in the MSC regarding to neural markers such as Nestin, Neurofilament 68 kDa, GLAST and GLT1 (data not shown), suggesting that these cells remained undifferentiated.

4. Discussion

Using the organotypic culture model, we have shown here that factors secreted by MSC are toxic, instead of neuroprotective, to hippocampal cells. Interestingly, this effect was specific to MSC, since we were not able to see cell death after exposure of brain slice cultures to media conditioned with other cell types (U87, H647 and HEK293 cell lines). In addition, we observed that this effect was not species-specific, since either media conditioned

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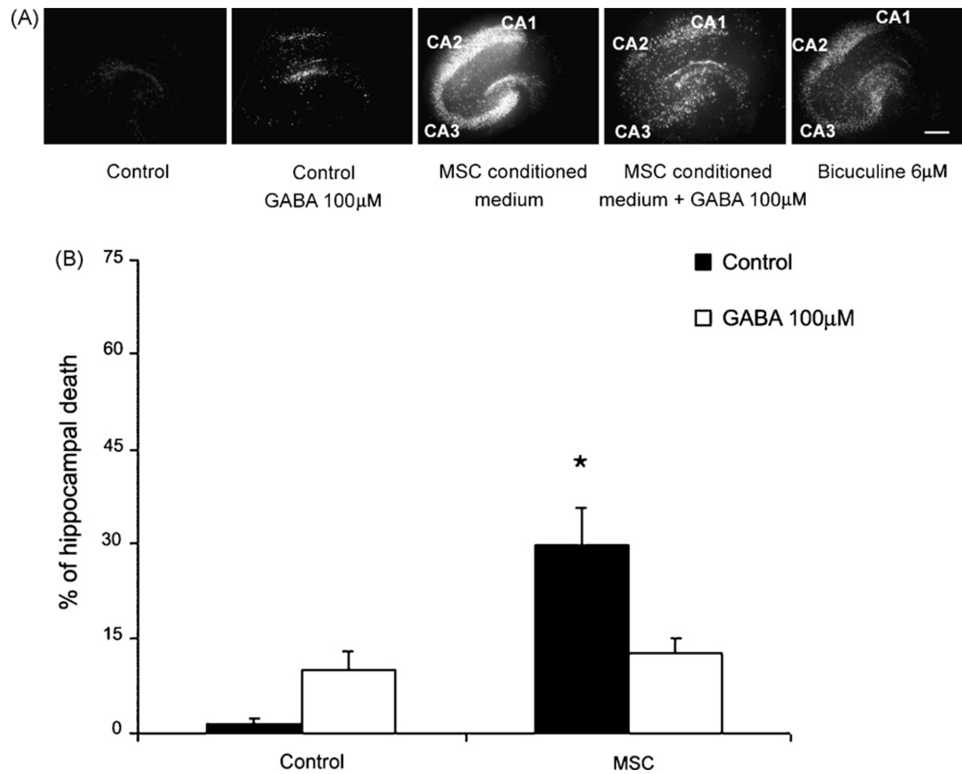

Fig. 3. Toxicity induced by MSC conditioned medium in organotypic hippocampal slice cultures can be attenuated by GABA (100 μM) treatment. (A) Representative pictures of PI incorporation in hippocampus after a 24-h exposure to MSC conditioned medium and GABA treatment. Scale bar: 0.5 mm. (B) Quantification of PI incorporation after the treatments. Bars represent the mean ± S.E.M., $n = 6$. *Significantly different from the other groups, $p < 0.05$ (one-way ANOVA followed by Tukey's test).

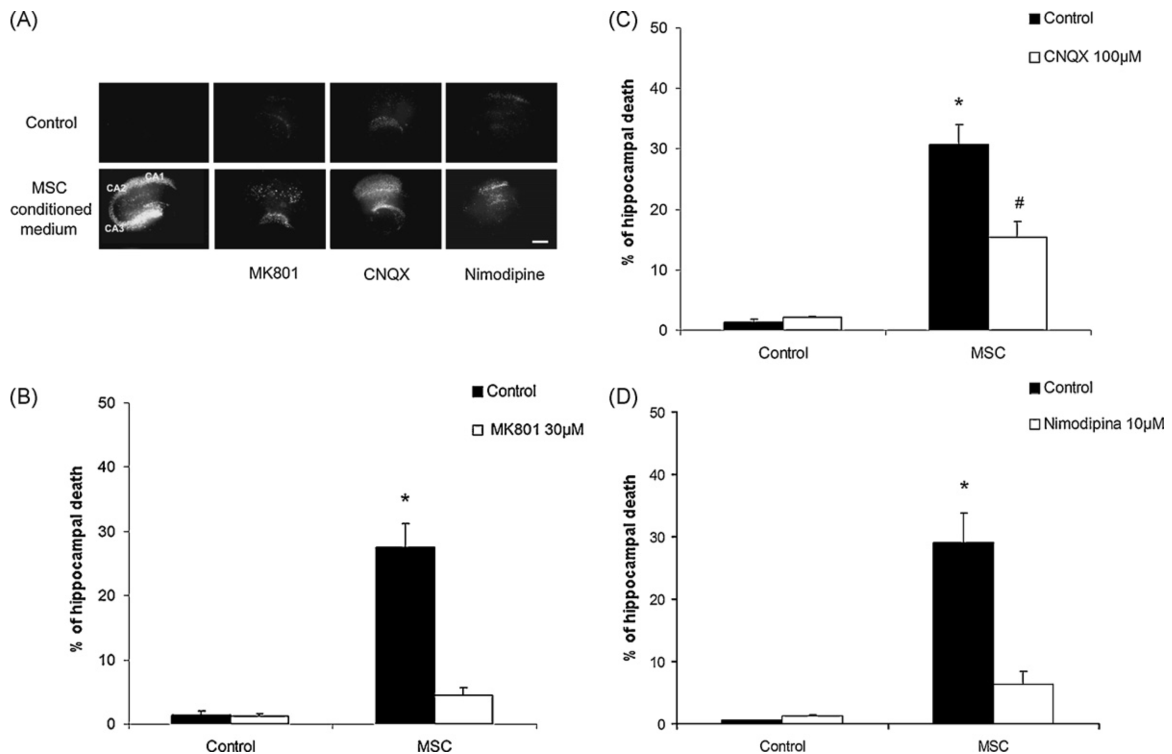

Fig. 4. Toxicity induced by MSC conditioned medium in organotypic hippocampal slice cultures can be attenuated by MK801 (30 μM), CNQX (100 μM) and nimodipine (10 μM) treatment. (A) Representative pictures of PI incorporation in hippocampus after a 24-h exposure to MSC conditioned medium or/and the drugs. Scale bar: 0.5 mm. (B) Quantification of PI incorporation after MK801 treatment; (C) represents the quantification of PI incorporation after CNQX treatment and (D) represents the quantification of PI incorporation after nimodipine treatment. Bars represent the mean ± S.E.M., $n = 9$ (MK801), $n = 6$ (CNQX), or $n = 6$ (nimodipine). *Significantly different from the other groups, #different from control group; $p < 0.05$ (one-way ANOVA followed by Tukey's test).

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with rat- or mouse-derived MSC had the same result on rat hippocampal cells in culture. Exposure of these cultures to conditioned medium from MSC plus OGD had an additional effect on cell death.

It is important to mention here that cells between the 10th and 25th passages were used in this work since only after 10 passages mouse MSC cultures can be described as free of terminally differentiated hematopoietic cells (da Silva Meirelles and Nardi, 2003). Also, the effect of prolonged cultivation on the capacity of these cells to differentiate was already determined, showing that MSC cultures with more than 32 passages still maintain their stem cell capacity, differentiating in osteoblasts and adipocytes (da Silva Meirelles et al., 2006).

It is well known that some factors secreted by MSC such as NGF, BDNF and GDNF are neuroprotective (Chopp and Li, 2002; Horita et al., 2006), but little is known about factors that could be toxic to the brain tissue. Regarding side-effects of neurotrophic factors, it has been shown that pre-treatment with GDNF can increase neuronal cell loss after OGD in hippocampal slice cultures by reverse transport of glutamate due to the upregulation of glutamate transporters (Bonde et al., 2003). Considering that MSC also secretes GDNF, it could be reasonable to think that it might be involved in aggravating cell death caused by OGD in hippocampal slice cultures.

To our knowledge, this is the first study showing that MSC products can be harmful to neural cells. Cavaliere et al. (2006) showed that factors released by the subventricular zone (SVZ), when in contact with organotypic hippocampal tissue, were also toxic to slices, and increased the damage caused by OGD. Considering that most neural stem cells found in the adult brain are located in the SVZ, it is reasonable to consider that stem cells can in fact release harmful factors, at least in culture. When the same authors treated organotypic cortical slice cultures with SVZ secreted factors, they observed neuroprotection rather than toxicity after OGD (Cavaliere et al., 2006). Surprisingly, no effect was seen in cortical slices, suggesting that the toxic factors are not a broad glutamatergic receptor or ion channel activator but rather affect a target exclusive to the hippocampus. In our study, MSC conditioned medium was not able to protect cortical slice cultures from cell death induced by OGD, as already observed in hippocampal slice cultures. Others have shown a neuroprotective effect of medium conditioned with bone marrow stromal cells in neuronal cultures exposed to staurosporine, but the differences in culture conditions and lesion model makes the comparison difficult (Isele et al., 2007).

The similarities of the effects of bone marrow- and lung-derived MSC supports the hypothesis already put forward by Nardi's group that each organ contains their stem cell population closely interacting with the capillaries and that these stem cells have a shared origin and maintain similar properties (Nardi, 2005; da Silva Meirelles et al., 2006).

Although we took great care with the conditioning procedure, it is reasonable to think that MSC depleted the medium of a general nutritional factor necessary for the survival of the hippocampal cells. Medium conditioned by three widely used cell lines did not show toxic effects, indicating that this effect is not a common by-product of the cellular metabolism. Also arguing against the depletion hypothesis is the observation that boiling of the conditioned media abolished the toxic effect, suggesting that it was not a depleted factor, but something heat sensitive that was secreted in this media by MSC that was responsible for cell death.

Since the inhibitory neurotransmitter γ-aminobutyric acid (GABA) counteracts glutamate receptor-mediated depolarization, enhanced GABAergic neurotransmission has been proposed as being neuroprotective. GABA receptor activation has already been shown to be neuroprotective after NMDA-induced and β-amyloid-induced neurotoxicity (Kristensen et al., 2003; Paula-Lima et al., 2005) and after cerebral ischemia (Zhang et al., 2007). Using GABA to stimulate GABAergic transmission we also observed protection of hippocampal cultures after their exposure to conditioned medium from bone marrow isolated MSC. These experiments were performed because, as described by Han and collaborators, bicuculline treatment induce the same profile in propidium iodide incorporation that we have observed here, selective to neurons in CA1, CA2 and CA3 (*Cornus Amonius*) areas (Han et al., 2004). Once protection could be seen in our model after GABA receptors stimulation, we suggest that these receptors are involved in cell death induced by MSC conditioned medium, probably being reversibly blocked by the MSC-produced toxic factors.

In an attempt to investigate whether MSC conditioned medium-induced cell death also involved calcium toxicity, we chose MK801 (a NMDA receptor antagonist) and nimodipine (a specific L-type calcium channel blocker) to minimize calcium effects. Strong neuroprotection was observed after exposure of hippocampal cultures to MSC conditioned medium in the presence of MK801 and nimodipine. Arguing in favor of neuronal activity being responsible for the cell death, blockage of AMPA/kainate receptors with CNQX also reduced cell death induced by MSC secreted factors, but to a lesser extent, indicating that Ca^{2+} entry is the key factor for this toxicity. It is already known that AMPA receptors are also Ca^{2+} permeable channels, what partially explains its neuroprotection after excitotoxicity (Arundine and Tymianski, 2003).

The results presented in this work suggest that a classic excitotoxic mechanism is occurring in the MSC conditioned medium-induced toxicity, probably involving membrane depolarization and calcium toxicity. Once intracellular calcium is elevated, it may overactivate proteases, lipases, phosphatases, and endonucleases that either directly damage cell structure or induce the formation of oxidative free radicals that mediate cell death. Also, sodium influx through these receptors may be causing a toxic cell swelling in hippocampal cultures, leading to the cell death (Arundine and Tymianski, 2003; Mattson, 2007).

Several groups have already shown an improvement in recovery using animal models that received MSC after stroke, mainly after transient medial cerebral artery occlusion (MCAO) (Chen et al., 2001; Chopp and Li, 2002; Zhao et al., 2002; Horita et al., 2006; Mendez-Otero et al., 2007; Guzman et al., 2008), but the exact mechanism by which these cells are acting after lesion is still unknown. The factors secreted by these cells seem to be the major players in this improvement, basically inducing angiogenesis, neurogenesis, synaptogenesis and decreasing apoptosis (Chopp and Li, 2002). Beneficial effects are suggested after injection of these cells in focal ischemia protocols, but not much is known about the side effects of administering them in vivo. It is important to mention here that the OGD protocol used in the present work mimics global ischemia, differently from focal transient ischemia (MCAO), were neuroprotection after stroke is well described (Guzman et al., 2008). Also, our protocol mimics the immediate treatment of stroke with MSC secreted factors, differently from the majority of preclinical and clinical protocols that show neuroprotection after injecting cells within hours, days or weeks after MCAO (Chopp and Li, 2002; Mendez-Otero et al., 2007; Guzman et al., 2008).

In spite of the urgency in having an efficient treatment to stroke patients, we recommend caution in clinical trials, since there is still a lack of understanding of the effect of these cells on brain tissue. Therefore, more studies are necessary before we go to clinic protocols, making sure that this cell does not have the potential to cause adverse effects. To our knowledge these is the

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first report showing that, at least in vitro, MSC can cause cell death in the brain tissue.

