Pontifícia Universidade Católica do Rio Grande do Sul

Faculdade de Biociências

Programa de Pós-Graduação em Biologia Celular e Molecular

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Efeito terapêutico de células-tronco mesenquimais no tratamento da sepse experimental

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“A ciência nunca resolve

um problema sem criar

pelo menos outros dez”

George Bernard Shaw

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SUMÁRIO

LISTA DE ABREVIATURAS

ALT - Alanina Aminotransferase

AMs - Antimicrobianos

AST - Aspartato Aminotransferase

ATP - Adenosina Trifosfato

DCs – Células Dendríticas

FiO_2 - Fração Inspirada de Oxigênio

IFN-γ – Interferon gama

IL-1 – Interleucina 1

IL-4 – Interleucina 4

IL-6 – Interleucina 6

IL-8 – Interleucina 8

IL-10 – Interleucina 10

IL-12 – Interleucina 12

IL-13 – Interleucina 13

MCP-1 – Proteína quimiotática de monócito 1

MSCs – Células-Tronco Mesenquimais

NADP – Nicotinamida Adenina Dinucleotídeo Fosfato

PAF – Fator Ativador Plaquetário

PAM - Pressão Arterial Média

PAs - Pressão Arterial Sistólica

PaO_2 - Pressão Parcial de Oxigênio Arterial

SRIS - Síndrome da Resposta Inflamatória Sistêmica

Th2 – Células T-Helper 2

TNF-α - Fator de Necrose Tumoral Alfa

TGF-β - Fator de Crescimento Tumoral Beta

UTI - Unidade de Terapia Intensiva

RESUMO

Sepse, uma condição médica que afeta 18 milhões de pessoas por ano no mundo, é caracterizada por um estado inflamatório generalizado causado por uma infecção. A ativação generalizada de vias de coagulação e inflamação evolui para disfunção de múltiplos órgãos, o colapso do sistema circulatório (choque séptico) e morte. Apesar de décadas de pesquisas e numerosas experimentações clínicas, pouco progresso tem sido observado no desenvolvimento de novos tratamentos e as taxas de mortalidade são praticamente as mesmas nos últimos 20 a 30 anos. Como tal, a sepse continua sendo um difícil adversário para cirurgiões e seus pacientes, logo, a busca por novas alternativas terapêuticas torna-se estritamente essencial. Recentemente as células-tronco têm emergido como uma terapia promissora para uma variedade de patologias, incluindo doenças cardiovasculares, neurodegenerativas, doença vascular periférica, doença renal, e várias outras. Seus efeitos benéficos estão relacionados principalmente às suas capacidades de se conectarem a lesões e inflamações, para atenuar a resposta inflamatória e acelerar a cicatrização de tecidos e de neoangiogênese devido a estímulos nocivos. Considerando esse potencial terapêutico, o presente estudo teve por objetivo avaliar se essas células poderiam conduzir a resposta imune de volta ao equilíbrio, atenuando a fisiopatologia da sepse e dessa forma aumentando o tempo de sobrevida em camundongos, utilizando um modelo de sepse experimental. Os resultados demonstraram que o tratamento com as células-tronco mesenquimais foi capaz de aumentar o tempo de sobrevida dos animais em estudo. Esse efeito observado deve-se à capacidade destas células de modularem a resposta imune, proporcionando uma menor lesão tecidual e a diminuição de células apoptóticas. Essas descobertas demonstram que as células-tronco mesenquimais têm potencial terapêutico e podem funcionar futuramente como um possível tratamento para a sepse.

Palavras chave: Sepse; Células-tronco mesenquimais; Imunomodulador; Inflamação.

ABSTRACT

Sepsis, a medical condition that affects 18 million people per year worldwide, is characterized by a generalized inflammatory state caused by infection. The widespread activation of coagulation pathways and inflammation progresses to multiple organ failure, the collapse of the circulatory system (septic shock) and death. Despite decades of research and numerous clinical trials, little progress has been made in developing new treatments and mortality rates are virtually the same in the last 20 to 30 years. As such, sepsis remains a difficult opponent for surgeons and their patients, so the search for new therapeutic alternatives becomes strictly essential. Recently stem cells have emerged as a promising therapy for a variety of diseases, including cardiovascular diseases, neurodegenerative disorders, peripheral vascular disease, renal disease, and several others. Its beneficial effects are due mainly to their ability to connect to injury and inflammation, to attenuate the inflammatory response, and accelerate tissue healing and neoangiogenesis due to noxious stimuli. Considering this therapeutic potential, this study aimed to evaluate whether these cells could lead to immune response back into balance, reducing the pathophysiology of sepsis and thereby increase the survival time in mice using an experimental model of sepsis. Our results demonstrated that treatment with mesenchymal stem cells was able to increase survival time of the animals that were tested. This effect is due to the ability of these cells to modulate the immune response providing