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CAPÍTULO 2

MESENCHYMAL STEM CELL CONDITIONED MEDIUM INDUCES NEUROINFLAMMATION IN ORGANOTYPIC CULTURES OF RAT HIPPOCAMPUS

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Periódico: **Neuroscience**

Status: **a ser submetido**

Mesenchymal stem cell conditioned medium induces neuroinflammation in organotypic cultures of rat hippocampus

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List of abbreviations

COX-2 – Cyclooxygenase 2

DCF-DA - Dihydrodichlorofluorescein diacetate

FBS – Fetal Bovine Serum

GFAP – Glial Fibrillary Acid Protein

HBSS - Hank’s Balanced Salt Solution

HEPES – N-(2-hydroxyethyl)piperazine-1-ethanosulphonic acid

IL-6 - Interleukin 6

IL-10 - Interleukin 10

IL-1β - Interleukin 1β

iNOS – Inducible nitric oxide synthase

L-NAME - N_{ω}-Nitro-L-arginine methyl ester hydrochloride

LPS - Lipopolysaccharide

MEM – Minimal Essential Medium

MSC – Mesenchymal stem cells

NO – Nitric oxide

PI – Propidium iodide

ROS – Reactive oxygen species

TH1 – T helper lymphocyte 1

TNFα - Tumor Necrosis Factor α

TROLOX - 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic Acid

Abstract

Cell therapy using bone marrow-derived mesenchymal stem cells (MSC) seems to be a new alternative for the treatment of neurodegenerative diseases. In spite of several good and promising results with the use of these cells, their side effects are still unknown. In a previous work we have shown that MSC conditioned medium is toxic to hippocampal slice cultures and aggravates the cell death induced by oxygen and glucose deprivation. In an attempt to understand why hippocampal cells are dying in response to MSC, we investigated whether the inflammatory response could be involved in this phenomenon. Rat organotypic hippocampal cultures were exposed for 24 h to conditioned medium from MSC isolated from rat bone marrow. A marked glial activation was showed after exposure of hippocampal cultures to MSC conditioned medium, as evidenced by GFAP and Isolectin B_4 increase. Tumor necrosis factor α (TNFα) and interleukin-6 (IL-6) levels were also increased in the culture medium, as well as DCF-DA oxidation and iNOS immunocontent. Antioxidants and anti-inflammatory drugs reduced cell death in hippocampal organotypic cultures after their exposure to MSC conditioned medium, as evidences by propidium iodide incorporation. The results suggest that MSC secreted factors trigger oxidative stress and inflammation in organotypic cultures of hippocampus.

Keywords: bone marrow mesenchymal stem cells, organotypic hippocampal culture, oxidative stress, neuroinflammation, cell death, neurotoxicity.

Introduction

Neurodegenerative diseases affect a wide spectrum of the population and, in most cases, lead to physical and/or mental incapacity, involving memory, cognition, language and personality. They include cerebrovascular disorders, epilepsy, Alzheimer's disease, Parkinson's disease and multiple sclerosis (Price, 1999). Each of these diseases produces different clinical manifestations and involves cells in different brain regions, but normally share microglial and astroglial activation, being neuroinflammation an important component in increasing neuronal damage (Sriram and O'Callaghan, 2007). Most of these diseases do not have efficient therapies and only symptomatic treatments are available.

It is well known that brain inflammation is a double-edged sword. When it is controlled and well balanced, the inflammation significantly contributes to repair of damaged tissue after brain injury. However, excessive inflammation damages surrounding healthy tissues and is now considered to be actively involved in both acute and chronic neurological disorders. Inflammatory responses in the brain are associated mainly with microglial and astrocytic activation, leading to the release of pro-inflammatory and cytotoxic factors that accelerate neurodegeneration (Park et al., 2007).

Several *in vivo* and *in vitro* models have been used to study the mechanism underlying neuronal degeneration and to evaluate the potential neuroprotective effect of pharmacological treatments (Noraberg et al., 2005). Among the *in vitro* tools used to study neurodegenerative disease models, organotypic hippocampal slice cultures offer great advantages because they mimic closely *in vivo* conditions. Slices in culture maintain their cell architecture and interneuronal connections, and neurons physiologically mature in culture, allowing an extended survival study (Gähwiler et al., 1997, Tavares et al., 2001). These cultures have been widely used in our group to study lesions caused by oxygen and glucose deprivation (Valentim et al., 2003; Horn et al., 2005; Zamin et al., 2006; Horn et

al., 2009) and to investigate the toxicity induced by β-amyloid peptides (Nassif et al., 2007; Frozza et al., *in press*).

In recent years, there has been increasing interest in the use of mesenchymal stem cells (MSC) as cell therapy for several medical problems. Stem cells, due to their self-renewal, differentiation potentials, and secretion of potential neuroprotective factors, bring new hope for patients that suffer from neurodegenerative diseases. Experimental studies suggest that stem cells would support cerebral plasticity via growth factor production and stimulation of endogenous mechanisms of local repair (Chopp and Li, 2002). These cells have been shown to secrete trophic factors, including glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and vascular endothelial growth factors (VEGF) (Chen et al., 2002), as well as cytokines such as IFN-γ, TNFα, TGFβ, IL-4 and IL-6 (Karnoub et al., 2007). Many reports have already described MSC as having immunosuppressive properties (Uccelli et al., 2006; Chamberlain et al., 2007), specifically modulating T-cell functions (Bartholomew et al. 2002), impairing maturation and function of dendritic cells (Jiang et al., 2005) and inhibiting B-cell proliferation (Corcione et al., 2006). Therefore, MSC are believed to be candidates for the treatment of several pathologies, including neurodegenerative diseases (Kan et al., 2007). Until now, few side effect of these cells were reported (Karnoub et al., 2007).

In a previous work we have shown that MSC secreted factors induce cell death in hippocampal cultures in an excitotoxic manner and aggravate lesion caused by oxygen and glucose deprivation (Horn et al., 2009). In an attempt to understand why and how hippocampal organotypic cultures are affected, the aim of this study was to investigate whether the factors secreted by cultured MSC were able to trigger oxidative stress and inflammatory responses in organotypic hippocampal cultures.

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Experimental Procedures

Materials

Propidium iodide (PI), DCF-DA, LPS, Hepes, L-NAME, TROLOX® and Isolectin B_4 peroxidase conjugated were purchased from Sigma (St. Louis, MO, USA). Millicell culture inserts were obtained from Millipore (Millicell®-CM, 0.4 µm, Millipore®, Bedford, MA, USA) and 6-well culture plates were from TPP (tissue culture test plates TPP®, Switzerland). Culture medium, HBSS, fungizone, Trypsin/EDTA solution, horse heat inactivated serum and fetal bovine serum (FBS) were obtained from GIBCO (Grand Island, NY, USA). Gentamicin was from Schering-Plough (Rio de Janeiro, Brazil). Ascorbic acid was from Nuclear, Indomethacin from ICN Biochemicals (Aurora, Ohio, USA) and Dexamethasone-acetate from EFFECHEM (Milan, Italy). GFAP antibody was from Dako Chemicals (Carpenteria, CA, USA) and iNOS antibody was from ABCAM (Cambridge, UK). ELISA kits were from R&D Systems (Minneapolis, MN, USA). Anti-rabbit IgG peroxidase-conjugated and reagents to detect chemioluminescence (ECL) were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Hybond-C nitrocellulose membranes were from Hybond™ ECL™ (Hybond™ ECL™ nitrocellulose membrane, Amersham Biosciences, Freiburg, Germany). X-ray films were purchased from Kodak (Kodak X-Omat, Rochester, NY, USA). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) or Merck (Darmstadt, Germany).

Organotypic hippocampal slice cultures

All animal use procedures were approved by the local Animal Care Committee and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Organotypic hippocampal slice cultures were prepared according to the method of Stoppini et al. (1991) with some modifications (Valentim et al., 2003; Horn et al., 2005). Briefly, 400 µm thick

hippocampal slices were prepared from 6-8-day-old male *Wistar* rats using a McIlwain tissue chopper and separated in ice-cold Hank's balanced salt solution (HBSS), pH 7.2. The slices were placed on Millicell® culture membranes and the inserts were transferred to a 6-well culture plate. Each well contained 1 ml of tissue culture medium consisting of 50% minimum essential medium (MEM), 25% HBSS, 25% heat inactivated horse serum supplemented with (mM, final concentration): glucose 36, HEPES 25 and $NaHCO_3$ 4; fungizone 1% and gentamicin 0.1 mg/ml, pH 7.3. Organotypic cultures were maintained in a humidified incubator gasified with a 5% CO_2 atmosphere at 37°C for 14 days. Culture medium was changed twice a week.

Mesenchymal stem cell culture

Mesenchymal stem cell cultures were prepared according to the method described by da Silva Meirelles and Nardi (2003) and Nardi and da Silva Meirelles (2006). Briefly, bone marrow was obtained from adult *Wistar* rats, which were anesthetized and killed by cervical dislocation. Bone marrow was flushed out of tibias and femurs. After washing by centrifugation at 400 *x g* for 10 min and counting viable cells with trypan blue, the cells were resuspended in DMEM with 10% FBS and 10 mM HEPES in a final concentration of 5 X 10^6 viable cells per ml. To initiate the cultures, cells were plated in 6-well tissue culture dishes at 3.5 ml/well (1.94 X 10^6 cells/cm^2) and kept in a humidified 5% CO_2 incubator at 37° C for 72 h, when non-adherent cells were removed by changing the medium. Confluent cultures from MSC were incubated with 0.25% trypsin solution containing 0.01% EDTA for detachment and maintained in culture by changing the medium every 3-4 days.

Medium conditioning procedure

For all the experiments, cells between the 10th and 20th passages were seeded in 6-well plates in DMEM with 10% FBS and 10 mM HEPES in a density of 30,000 cells/well 2 days before the experiment with the organotypic culture. Twenty-four hours before the experiment, the medium was changed and conditioned during the next 24 h. Organotypic cultures were placed in this medium and is important to note that although MSC were still present in the culture plate, they had no contact with the organotypic culture. Only the secreted factors reached the tissue.

When indicated, Ascorbic acid (500 µM), TROLOX (500 µM), L-NAME (500 µM), Indomethacin (100 µM) or Dexamethasone-acetate (100 µM) were added 2 hours before the slices had contact with the MSC conditioned media and maintained during all the incubation period (24 h). Except for indomethacin, which was dissolved in calcium carbonate 3%, all the other drugs were dissolved in water.

Quantification of cell death

Cellular damage was assessed by fluorescent image analysis of propidium iodide (PI) uptake (Noraberg et al., 1999; Horn et al., 2005; Noraberg et al., 2005). PI 5 µM was added 1 h before the end of the recovery period and cultures were observed with an inverted microscope (Nikon Eclipse TE 300) using a standard rhodamine filter. Images were captured and analyzed using Scion Image software (www.scioncorp.com). The area where PI fluorescence was detectable above background levels was determined using the "density slice" option of Scion Image software and compared to the total slice area to obtain the percentage of damage.

Western blotting analysis

After obtaining fluorescent images, cells were homogenized in lyses buffer, aliquots were taken for protein determination and β-mercaptoethanol was added to a final concentration of 5%. Proteins were resolved (75 μg per lane) on 8% or 12% SDS-PAGE. After electrophoresis, proteins were electro transferred to nitrocellulose membranes using a semi-dry transfer apparatus. Membranes were incubated for 60 min at 4ºC in blocking solution (Tris-buffered saline containing 5% powdered milk and 0.1% Tween-20, pH 7.4) and further incubated with the appropriate primary antibody dissolved in the blocking solution overnight at 4ºC. Primary antibodies against GFAP (1:2000) and iNOS (1:1000) were used. The membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit antibodies (1:1000) for 2 h. The chemioluminescence was detected using X-ray films that were scanned and analyzed using the Optiquant software (Packard Instruments). Data are expressed as percentage of control cultures.

Isolectin-B_4 binding to detect microglial cells

In the same way as described above, proteins were resolved (75 μg per lane) on 8% SDS-PAGE and, after electrophoresis, they were electro transferred to nitrocellulose membranes using a semi-dry transfer apparatus. Membranes were incubated overnight at 4ºC in albumin solution (5% albumin and 2% Tween in PBS, pH 7.4). Isolectin B_4 peroxidase conjugated (Sigma) was incubated in PBS containing 0,05% Tween overnight in a final concentration of 0.125 μg/mL. The chemioluminescence (ECL, Amersham Pharmacia Biotech) was detected using X-ray films that were scanned and analyzed using the Optiquant software (Packard Instruments). LPS 2 μg/ml was incubated for 24 h in organotypic hippocampal cultures to be used as a positive control.

Determination of TNFα, IL-6 and IL-10 levels

After 24 h of incubation of organotypic hippocampal cultures with MSC conditioned medium, media were collected, rapidly frozen and stored at -20°C for later measurement of TNF-α, IL-6 and IL-10 levels using specific enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (R&D Systems). Also here LPS 2 μg/ml in organotypic hippocampal cultures for 24 h was used as a positive control.

ROS measurement

After 14 days in culture, hippocampal slices were washed with HBSS pH 7.4, carefully removed from the culture membrane and placed in 96-well black plates (one slice per well) containing control or conditioned medium with 30 μM DCF-DA (2,7-dihydrodichlorofluorescein diacetate). Slices were incubated at 37°C in the dark for 30 minutes. This fluorophore difuses through cell membrane and is subsequently enzimatically deacetylated by intracellular esterases to the non-fluorescent DCF-H. Some oxidizing molecules as peroxynitrite, nitric oxide and hydrogen peroxide have been reported to convert DCF-H to the highly fluorescent DCF (Possel et al., 1997). After the time of incubation, the intensity of DCF fluorescence was measured during 60 minutes with an excitation wavelength of 485 nm and an emission wavelength of 535 nm (Hitashi F2000 Fluorescence Spectrophotometer, Japan). LPS 2 μg/ml was used as a positive control.

Statistical analysis

Data are expressed as mean ± S.D. Student's t-test or One-way analysis of variance (ANOVA) were applied to the means to determine statistical differences between experimental groups. Post hoc comparisons were performed by Tukey's test. Differences between mean values were considered significant when $p<0.05$.

Results

MSC conditioned medium induces glial activation in organotypic hippocampal cultures

MSC conditioned medium induced a marked increase in Isolectin B_4 binding in organotypic hippocampal cultures samples, a hallmark of microglial activation (Figure 1A). LPS 2 μg/mL was used as a positive control. To investigate microglial activation prevention, both Indomethacin 100 μM or TROLOX 500 μM were co-incubated with MSC conditioned medium and decreased the MSC medium-induced microglial activation, as evidenced by a decrease in Isolectin B_4 binding (Figure 1A).

Also, exposure of hippocampal organotypic cultures to MSC conditioned medium caused astrocytic activation, as evidenced by the 25% increase in GFAP immunoreactivity in these cultures when compared to control cultures (Figure 1B).

MSC conditioned medium induces oxidative stress in organotypic hippocampal cultures

The generation of oxidative stress in organotypic cultures was investigated using the cell permeable fluorogenic compound DCF-DA. As observed in Figure 2, MSC conditioned medium induces an increase in free radicals generation in organotypic hippocampal cultures. LPS 2 μg/ml was used as a positive control and showed a very pronounced increase in oxidative stress (Figure 2A). A 52% increase in iNOS immunocontent was also observed in organotypic hippocampal cultures exposed to MSC conditioned medium, suggesting that this protein may be involved in NO (nitric oxide) generation in this condition (Figure 2B).

Exposure of organotypic hippocampal cultures to the medium conditioned with bone marrow-derived rat MSC caused also a marked increase in cell damage, as shown by the intense fluorescence due to incorporation of PI in hippocampus after 24 h of contact with this medium (Figure 3A). Quantification of PI fluorescence showed that the exposure

of cultures to conditioned medium from MSC caused around 35% of damage in hippocampus after a 24 h period, a significant increase when compared to control cultures. As observed in Figure 3A, damage was mainly in CA1, CA2 and CA3 areas of hippocampus. In an attempt to investigate whether oxidative stress caused by ROS generation (Figure 2) could be involved in the observed increase in cell death, we used antioxidants to decrease ROS effects. When cultures were incubated with MSC conditioned medium and the antioxidants Ascorbic acid (500 μM) (Figure 3B), TROLOX (500 μM) (Figure 3C), and the iNOS inhibitor L-NAME (500 μM) (Figure 3D) cell death was decreased from around 34% to 10%, 13% and 6%respectively, suggesting that ROS production is essential for the MSC conditioned medium-induced cell death.

MSC conditioned medium triggers inflammatory responses in organotypic hippocampal slice cultures

To investigate a possible role of inflammation in hippocampus in response to MSC secreted factors, we investigated some key pro-inflammatory cytokines production. TNFα and IL-6 levels were highly increased in the culture medium after exposure of hippocampal culture to MSC conditioned medium for 24 h (Figure 4A and 4B). The anti-inflammatory cytokine IL-10 did not show any alteration in this condition (Figure 4C). Also here we used LPS 2 μg/ml as a positive control and it was observed that MSC conditioned medium induced the same amount of TNFα increase than LPS. We did not observe any detection of IL-1β after 1 h and 24 h of exposure of hippocampal cultures to MSC conditioned medium (data not shown).

Once it was observed that oxidative stress and inflammation were triggered by MSC conditioned medium, we decided to investigate if anti-inflammatory drugs such as the steroidal dexamethasone (Figure 5A and 5B) and the non-steroidal indomethacin (Figure

5A and 5C) were able to protect the organotypic hippocampal cultures from cell death. Both anti-inflammatory drugs reduced cell death induced by conditioned medium from MSC in a dose dependent way.

Discussion

Using the organotypic culture model, we have shown here that factors secreted by MSC induce glial activation, oxidative stress and inflammatory responses in hippocampal slice cultures. These results were evidenced by an increase in GFAP immunocontent and Isolectin B_4 binding, increase in DCF-DA oxidation and iNOS immunocontent and increase in TNFα and IL-6 levels. Both anti-inflammatory and antioxidants drugs were able to reduce cell death induced by MSC conditioned medium.

It is well known that inflammation itself does not necessarily cause neuronal death. Also, it is equally clearly demonstrated that inflammation can cause neuronal death when combined with particular circumstances as, for example, glutamate-induced excitotoxicity (Brown 2007), that seems to be the case in our model.

Microglial cells have a close interaction with neurons and play an important role in host defense. As the primary immune cell in CNS, they play a dual role in cellular response to neuronal injury: a pathogenic role that initiates inflammation and exacerbates degeneration and a neuroprotective role (Sriram and O'Callaghan, 2007). Reactive microglia secretes a variety of molecules, such as glutamate, cytokines (TNFα, TGFβ, IL-1β, IL-6, IL-10, IL-12, IL-16), chemokines (CCL2, CCL3, CCL4, CCL5), matrix metalloproteinases (MMP-2, MMP-3, MMP-9), free radicals (superoxide, NO) and eicosanoids (PGD_2, leukotriene C_4) that can act synergistically with the lesion to exacerbate cell damage (Rock et al., 2004). Also, these cells start to express a new range of genes and proteins, including iNOS, COX-2 and MHC class II and complement (Brown 2007). The increase in Isolectin B_4 binding observed in the present work suggests that

microglia is activated in organotypic hippocampal cultures exposed to MSC conditioned medium. This activation is probably increasing iNOS, NO and other ROS production, cytokines production, and glutamate release in the culture medium, culminating in the cell death observed in hippocampal cultures.

After several conditions such as brain injury, for example, astrocytes undergo astrogliosis, that is characterized by an increase in GFAP, vimentin and nestin expression, by cellular hypertrophy, by process extension and interdigitation, by cell proliferation and by secretion of a variety of cytokines. In addition, reactive astrocytes form a glial scar which obstructs neurite outgrowth and axonal regeneration and communication (Liberto et al., 2004). The most prototypic biochemical change during astrogliosis is GFAP up-regulation that is almost universally recognized as a marker of reactive astrocytes. Another protein associated with astrogliosis is IL-6, which during brain injury, inflammation and diseases including stroke and Alzheimer has its level elevated (Gadient and Otten, 1997; Gao et al., 2008). Reactive astrocytes may also exacerbate tissue damage by releasing of pro-inflammatory cytokines such as TNFα and by producing and releasing arachidonic acid metabolites, NO, and reactive oxygen species (ROS). It is important to mention here that in spite of microglia producing much higher levels of these cell damaging intermediates than astrocytes, they certainly contribute to cell death (Liberto et al., 2004). Activated astrocytes and microglial cells were described to be able to release TNFα and IL-6, but we can not role out that the MSC could be responsible for the release of these factors in the culture medium.

In spite of some reports describing MSC as having immunosuppressive properties (Uccelli et al., 2006; Chamberlain et al., 2007), its effects normally impinge upon T-cell functions (Bartholomew et al. 2002), dendritic cells (Jiang et al., 2005) and B-cells (Corcione et al., 2006), but these event have little effect in the brain tissue. Gao and

collaborators have shown that bone marrow stromal cells reduce astrocytic activation *in vitro*, using astrocytes culture and GFAP and IL-6 decrease to support their conclusions. However, they have used only one cell type (astrocytes), mouse bone marrow stromal cells, and they do not describe the passage they used these cells, making the comparison difficult (Gao et al., 2008). It was already demonstrated by two independent groups that *in vivo* injection of MSC in hippocampus activates microglia, and probably this activation is responsible for killing MSC after transplantation (Coyne et al., 2006; Ohtaki et al., 2008). Our result also suggest microglial activation in response to MSC secreted factors and further studies are necessary to understand which mecanisms are involved in this activation.

It was already demonstrated by Djouad and collaborators that environmental parameters might influence the properties of MSC. In a model of collagen-induced arthritis in mouse, inflammation seems to reverse immunosuppression, associated with MSC increase in TH1 helper responses. Interestingly, in the presence of TNFα , MSC were unable to inhibit proliferation of allogenic T cells and started to secrete high amounts of IL-6 in the culture medium, worsening inflammation (Djouad et al., 2005). In our experiments, we have shown an increase in TNFα and IL-6 levels in the culture medium. This increase in TNFα in the culture medium could be the responsible for the MSC pro-inflammatory behavior observed in our work and could also help to explain IL-6 increase.

MSC conditioned medium-induced toxicity was reduced when indomethacin was added to the culture medium, probably by its action in decreasing Cox-2 expression and prostaglandin release. Eligini and collaborators showed that TNFα increase Cox-2 expression (Eligini et al., 2005) increasing inflammation, partially explaining why indomethacin can protect hippocampal cultures from MSC conditioned medium. The toxicity can also be reduced by addition of dexamethasone to the culture medium, that was

already shown to directly inhibit Cox-2 and iNOS expression and consequently NO production (Hämäläinen et al., 2007). Nitric oxide (NO) generated from iNOS appears to be a key mediator of glial-induced cell death. The high sensitivity of neurons to NO is partly due to NO causing inhibition of respiration, rapid glutamate release from both astrocytes and neurons, and subsequent excitotoxic death of the neurons via the NMDA receptor (Bal-Price and Brown, 2001; Brown 2007). It was already demonstrated that high TNFα levels can increase iNOS expression, leading to NO and peroxynitrite prodution and consequently to neuronal death (Combs et al., 2001). Also, it is described that TNFα induces neurotoxicity via direct glutamate release from activated microglia (Takeuchi et al., 2006) and from activated astrocytes (Bezzi et al., 2001). So, we conclude that TNFα increase and iNOS increase due to glial activation in our model reinforce the excitotoxic hypothesis, suggesting that glutamate release by microglia and astrocytes could be one of the ways of MSC conditioned medium to induce cell death in hippocampal cultures. In a previous work we already described that blockage of NMDA receptors, as well as AMPA receptors and Ca^{2+} dependent voltage channels reduced cell death induced by MSC conditioned medium in organotypic hippocampal cultures (Horn et al., 2009), suggesting that excitotoxic-like mechanisms are responsible for the observed cell death. We can not affirm from were exactly this glutamate is coming from: microglial cells, astrocytes, neurons or from the MSC that, in spite of having no contact with the hippocampal tissue are still present in the system and certainly secreting factors during the 24 h incubation period.

It is important to mention here that cells between the 10th and 20th passages were used in this work because it was already demonstrated that only after 10 passages mouse MSC cultures can be described as free of terminally differentiated hematopoietic cells (da Silva Meirelles and Nardi, 2003). Also, the effect of prolonged cultivation on the capacity of these cells to differentiate was already determined, showing that MSC cultures with more

than 32 passages still maintain their stem cell capacity, differentiating in osteoblasts and adipocytes (da Silva Meirelles et al., 2006).

In spite of a number of promising results and the urgency in having an efficient treatment to patients that suffer from neurodegenerative diseases, we caution on the use of MSC in clinical trials, once there is still a lack of understanding of the effect of these cells on brain tissue. Therefore, more studies are necessary before we go to clinical protocols, making sure that these cells do not have the potential to cause severe adverse effects. Also, one should take into account disease-specific mechanisms as well as environmental factors that are likely to significantly affect the biological properties of MSC. Our results clearly show that these cells can cause inflammatory response in the hippocampal tissue and so, we suggest caution mainly in diseases were the inflammatory component is already present.

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Figure Legends:

Figure 1: MSC conditioned medium induces glial activation in organotypic hippocampal cultures. **A:** Representative pictures showing Isolectin B_4 binding in organotypic hippocampal cultures samples after 24 h of exposure to MSC conditioned medium. LPS 2μg/ml was used as a positive control and Indomethacin (100μM) and TROLOX (500μM) were used to prevent microglial activation. **B:** GFAP immunoreactivity in organotypic hippocampal culture after 24 h of exposure to MSC conditioned medium. Quantification of GFAP immunocontent considering control cultures as 100%. Bars represent the mean ± S.D., n=8 animals, $p<0.05$ (Student's t-test). * Significantly different from control cultures.

Figure 2: MSC conditioned medium induces oxidative stress and iNOS increase in organotypic hippocampal slice cultures. **A:** Quantification of DCF-DA oxidation in slices in the first hour of exposure to MSC conditioned medium. LPS 2μg/ml was used as a positive control. Bars represent the mean ± S.D., n=5 animals, $p<0.05$ (one-way ANOVA followed by Tukey's test). * Significantly different from control cultures. # significantly different from all the other groups. **B:** iNOS immunocontent in organotypic hippocampal cultures after 24 h of exposure to MSC medium. Bars represent the mean ± S.D., n=6 animals, $p<0.05$ (Student's t-test). * Significantly different from control culture.

Figure 3: Cell death induced by MSC conditioned medium can be attenuated by antioxidants. **A:** Representative pictures of organotypic hippocampal cultures exposed for 24 h to control or conditioned medium in the presence or absence of the antioxidants. **B**, **C** and **D** show the quantification of propidium iodide incorporation after

treatment with Ascorbic acid 500 μM (**B**), TROLOX 500 μM (**C**) and L-NAME 500 μM (**D**). Bars represent the mean ± S.D., n=6 animals, $p<0.05$ (one-way ANOVA followed by Tukey's test). Magnification: 40x; scale bar: 0.5 mm. * Significantly different from control cultures. # Significantly different from all the other groups.

Figure 4: MSC conditioned medium triggers inflammatory responses in organotypic hippocampal cultures. (**A**) TNFα (**B**) IL-6 and (**C**) IL-10 levels in the culture medium 24 h after exposure of organotypic hippocampal cultures to MSC conditioned medium. Bars represent the mean ± S.D., n=5 experiments (one-way ANOVA followed by Tukey's test). *** Significantly different from hippocampal culture medium and MSC medium, $p<0.001$. ** Significantly different from all other groups, $p<0.01$. # Significantly different from all the other groups, p<0,05.

Figure 5: Toxicity induced by MSC conditioned medium in organotypic hippocampal slice cultures can be reduced by anti-inflammatory drugs. **A:** Representative pictures showing propidium iodide incorporation after exposure of organotypic hippocampal cultures to MSC conditioned medium in the absence or presence of indomethacin or dexamethasone. **B:** Quantification of Dexamethasone 50 μM and 100 μM treatment in organotypic hippocampal cultures exposed to MSC conditioned medium. **C:** Quantification of Indomethacin 20 μM and 100 μM treatment in organotypic hippocampal cultures exposed to MSC conditioned medium. Bars represent the mean ± S.D., n=5 animals, $p<0.05$ (one-way ANOVA followed by Tukey's test). Magnification: 40x; scale bar: 0.5 mm. * Significantly different from control cultures and from MSC medium treated with dexamethasone 100 μM or indomethacin 100 μM.

Figure 1

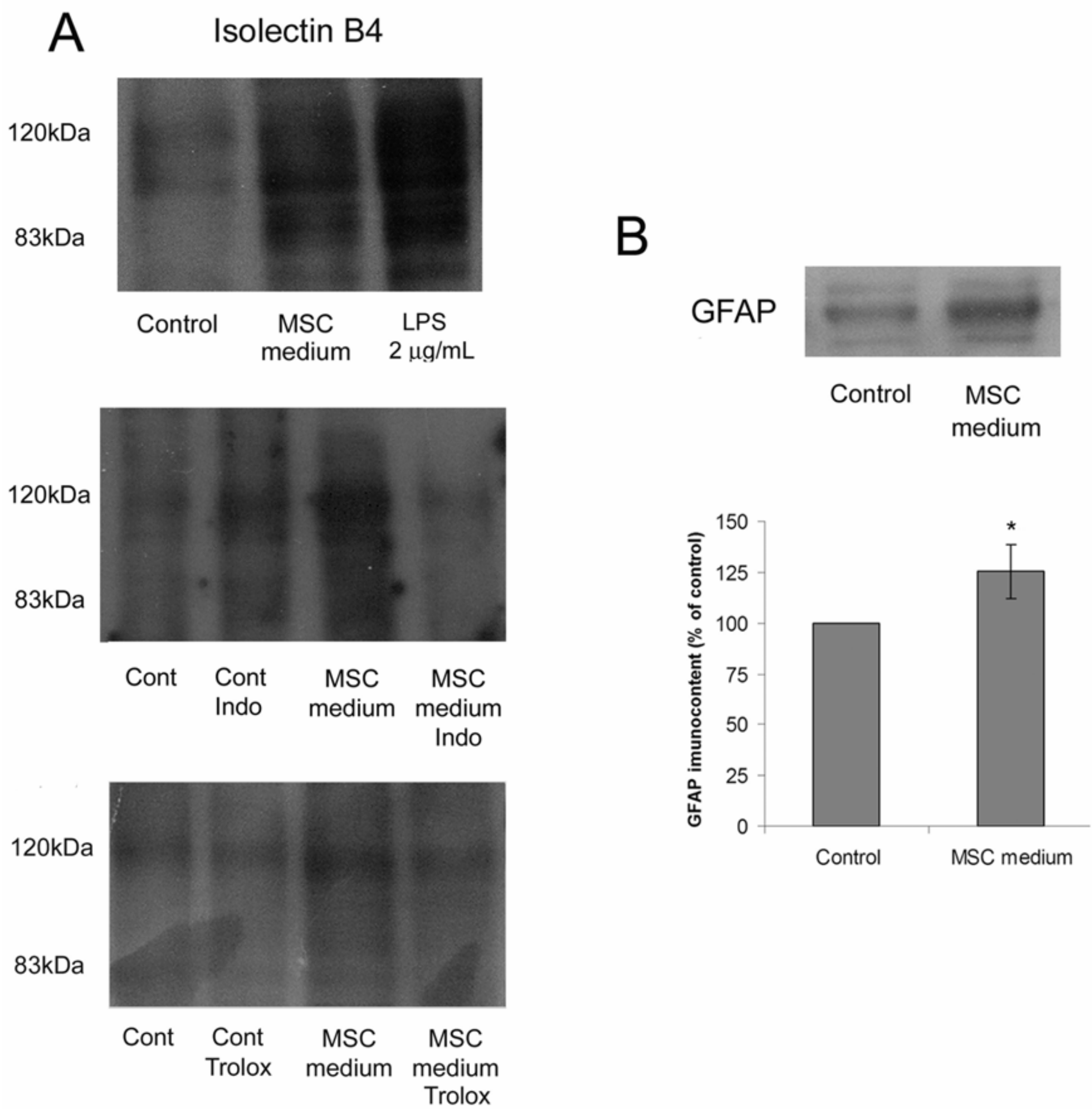
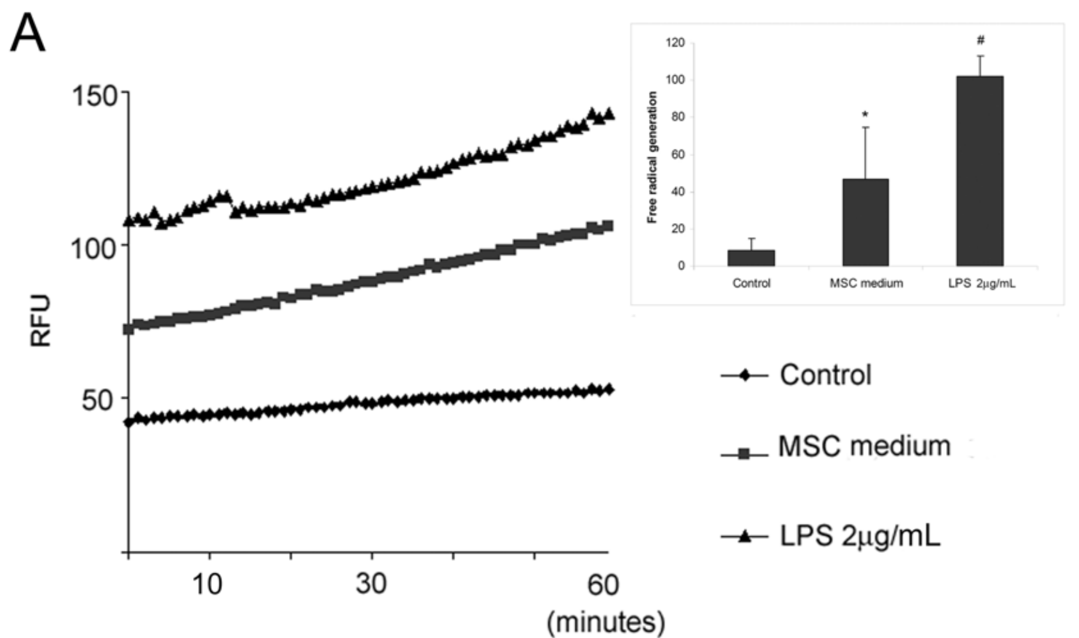


Figure 2



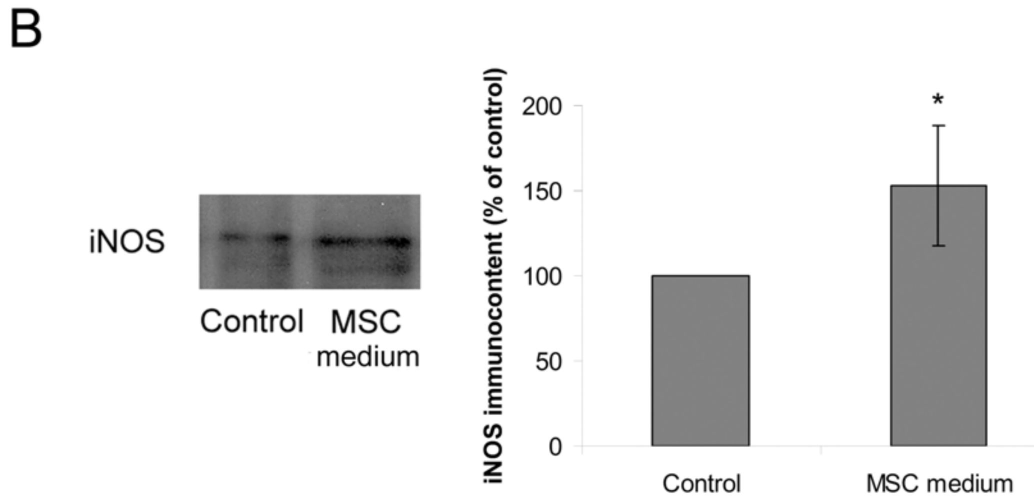
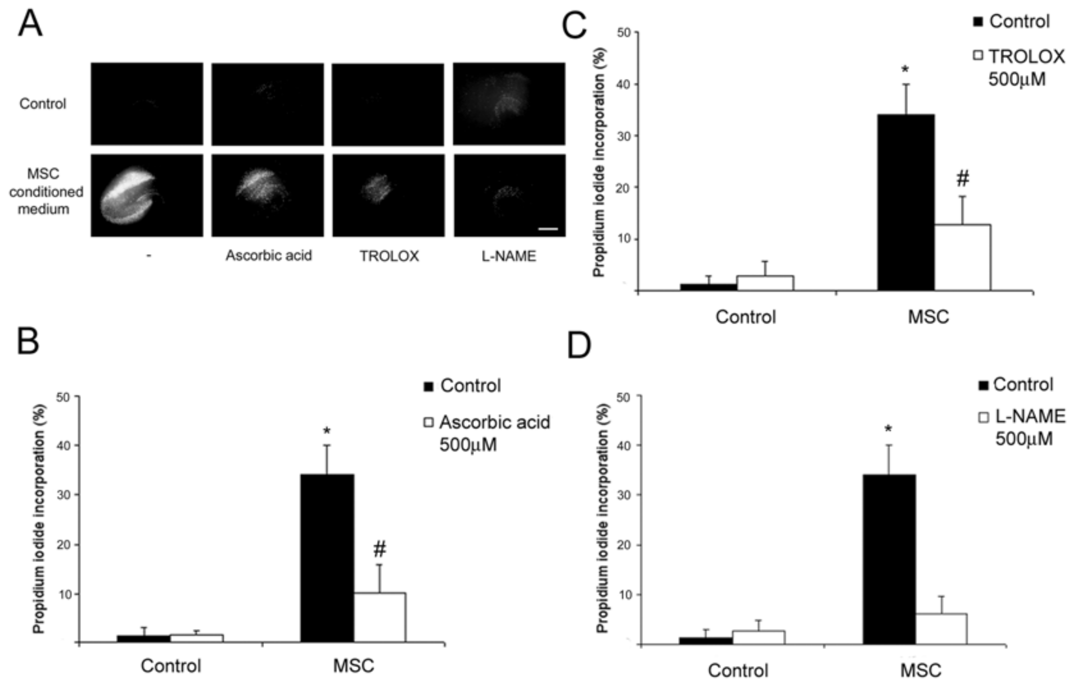


Figure 3



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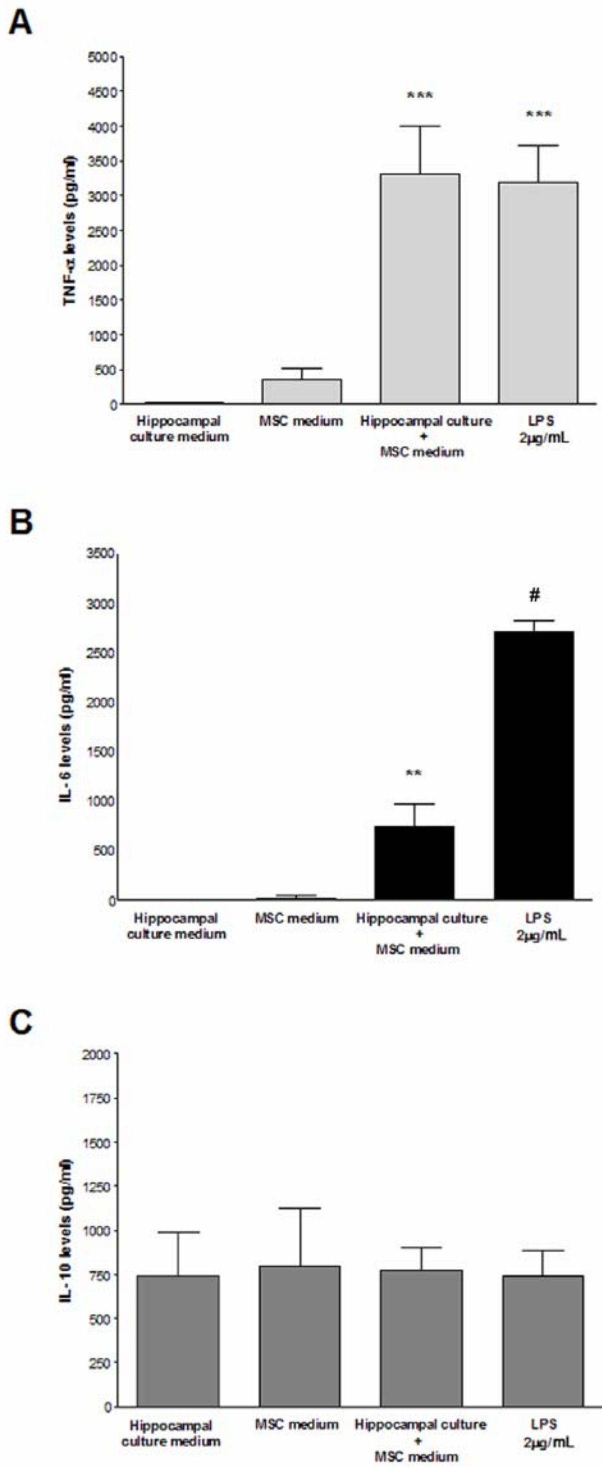
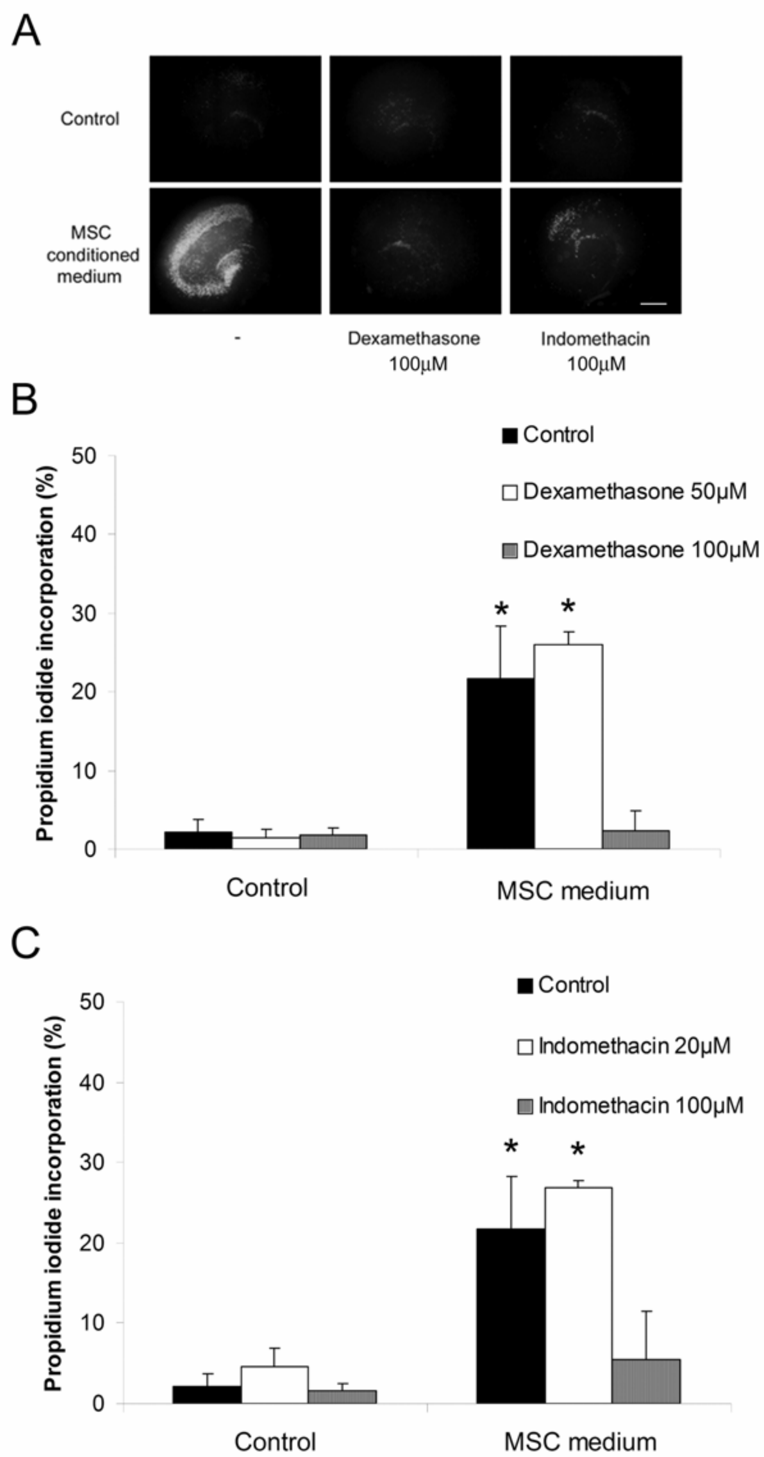


Figure 5



CAPÍTULO 3

FACTORS SECRETED BY INJURED ORGANOTYPIC HIPPOCAMPAL CULTURES DO NOT INDUCE MESENCHYMAL STEM CELLS DIFFERENTIATION

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FACTORS SECRETED BY INJURED ORGANOTYPIC HIPPOCAMPAL CULTURES DO NOT INDUCE MESENCHYMAL STEM CELLS DIFFERENTIATION

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ABSTRACT

In recent years, several studies have shown that bone marrow-derived mesenchymal stem cells (MSC) can transdifferentiate into neurons and astrocytes under some *in vitro* conditions. In an attempt to study MSC response under lesion-stimulated conditions, we investigated whether soluble factors derived from rat organotypic hippocampal cultures that were exposed or not to oxygen and glucose deprivation (OGD) were able to induce a neuro-glial phenotype in rat MSC. The cells were exposed to conditioned medium from organotypic hippocampal slices for 24 or 72 h, without contact with the brain tissue. Propidium iodide staining and caspase 3 immunoblots showed that hippocampal conditioned medium from cultures exposed or not to OGD do not induce cell death in MSC. There is an increase in proliferation in these cells when they are exposed to the injury-induced conditioned medium, but there is not expression of the neural markers Nestin, NF68kDa, GLT-1 and GLAST in these conditions. Our results suggest that, in our model, MSC increase proliferation in response to an environment that contains soluble factors secreted by the lesioned hippocampus, but transdifferentiation of the cells in neural cells does not occur.

Key words: Mesenchymal stem cells, Organotypic hippocampal culture, Conditioned medium, Transdifferentiation.

Abbreviations:

BHA – Butylated hydroxyanisole
BME - β-mercaptoethanol
CNS - Central Nervous System
MSC – Mesenchymal stem cells
NSC – Neural stem cells
OGD – Oxygen and glucose deprivation
PI – Propidium iodide
RA – Retinoic acid

INTRODUCTION

Among the adult stem cells, bone marrow mesenchymal stem cells (MSC) may represent the best hope for autologous stem cell-based replacement therapies because, in addition to their multipotency and accessibility, these cells should not elicit graft versus host disease [1]. For this reason, the MSC is one of the most extensively studied adult stem cells with respect to transdifferentiation potential, also toward neural differentiation [2], aiming the development of therapeutics for neurodegenerative diseases [3].
Several protocols to induce the transformation of MSC into neural lineages have relied on exposure of these cells to retinoic acid (RA) and cytokine cocktails, butylated hydroxyanisole (BHA), β-mercaptoethanol (BME), and dimethyl sulphoxide (DMSO) [4]. These protocols are considered very aggressive and recently it has been demonstrated that the "neuronal phenotype" observed after this treatments is only an artifact. The neuronal-like morphology induced by DMSO, for example, is believed to be simply cell shrinkage with retraction of the majority of existing cell extensions, leaving only few, fine neurite-like processes [5]. The expression of differentiation markers, such as β-tubulin III, neuron-specific enolase, neurofilaments, NeuN, and MAP-2, observed by several groups, seems to be due to an aberrant general gene expression that is observed in MSC exposed to these agents [4, 5, 6].
Several *in vivo* and *in vitro* experimental models have been used to study the mechanism underlying neuronal degeneration and to evaluate the potential neuroprotective effect of pharmacological treatments [7]. Among the *in vitro* tools used to study ischemia-induced injuries, organotypic hippocampal slice cultures, combined with oxygen and glucose deprivation (OGD), offer great advantages because they mimic closely *in vivo* conditions [8, 9, 10].
In this context, the aim of our study was to investigate whether rat MSC exposed to conditioned medium from hippocampal slice cultures that were exposed or not to OGD were dying, proliferating or expressing neural markers in response to factors released by the injured slices.

MATERIALS AND METHODS

Organotypic hippocampal slice cultures

All animal use procedures were approved by local Animal Care Committee and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Organotypic hippocampal slice cultures were prepared according to the method of Stoppini et al., 1991 [11] with modifications [9]. Briefly, 400 µm thick hippocampal slices were prepared from 6-8-day-old male *Wistar* rats using a McIlwain tissue chopper and separated in ice-cold Hank's balanced salt solution (HBSS), pH 7.2. The slices were placed on Millicell culture membranes (Millipore) and the inserts were transferred to a six-well culture plate. Each well contained 1 ml of tissue culture medium consisting of 50% minimum essential medium (MEM, Gibco), 25% HBSS (Gibco), 25% heat inactivated horse serum (Gibco) supplemented with (mM, final concentration): glucose 36, HEPES 25 and $NaHCO_3$ 4; fungizone 1% and gentamicin 0,1 mg/ml, pH 7.3. Organotypic cultures were maintained in

a humidified incubator gasified with a 5% CO_2 atmosphere at 37°C for 14 days. Culture medium was changed twice a week.

Mesenchymal stem cell culture

Mesenchymal stem cell cultures were prepared according to the method described by da Silva Meirelles and Nardi in 2003 [12] and Beyer Nardi and da Silva Meirelles in 2006 [13]. Briefly, bone marrow cells were obtained from adult *Wistar* rats. The animals were killed by cervical dislocation and bone marrow was flushed out of tibias and femurs. After washing by centrifugation at 400 X g for 10 min and counting viable cells with trypan blue, the cells were resuspended in DMEM (Gibco) with 10% FBS (Gibco) and 10 mM HEPES (Sigma) in a final concentration of 5 X 10^6 viable cells per ml. To initiate the cultures, cells were plated in six-well tissue culture dishes at 3.5 ml/well (1.94 X 10^6 cells/cm^2) and kept in a humidified 5% CO_2 incubator at 37° C for 72 h, when non-adherent cells were removed by changing the medium. Cells were maintained in culture by changing the medium every 3-4 days.
For our experiments, cells between the 10th and 25th passages were seeded in six-well plates in DMEM with 10% FBS and 10 mM HEPES in a density of 30,000 cells/well two days before the contact with the organotypic hippocampal culture medium. These cells stayed 24 h in contact with hippocampal cultures that were exposed or not to OGD conditions. It is important to mention here that only the conditioned medium from the hippocampal tissue was playing a role in the response observed here, once MSC and the tissue did not have contact with each other.

Oxygen and glucose deprivation (OGD) - lesion model

The induction of OGD was based on the method described by Strasser and Fischer in 1995 [14], with some modifications [9, 10]. Cultures were carefully rinsed twice with OGD medium composed of: $CaCl_2$ 1.26 mM, KCl 5.36 mM, NaCl 136.9 mM, H_2PO_4 0.34 mM, $MgCl_2$ 0.49 mM, $MgSO_4$ 0.44 mM, HEPES 25 mM, pH 7.2. Slices were left in 1 ml of this medium for 15 minutes to deplete glycogen stocks, and then changed to the same medium previously bubbled with nitrogen for 30 minutes. The cultures were transferred to an anaerobic chamber at 37°C in which the oxygen was replaced by nitrogen, and left in these conditions for 60 minutes. Slices were washed twice with HBSS and placed in a six-well plate containing the MSC in DMEM/10% FBS medium (conditioned medium) or only DMEM/10% FBS (control medium).

Quantification of cell death

Cellular damage in MSC was assessed by fluorescent image analysis of propidium iodide (PI) (Sigma) uptake. PI 5 μM was added 1 h before the end of the recovery period and cultures were observed with an inverted microscope (Nikon Eclipse TE 300) using a standard rhodamine filter. Images were captured and analyzed using the software Photoshop version 7.0. A total of 1000 cells were counted in each treatment and the cells positive to PI incorporation were considered death.

Cell counting

At the end of 24 h of exposure, the medium was removed and cells were washed with phosphate-buffered saline (PBS) and incubated with 0.25% trypsin/EDTA solution to detachment. Cells were immediately counted in a hemocytometer.

Western Blot analysis

Cells were homogenized in lyses buffer and aliquots were taken for protein determination. Proteins were resolved (50 µg per lane) on 8% or 10% SDS-PAGE and electro transferred to nitrocellulose membranes using a semi-dry transfer apparatus. Membranes were incubated for 60 min at 4ºC in blocking solution (Tri-buffered saline containing 5% powdered milk and 0.1% Tween-20, pH 7.4) and further incubated with the appropriate primary antibody overnight at 4ºC. Primary antibodies against the following proteins were used: anti-cleaved caspase-3 (Asp175) (1:200; Cell Signaling); anti-caspase-3 (1:1000; Cell Signaling), anti-nestin (1:1000; Chemicon), anti-neurofilament 68kDa (1:1000; Sigma), anti-GLAST (1:500), anti-GLT1 (1:500) (both kindly provided by Dr D. Pow, University of Newcastle, Australia) and anti-β-actin (1:1000; Cell Signaling). The membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies (1:1000; Amersham Pharmacia Biotech) for 2 h. The chemioluminescence (ECL, Amersham Pharmacia Biotech) was detected using X-ray films that were scanned and analyzed.

Statistical analysis

Data are expressed as mean ± S.D. One-way analysis of variance (ANOVA) followed by Tukey's test was applied to the means to determine statistical differences and p value lower than 0.05 was considered significant.

RESULTS AND DISCUSSION

Conditioned medium from lesioned hippocampus has no toxic effect and induce MSC proliferation

To investigate a possible toxic effect from organotypic conditioned medium in MSC, these cells were incubated with PI to estimate cell death. MSC exposed to organotypic hippocampal cultures conditioned medium do not show increase in PI incorporation after a 24 h contact with this medium (Fig. 1A). Also, western blot analysis for caspase 3 detection does not show cleavage and activation of this protein in MSC after being exposed to hippocampal conditioned medium with or without lesion (Fig. 1B). Cellular morphology did not change, even when MSC were exposed to medium from the injured organotypic cultures (Fig. 1A and 1C).

Expansion capacity was investigated to verify if the number of MSC could change in injury-stimulated conditions and showed that cells exposed to hippocampal factors derived from an injured culture increased cell proliferation (Fig. 1D). Taken together, our results suggest that conditioned medium from hippocampal cultures submitted or not to injury do not induce cell death or loss of proliferation capacity in rat bone marrow isolated MSC.

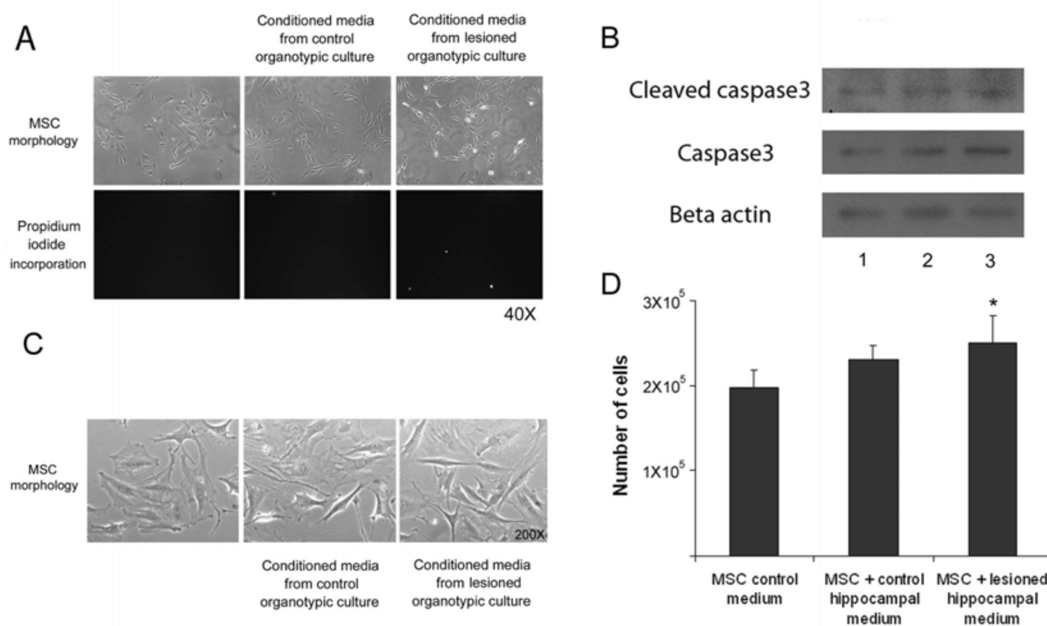

Fig. 1: MSC survive and maintain proliferation in response to hippocampal conditioned medium. **A:** Representative pictures of MSC cellular morphology and propidium iodide uptake after the contact for 24 h with the secreted factors from normal or injured hippocampus. **B:** Caspase 3 is not activated after MSC exposure to hippocampal conditioned medium. 1- MSC exposed to control culture medium; 2- MSC exposed to hippocampal culture conditioned medium; 3- MSC exposed to hippocampal culture + OGD conditioned medium. **C:** High magnificence (200X) pictures showing cellular morphology of MSC exposed to hippocampal conditioned medium. Pictures are representative of at least 3 independent experiments. **D:** Cell number after 24 h of exposure to hippocampal conditioned medium. Data represent mean ± S.D., One-Way ANOVA followed by Tukey's test, $p<0.05$, $n=4$.

MSCs do not express neural markers in response to conditioned medium from organotypic hippocampal cultures

To investigate whether MSC were differentiating in neural cells after the contact with factors secreted by the nervous tissue, we performed a western blot analysis for the neural markers Nestin, Neurofilament, GLT1 and GLAST glutamate transporters. As shown in Fig. 2, none of these markers were detected in MSC after 72 h of exposure to conditioned medium from hippocampal cultures submitted or not to OGD. Positive control used here was hippocampal tissue (for Neurofilament, GLT1 and GLAST) or C6 glioma cell line (for nestin). Beta actin was used as an internal control.

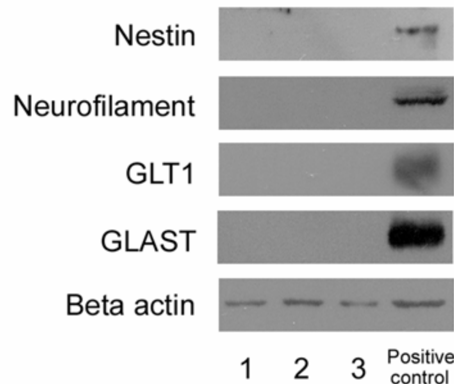

Fig. 2: MSC exposed for 72 h to hippocampal conditioned medium do not start to express neural markers. 1- MSC exposed to control culture medium; 2- MSC exposed to hippocampal culture conditioned medium; 3- MSC exposed to hippocampal culture + OGD conditioned medium. The pictures are representative of at least 4 different experiments.

Some authors have already demonstrated that, even considering germ layer different origin, neurons can be generated *in vitro* from MSC isolated from rat, mouse and human [15,16]. These studies generally use protocols of inducing differentiation based on BME, DMSO, BHA and retinoic acid combined with growth factors. These protocols are being strongly questioned once some researches believe that the observations are only an artifact [4, 5, 17]. In this work we decided to test a condition based only in neural cells secreted factors to investigate if transdifferentiation can occur.

A study was already performed using soluble factors derived from different brain regions without any lesion (cortex, cerebellum and hippocampus) to investigate if MSC could present a neuronal-like phenotype when exposed to brain extracts [18]. It was shown that factors derived from hippocampal extract were capable to induce a neuronal morphology and expression of neuronal markers in cultured MSC, in addition to promote expansion and survival of these cells. Unfortunately it is difficult to compare the results obtained here with the ones from Rivera and collaborators, once the protocols differ widely. They used brain extracts, MEM medium with only 0.5% FBS and lower MSC passages than we have used in our experiments. Also, our interest was to study MSC alteration after injury condition, a different approach than the one from Rivera and collaborators.

Abouelfetouh and collaborators showed in 2004 that MSC plated in contact with the organotypic culture of hippocampus show neuronal markers and the quantity of cells with neuronal morphology was increased after retinoic acid treatment [19]. Interestingly, cells plated in the same culture membrane, but not over the hippocampal slice, were incapable to differentiate, suggesting that the contact of MSC with the tissue is essential for differentiation. Our results point in the same direction from those from Abouelfetouh and collaborators, once only the use of conditioned medium was unable to change MSC morphology and phenotype. We are not sure if transdifferentiation of MSC can in fact occur, but if it occurs we also believe that contact with hippocampal cells is necessary. Our experiments show for the first time that not even the presence of an environment were hippocampal cultures were lesioned was able to induce any transdifferentiation of MSC.

It is important to mention here that cells between the 10th and 25th passages were used in this study because it was already demonstrated by Nardi's group that only after 10 passages mouse MSC cultures can be described as free of terminally differentiated hematopoietic cells [12]. Also, the effect of prolonged cultivation on the capacity of these cells to differentiate was determined, showing that MSC cultures with more than 32 passages still maintain their stem cell capacity, differentiating in osteoblasts and adipocytes [20].

Recent evidence using GFP positive MSC transplanted into rat adult brains suggest that these cells are rejected by an inflammatory response and transfer donor labels, in this case BrdU, to host neurons and glia, questioning the pluripotency of MSC in generating neurons and glial cells [21]. In our controlled system we have shown that MSC proliferate in injury-stimulated conditions, but certainly the uncontrolled inflammatory response needs to be taken into account *in vivo*. The same word of caution against the interpretation of studies showing the pluripotency of cells after grafting them to the brain was discussed by Burns and collaborators, who demonstrated that even after transplantation of dead cells or BrdU labeled fibroblast it is possible to find BrdU positive neurons and astrocytes [22].

Animal studies have already shown that bone marrow stromal cells injection after cerebral ischemia in rats improves their functional deficit with very rare differentiation of the stem cells [3, 23]. Also in other injuries, such as stab wound, the results suggest that, *in vivo*, bone marrow derived cells failed to transdifferentiate into neural cells [24]. Even in the studies where the authors suggest neuronal differentiation of these cells, there is still a doubt whether these new neurons are physiologically functional [6, 17]. The neuroprotection observed upon injection of MSC after injuries seems to be mainly explained by secreted factors that help the tissue to recover and not a direct differentiation of these cells into neurons [3]. At the moment there seems to be uncertainty if transdifferentiation of MSC into neurons really occurs and if it is biologically possible. Many issues needs to be further examined and clarified before the therapeutic use of this cells can be ordinarily used.

Even if MSC can not transdifferentiate into functional cells in the nervous system, we believe that they could be useful for the treatment of some specific diseases. The use of engineered stem cells as cell factories for delivery of protective biologically active compounds, such as growth factors and protective or anti-inflammatory chemokines/cytokines could be used in early stages of these diseases. Much research on this field is still necessary until stem cells can be approved for using in clinical approaches.

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DISCUSSÃO

Os resultados obtidos nessa tese mostram que o meio condicionado por MSC isoladas de medula óssea de ratos é capaz de induzir a morte celular em regiões específicas do hipocampo. Esse meio também é capaz de disparar respostas inflamatórias e induzir estresse oxidativo nas culturas organotípicas de hipocampo. Ainda, agrava a lesão induzida por privação de oxigênio e glicose, fazendo com que células de todas as regiões do hipocampo morram. Esse conjunto de resultados nos permite sugerir que as MSC possam ter um efeito adverso no tecido nervoso, atribuído a algun(s) fatore(s) que elas podem secretar.

Dentre os poucos trabalhos que já demonstraram possíveis efeitos adversos do uso das MSC temos o trabalho desenvolvido por Djouad e colaboradores, onde os autores mostram que a administração tanto subcutânea quanto intravenosa de MSC em camundongos favoreceu o desenvolvimento de tumor de melanoma, sendo esse efeito atribuído à capacidade imunossupressora sistêmica dessas células, tornando-as perigosas, uma vez que podem inibir a resposta antitumoral do paciente (Djouad et al., 2003). Karnoub e colaboradores mostraram em 2007 que MSC humanas aumentam o potencial metastático de células de tumor de mama, provavelmente por seu efeito parácrino nessas células, secretando citocinas como CCL5, que aumentam a invasividade das células tumorais (Karnoub et al., 2007). Ainda, Fazel e colaboradores mostraram em 2008 que, apesar de melhorarem a função cardíaca num modelo de infarto do miocárdio, MSC geneticamente modificadas para aumentarem a expressão de SCF (fator de célula tronco) induziram a formação de fibrosarcomas e de

metástases em 4 dos 20 animais injetados (Fazel et al., 2008). Não encontramos na literatura nenhum estudo sugerindo um possível efeito adverso dessas células no SNC.

Os estudos *in vitro*, apesar das suas limitações, são ferramentas importantes para o estudo controlado de interações entre células e de toxicidade de substâncias. A cultura organotípica de hipocampo é amplamente utilizada para esse fim (Sundstrom et al., 2005) e muitos estudos de morte celular (Valentim et al., 2003; Horn et al., 2005; Cavaliere et al., 2006; Frozza et al., *in press*) e de ativação microglial e inflamação (Heppner et al., 1998; Skibo et al., 2000; Hailer et al., 2005; Strassburger et al., 2008) já foram validados e realizados nesse modelo, o que justifica sua utilização nesse trabalho. As MSC de medula óssea aqui utilizadas foram semeadas sempre entre a 10ª e a 25ª passagens, com o intuito de padronizar sua faixa de utilização, não permitindo que estivessem ainda contaminadas com macrófagos ou que fossem usadas em passagens muito avançadas (da Silva Meirelles e Nardi, 2003; da Silva Meirelles et al., 2006). Essa faixa de utilização nos pareceu uma alternativa segura para o desenvolvimento do trabalho. Sendo assim, decidimos usar culturas organotípicas de hipocampo e córtex de ratos e MSC de medula óssea e pulmão de ratos e camundongos para atingir os nossos objetivos.

Para facilitar a discussão dos resultados eles serão agrupados em três subcapítulos, o primeiro discutindo o porquê do meio condicionado pelas MSC agravar a lesão por POG, o segundo discutindo um possível mecanismo de ação dessas células no SNC e o terceiro falando sobre como as MSC respondem ao meio condicionado pelo SNC após uma lesão.

1) Os fatores secretados pelas MSC agravam a morte celular causada pela POG

As MSC secretam vários fatores de crescimento já descritos como neuroprotetores e tidos como benéficos, dentre eles BDNF, NGF, VEGF, HGF e GDNF (Chen et al., 2002; Chopp e Li, 2002). Entretanto, pouco se sabe até o momento sobre fatores secretados que possam ser maléficos às células do SNC. Dois grupos independentes mostraram em 2003 que o GDNF, até então tido como um fator de crescimento promissor para o desenvolvimento de uma possível terapia neuroprotetora (Kilic et al., 2003), é capaz de aumentar a morte neuronal induzida por isquemia tanto *in vitro* (Bonde et al., 2003) quanto *in vivo* após oclusão da artéria cerebral média (Arvidsson et al., 2003), introduzindo uma nota de cautela na utilização desse fator de crescimento. Os autores sugerem que o GDNF seja capaz de regular positivamente os transportadores de glutamato, que num momento de falência energética como na isquemia cerebral funcionam liberando glutamato para a fenda sináptica, aumentando a excitotoxicidade. Esse bloqueio na captação do glutamato parece ser o responsável pela toxicidade induzida pelo GDNF após a isquemia (Bonde et al., 2003). Como observamos em nossos resultados que o mecanismo de excitotoxicidade é disparado pelo fatores secretados pelas MSC, não descartamos que um desses fatores possa ser o GDNF. Assim como para exemplo do GDNF, acreditamos que estudos possam mostrar em um futuro próximo que todos esses fatores podem ser protetores ou não ao SNC, dependendo do tipo celular, da lesão e da situação biológica que o SNC encontra-se naquele momento. Assim, considerando as MSC como “fábricas” de fatores tróficos, capazes de alterar seu padrão de secreção desses de acordo com o nicho em que se encontram, temos a plena convicção de que conhecer os

fatores secretados e como eles podem ser alterados torna-se fundamental para uma possível utilização dessas células em terapias.

No capítulo 1 desse trabalho vimos que o efeito tóxico do meio condicionado parece ser uma característica das MSC, não importando se são isoladas de medula óssea ou de pulmão ou se são obtidas de ratos Wistar ou camundongos C57Bl6. O meio condicionado pelas MSC sempre causou morte no hipocampo e agravou a lesão por POG. Um estudo desenvolvido por Cavaliere e colaboradores mostra que o meio condicionado por culturas organotípicas de zona subventricular é capaz de aumentar o dano causado pela POG em culturas organotípicas de hipocampo em torno de 50%. Considerando que a zona subventricular é rica em células tronco neurais (NSC), imaginamos que essas células possam ter um perfil de secreção de fatores semelhante às MSC, apresentando por isso a mesma toxicidade (Cavaliere et al., 2006). Mesmo não tendo testado diretamente fatores secretados por NSC em nosso modelo, parece-nos que o mecanismo de toxicidade induzido por essas células também com características "tronco" possa ser semelhante.

Um ponto bastante intrigante é tentar entender por que há uma vulnerabilidade apenas das células das regiões CA1, CA2 e CA3 do hipocampo aos fatores secretados pelas MSC. O que exatamente há de peculiar nessas regiões não está descrito na literatura, mas sabe-se que as regiões dentro do próprio hipocampo respondem de forma diferente a diferentes lesões. Frente à isquemia cerebral, por exemplo, as regiões formadas por neurônios piramidais CA1 são mais sensíveis, sendo os neurônios granulares do DG resistentes (Schmidt-Kastner e Freund, 1991; Horn et al., 2005), característica essa também

evidenciada nos resultados aqui apresentados (Capítulo 1, figura 1A). Outras doenças como esquizofrenia, por exemplo, afetam seletivamente as células em CA3, que também apresenta maior sensibilidade aos corticosteróides e ao cainato (Kolomeets et al., 2007). Sabe-se ainda que o DG sofre mais rapidamente os efeitos da inibição da via de sinalização PI3K (Horn et al., 2005) e que um antagonista do receptor GABA, a bicuculina, mata seletivamente as regiões CA2 e CA3 (Han et al., 2005). Essas diferenças são geralmente atribuídas a diferentes sensibilidades ao estresse oxidativo (Wang et al., 2005), à excitotoxicidade dos receptores NMDA (Gee et al., 2006), à acidose (Cronberg et al., 2005) e também a diferenças na permeabilidade do poro mitocondrial (Mattiasson et al., 2003) e no retículo endoplasmático (Kosuge et al., 2008) das diferentes regiões. Como não foi possível identificar e caracterizar qual ou quais os fatores secretados pelas MSC responsáveis pela toxicidade observada, e diante das diferenças existentes entre as diferentes áreas do hipocampo citadas acima, qualquer especulação que justifique tal efeito torna-se difícil.

Muitos dos trabalhos citados na literatura mostrando o efeito benéfico da administração de MSC em modelos animais e humanos de isquemia cerebral são baseados em resultados da melhora em parâmetros neurológicos e comportamentais (Chen et al., 2001; Borlongan et al., 2004; Bang et al., 2005; Kurozumi et al., 2005; Nomura et al., 2005; Horita et al., 2006; Onda et al., 2008). Avaliando-se cuidadosamente esses estudos, percebemos que a melhora após a utilização dessas células é obtida após uma isquemia focal, geralmente com oclusão da ACM, sendo que muitos trabalhos utilizam MSC modificadas geneticamente para aumentar a produção de algum fator de crescimento

(Kurozumi et al., 2005; Nomura et al., 2005; Horita et al., 2006; Onda et al., 2008). Há apenas um estudo na literatura mostrando o efeito benéfico das MSC humanas após uma isquemia global em camundongos. Os autores atribuem esse efeito às propriedades imunomoduladoras das MSC (Ohtaki et al., 2008). O modelo de POG utilizado nesse trabalho é um modelo de isquemia global, uma vez que todo o hipocampo permanece sem glicose e oxigênio por um determinado período. O fato de ser um modelo global, aliado ao fato de estarmos trabalhando com um modelo *in vitro*, poderia explicar porque nossos resultados vão de encontro aos resultados benéficos da terapia celular após isquemia cerebral focal já descritos na literatura. Além disso, a avaliação comportamental descrita nesses artigos não pode ser comparada com os parâmetros celulares aqui analisados.

Uma vez que a isquemia cerebral por si só causa excitotoxicidade e inflamação (Doyle et al., 2008), é fácil compreender porque na presença das MSC, que também são capazes de causar sua toxicidade disparando os mesmos mecanismos, esses efeitos se somam, levando à morte celular em todas as regiões do hipocampo.

2) Os fatores secretados pelas MSC induzem inflamação e excitotoxicidade

Nossos resultados mostraram que tanto um mecanismo excitotóxico quanto um mecanismo inflamatório são disparados no hipocampo por fatores secretados pelas MSC. Não é possível sabermos se esses eventos ocorrem em paralelo ou se um é a conseqüência do outro, mas certamente ambos são importantes para a toxicidade observada. Nos capítulos 1 e 2 desse trabalho vimos que a morte celular observada pode ser reduzida com o uso de antioxidantes como TROLOX e

ácido ascórbico, de antiinflamatórios como dexametasona e indometacina, de antagonistas AMPA (CNQX) e NMDA (MK801), do agonista GABA e do bloqueador de canais de cálcio dependente de voltagem nimodipina. Nesse contexto é difícil inferir o que é causa e o que é conseqüência, ou seja, qual desses eventos é primeiramente disparado pelos fatores secretados pelas MSC. Os mecanismos estão intimamente relacionados em vários pontos, e possivelmente sofrem uma retroalimentação positiva, como discutiremos a seguir.

Como já citado na introdução, os astrócitos e a microglia quando ativados secretam moléculas capazes de induzir a excitotoxicidade, o estresse oxidativo e a inflamação, como glutamato, TNFα, IL-1β, IL-6, prostaglandinas, NO e superóxido, (Block et al., 2007). Essas moléculas em níveis elevados são responsáveis pela neurotoxicidade, que pode ser disparada por vários fatores secretados pelas células. Utilizando o meio condicionado pelas MSC não é possível sabermos quais os fatores são os responsáveis pela ativação glial e pela toxicidade encontrada nesse trabalho, sendo a identificação desses fatores uma de nossas perspectivas.

Focaremos primeiramente no glutamato, molécula bem caracterizada como principal causadora da excitotoxicidade. Mesmo não tendo medido sua quantidade no meio condicionado, suspeitamos que ele esteja diretamente envolvido na morte celular observada em nosso modelo. É importante salientar que não acreditamos ser o glutamato o fator secretado pelas MSC que esteja sendo tóxico ao hipocampo, uma vez que a fervura do meio condicionado impediu seu efeito (Capítulo 1, figura 2), sugerindo que os fatores tóxicos sejam termolábeis. Acreditamos, sim, que esse glutamato seja secretado em função de algum

mecanismo já disparado pelo meio condicionado. Já foi demonstrado por Takeuchi e colaboradores em 2006 que as células microgliais ativadas liberam grandes quantidades de glutamato não por transportadores, mas por junções comunicantes, especialmente hemicanais conexina 32, sendo esse glutamato o fator mais neurotóxico que a microglia ativada pode liberar (Takeuchi et al., 2006). Essas células sintetizam glutamato a partir da glutamina extracelular na presença da enzima glutaminase e liberam-no pelas junções comunicantes (Yawata et al., 2008). Sabe-se também que astrócitos ativados liberam glutamato, sendo esse mecanismo dependente de aumento nos níveis de TNFα (Bezzi et al., 2001). A ativação glial evidenciada em nossos experimentos (Capítulo 2, figura 1) sugere que uma quantidade considerável de glutamato possa estar sendo liberada, culminando com a excitotoxicidade aqui observada. Esse aumento de glutamato ativaria os canais NMDA e AMPA (que quando inibidos protegem as células da morte, Capítulo 1, figura 4), facilitando a entrada de cálcio nas células neuronais. A despolarização da membrana abriria também os canais de cálcio dependentes de voltagem, colocando ainda mais cálcio para dentro da célula (processo esse inibido pela nimodipina, Capítulo 1, figura 4). Os efeitos tóxicos do cálcio são bem conhecidos e incluem a ativação de lipases, proteases e DNAses, que culminarão com a morte celular (Arundine e Tymianski, 2003; Mattson, 2007). O agonista GABA também foi capaz de reduzir a morte induzida pelo meio condicionado, possivelmente por seu potencial de inibir a despolarização da membrana neuronal, evitando assim a entrada de cálcio (Capítulo 1, figura 3).

A nimodipina, bloqueador de canais de cálcio dependentes de voltagem, foi eficaz na proteção das culturas aos efeitos tóxicos do meio condicionado (Capítulo 1, figura 4), provavelmente por seu efeito em evitar o aumento do cálcio intracelular e assim de seus efeitos deletérios. Porém, outro mecanismo de ação está sendo proposto para essa substância. Li e colaboradores mostraram que ela inibe a ativação microglial, inibindo assim a degeneração causada pela inflamação. A nimodipina é capaz de inibir a produção de NO, TNFα, IL-1β e PGE2 em um modelo de ativação microglial estimulada por LPS (Li et al., 2009). Sendo assim, acreditamos que em nosso modelo ambas as ações da nimodipina foram importantes, com ênfase no seu efeito em diminuir a resposta inflamatória.

O TNFα é uma citocina liberada pela microglia ativada, pelos astrócitos reativos e pelos neurônios. Em grandes quantidades sabe-se que é um importante causador de neurotoxicidade, basicamente por induzir a apoptose nas células através de seus receptores específicos e de alterar a integridade da barreira hemato-encefálica, facilitando a infiltração das células do sistema imunológico (Sriram e Callaghan, 2007). Zou e Crew mostraram que o TNFα é capaz de inibir a recaptação de glutamato pela glia, o que potencializa o efeito neurotóxico do glutamato. Ainda, a toxicidade de TNFα/glutamato pode ser bloqueada por antagonistas NMDA (Zou e Crew, 2005). Já foi demonstrado também que o TNFα é capaz de induzir um aumento do número de receptores AMPA/cainato responsívos a cálcio na membrana neuronal (Ogoshi et al., 2005), permitindo uma maior entrada desse íon na célula e aumentando assim sua vulnerabilidade à excitotoxicidade. Considerando os altos níveis de TNFα encontrados no meio de

cultura após a exposição das culturas organotípicas de hipocampo ao meio condicionado pelas MSC (Capítulo 2, Figura 4A) e que os antagonistas dos receptores NMDA e AMPA foram capazes de bloquear seu efeito tóxico (Capítulo 1, figura 4), sugerimos que um mecanismo semelhante esteja ocorrendo em nosso modelo. Além disso, já foi demonstrado por Eligini e colaboradores que altos níveis de TNFα são capazes de aumentar os níveis da enzima ciclooxigenase-2 (COX-2), responsável pela geração de prostanóides e propagação da resposta inflamatória, culminando também com a neurotoxicidade e morte neuronal (Eligini et al., 2005). A inibição de COX-2 já foi mostrada por vários autores como sendo neuroprotetora (Nakayama et al., 1998; Araki et al., 2001). Nossos resultados mostraram um claro efeito da indometacina, antiinflamatório não esteróide, em reduzir a morte celular induzida pelo meio condicionado pelas MSC (Capítulo 2, figura 5), sugerindo que essa ação possa ser via inibição da proteína COX-2, uma vez que essa droga age inibindo a expressão dessa enzima.

Uma outra função muito interessante já atribuída ao TNFα num modelo de artrite induzida por colágeno em ratos pode ser útil para interpretar nossos resultados. Djouad e colaboradores mostraram que a injeção de MSC nos animais com artrite não lhes conferiu nenhum benefício, ao contrário, essas células foram capazes de acentuar a resposta inflamatória do tipo TH1. O mecanismo sugerido para esse fenômeno foi que os altos níveis de TNFα são capazes de reverter o fenótipo imunossupressor das MSC, fazendo com que percam suas propriedades antiinflamatórias e passem a apresentar propriedades pró-inflamatórias. Além disso, as concentrações de IL-6 foram muito aumentadas pela presença de TNFα,

contribuindo para a resposta pró-inflamatória observada (Djouad et al., 2005). Os altos níveis de TNFα e de IL-6 observados no meio de cultura em nossos experimentos (Capítulo 2, figura 4) permitem-nos especular que sejam em parte responsáveis pela ausência do efeito imunossupresor das MSC. Talvez os fatores secretados por essas células não sejam diretamente pró-inflamatórios, mas uma vez que são capazes de induzir direta ou indiretamente a ativação glial e disparar a secreção dessas citocinas, o sistema pode entrar num ciclo vicioso de retro-alimentação positiva, aumentando a neurotoxicidade.

Em situações de estresse celular a proteína iNOS passa a ser expressa principalmente nas células microgliais e nos astrócitos, produzindo assim altas quantidades de NO, que conjugado ao ânion superóxido forma o peroxinitrito, composto altamente reativo também envolvido com a neurotoxicidade (Xie et al., 2002). Além disso, o NO por si só é capaz de induzir a morte celular de neurônios, principalmente por sua capacidade de inibição da cadeia respiratória e da liberação de glutamato e conseqüente excitotoxicidade por ele disparada (Bal-Price e Brown, 2001). Stewart e colaboradores mostraram em 2002 que esse efeito tóxico do NO pode ser inibido por MK801, antagonista NMDA, sugerindo novamente o envolvimento do glutamato na morte induzida por NO (Stewart et al., 2002). Já foi demonstrado também que a iNOS pode ter sua expressão aumentada em resposta a secreção de citocinas como TNFα, IL-6 e IL-1β (Chapple, 1997; Mingheti e Levi, 1998; Combs et al., 2001). Além disso, sabe-se que o NO é capaz de aumentar a expressão de COX-2, propagando assim a reação inflamatória, podendo esse efeito ser inibido pela indometacina (Salvemini

et al., 1993; Pauletti et al., 1998). Considerando que observamos um aumento de 50% no conteúdo da proteína iNOS em nossos resultados (Capítulo 2, figura 2B), que houve um aumento significativo na produção de espécies reativas (Capítulo 2, figura 2A) e que L-NAME e antioxidantes como ácido ascórbico e TROLOX foram capazes de reduzir a morte celular induzida pelo meio condicionado (Capítulo 2, figura 3), concluímos que o estresse oxidativo e suas conseqüências contribuem para a toxicidade induzida pelas MSC no hipocampo. Além disso, a proteção que observamos após a utilização de MK801 (Capítulo 1, figura 4) também pode ser explicada aqui pela presença de excesso de glutamato (agora de origem neuronal) causando excitotoxicidade disparada diretamente por NO.

É importante lembrarmos que espécies reativas estão sendo geradas por diferentes estímulos e possivelmente nos vários tipos celulares presentes no hipocampo. Onde exatamente os fatores secretados pelas MSC disparam o sinal não sabemos, mas suspeitamos que possa haver um mecanismo de retroalimentação muito eficiente, que culmina com a morte celular. Isso justificaria a eficiência das drogas antioxidantes utilizadas na redução da toxicidade observada. Além de diminuírem a geração de ROS pelas células gliais elas atuam também nos neurônios, diminuindo a geração de ROS oriunda da entrada de cálcio nas células, disparada pelo glutamato. Os antiinflamatórios também possuem uma importante função na redução direta do estresse oxidativo. A dexametasona, por exemplo, é capaz de inibir diretamente a expressão de iNOS e a geração de NO (Hämäläinen et al., 2008).

Um esquema com o que especulamos ser o mecanismo de toxicidade induzido pelo meio condicionado pelas MSC está apresentado na figura 6.

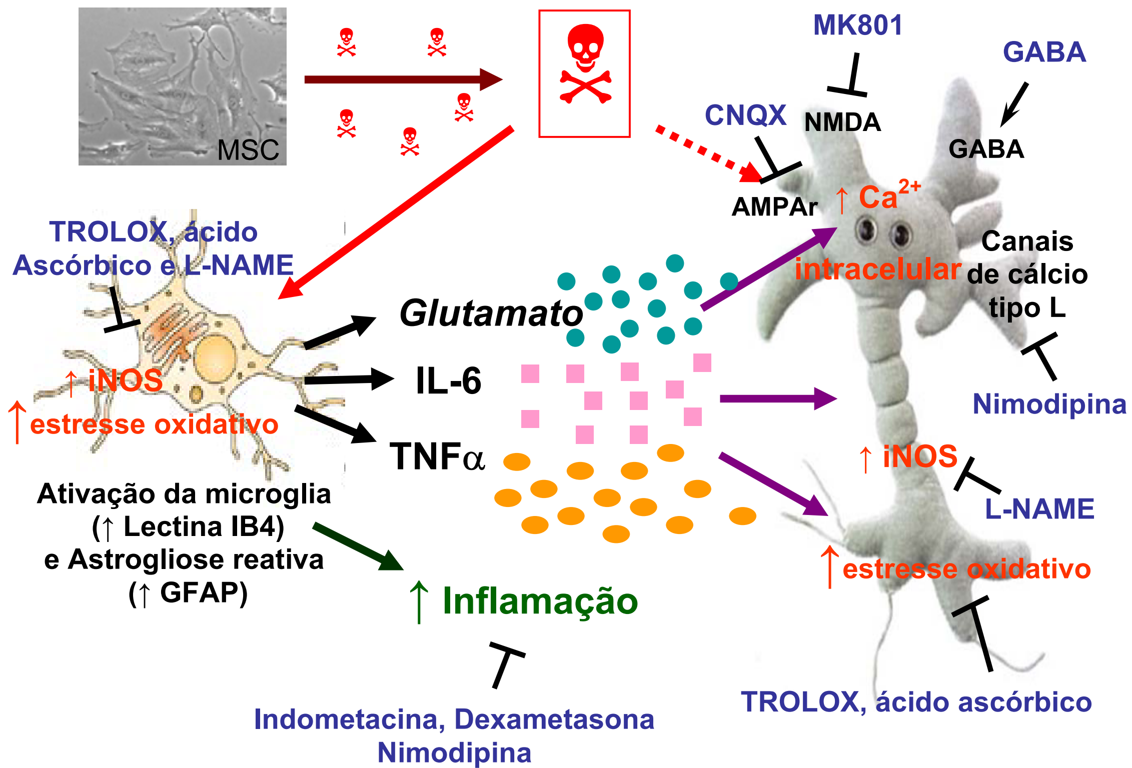

Figura 6: Esquema com o mecanismo proposto para a toxicidade do meio condicionado pelas MSC de medula óssea sobre o tecido nervoso.

3) Resposta das MSC ao meio condicionado pelo tecido nervoso

Além de estudar como o tecido nervoso responde aos fatores secretados pelas MSC, ficamos interessados também em investigar como as MSC respondem aos fatores secretados pelo tecido nervoso, principalmente quando esse tiver sofrido uma lesão. Os resultados obtidos nos permitem concluir que esses fatores não induziram morte celular ou diferenciação das MSC, mantendo essas a sua morfologia característica e o seu potencial proliferativo, que inclusive foi aumentado na presença do meio condicionado pelo hipocampo exposto à POG.

Como já comentado na introdução, a literatura nos traz vários trabalhos mostrando uma possível diferenciação das MSC em células neurais, principalmente neurônios. Os protocolos utilizados são bastante agressivos e atualmente muito questionados e considerados apenas artefatos da técnica (Lu et al., 2004; Neuhuber et al., 2004; Krabbe et al., 2005; Lu e Tymanski, 2005). Nossos resultados corroboram com os trabalhos acima citados, uma vez que vimos aqui que apenas na presença do meio condicionado pelo tecido nervoso, mesmo tendo sofrido lesão, as MSC não são capazes de diferenciar em novos neurônios. Assim, concordamos com a visão de pesquisadores como Michael Chopp e Gary Steinberg, que atribuem os possíveis efeitos benéficos dessas células apenas aos fatores por elas secretados, e não ao fato de diferenciarem em outras células e repovoarem o cérebro após serem transplantadas (Chopp e Li, 2002; Guzman et al., 2008).

O fato das MSC manterem sua capacidade proliferativa pode ser interpretado como um efeito benéfico ou perigoso, dependendo da situação. Se por um lado essas células forem implantadas no SNC para tratamento de doenças neurodegenerativas, e mantendo sua proliferação secretarão fatores neuroprotetores, elas poderão ter um efeito benéfico (Kan et al., 2007). Por outro lado, vimos que fatores neurotóxicos também podem ser secretados, tornando a perpetuação dessas células uma desvantagem. É importante salientar que os resultados do estudo aqui realizado foram uma constatação *in vitro* do potencial proliferativo das MSC e não podemos sugerir que o mesmo efeito seja obtido quando essas células forem implantadas *in vivo*. Coyne e colaboradores já mostraram que MSC transplantadas no cérebro de animais adultos ativaram a

microglia e foram rejeitadas pela resposta inflamatória, sendo que em 7 dias praticamente todas as MSC já estavam mortas (Coyne et al., 2006).

Com base nos resultados do trabalho aqui apresentado concluímos que o possível uso terapêutico dessas células para doenças do SNC deve ser exaustivamente estudado antes de sua utilização em pacientes, uma vez que o conjunto de efeitos adversos causados por essas células ainda é desconhecido. A necessidade de uma alternativa terapêutica para o tratamento de doenças incuráveis justifica a euforia vivida e a esperança de utilização das células tronco, mas acreditamos que muita cautela e experimentação animal são necessárias antes que se essas células sejam amplamente utilizadas em humanos.

Apesar dos possíveis efeitos colaterais e discussões aqui expostas, acreditamos sim que as MSC possam ser utilizadas para a terapia celular de algumas doenças que afetam o SNC, principalmente aquelas onde apenas uma população específica de neurônios está envolvida e num local restrito do sistema. Muito estudo ainda é necessário até que os fatores benéficos e os fatores prejudiciais secretados por essas células sejam identificados. Ao que parece, a engenharia genética será uma grande ferramenta na confecção das células ideais a serem transplantadas nos pacientes, reduzindo o risco de possíveis efeitos adversos. Sabendo que o ambiente onde essas células são transplantadas é capaz de modificar suas propriedades, muito estudo ainda é necessário até que entendamos suas características biológicas e como respondem aos diferentes estímulos, para daí sim podermos utilizá-las em tratamentos.

CONCLUSÕES

Os resultados apresentados nessa tese nos permitem concluir que:

1) O meio condicionado por MSC isoladas de medula óssea de ratos é tóxico para as regiões CA1, CA2 e CA3 de culturas organotípicas de hipocampo;
2) A lesão causada por POG em culturas organotípicas de hipocampo é agravada na presença do meio condicionado pelas MSC;
3) A morte celular induzida pelo meio condicionado pelas MSC possui características de uma morte excitotóxica;
4) O meio condicionado pelas MSC induz ativação glial nas culturas organotípicas de hipocampo, disparando o estresse oxidativo e a inflamação;
5) As MSC mantêm sua proliferação na presença do meio condicionado pelo hipocampo lesionado, não apresentando a expressão de marcadores clássicos de neurônios ou astrócitos num período de 72 h;
6) A utilização de MSC para tratamento de doenças que afetam o SNC pode possuir efeitos adversos, sugerindo cautela na utilização dessas células.

PERSPECTIVAS

Como continuação desse trabalho, pretende-se trabalhar com os seguintes objetivos:

- Medir a concentração de glutamato no meio condicionado pelas MSC;
- Investigar se o meio condicionado por quantidades menores de MSC é tóxico para o hipocampo e se é capaz de proteger da POG;
- Investigar se a mesma quantidade de MSC utilizada nesse trabalho induz inflamação ou toxicidade quando colocada <u>sobre</u> a fatia de hipocampo, tendo assim contato com essa;
- Investigar se o fato das MSC estarem em contato com as fatias hipocampais com ou sem lesão é capaz de induzir nelas um fenótipo de células neurais, investigando marcadores neuronais, astrocíticos e de oligodendrócitos;
- Investigar, *in vivo*, se a injeção dessas MSC no hipocampo de ratos é capaz de causar alterações significativas nos parâmetros inflamatórios e de toxicidade nas regiões CA1, CA2 e CA3, bem como em testes comportamentais de memória nesses animais;
- Isolar e caracterizar no meio condicionado quais os fatores secretados pelas MSC responsáveis pela indução de inflamação e toxicidade.

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ANEXO 1

Co-autoria de artigos publicados durante o doutorado:

1. **Estradiol protects against oxygen and glucose deprivation in rat hippocampal organotypic cultures and activates Akt and inactivates GSK-3β.** Cimarosti H, Zamin LL, Frozza R, Nassif M, **Horn AP**, Tavares A, Netto CA, Salbego C. *Neurochem Res*. 2005; 30: 191-199.

2. **The effects of estradiol on estrogen receptor and glutamate transporter expression in organotypic hippocampal cultures exposed to oxygen-glucose deprivation.** Cimarosti H, O'Shea RD, Jones NM, **Horn AP**, Simão F, Zamin LL, Nassif M, Frozza R, Netto CA, Beart PM, Salbego C. *Neurochem Res*. 2006; 31: 483-490.

3. **Protective effect of resveratrol against oxygen-glucose deprivation in organotypic hippocampal slice cultures: Involvement of PI3-K pathway.** Zamin LL, Dillenburg-Pilla P, Argenta-Comiran R, **Horn AP**, Simão F, Nassif M, Gerhardt D, Frozza RL, Salbego C. *Neurobiol Dis.* 2006; 24: 170-182.

4. **Beta-amyloid peptide toxicity in organotypic hippocampal slice culture involves Akt/PKB, GSK-3β, and PTEN.** Nassif M, Hoppe J, Santin K, Frozza R, Zamin LL, Simão F, **Horn AP**, Salbego C. *Neurochem Int.* 2007; 50: 229-235.

5. **A Comparative Study of beta-Amyloid Peptides Aβ1-42 and Aβ25-35 Toxicity in Organotypic Hippocampal Slice Cultures.** Frozza RL, **Horn AP,** Hoppe JB, Simão F, Gerhardt D, Comiran RA, Salbego CG. *Neurochem Res*. 2009; 34: 295-303.

6. **Effects of Chronic Restraint Stress and Estradiol Replacement on Glutamate Release and Uptake in the Spinal Cord from Ovariectomized Female Rats.** Crema LM, Vendite D, **Horn AP**, Diehl LA, Aguiar AP, Nunes E, Vinade L, Fontella FU, Salbego C, Dalmaz C. *Neurochem Res.* 2009; 34: 449-507.

7. **Boldine: a potential new antiproliferative drug against glioma cell lines.** Gerhardt D, **Horn AP**, Gaelzer MM, Frozza RL, Delgado-Cañedo A, Pelegrini AL, Henriques AT, Lenz G, Salbego C. *Invest New Drugs*. 2008; *in press*.

8. **Protective profile of oxcarbazepine against oxygen-glucose deprivation in organotypic hippocampal culture could involve PI3K cell signaling pathway.** Simão F, Zamin LL, Frozza R, Nassif M, **Horn AP**, Salbego CG. *Neurol Res*. 2008; *in press*.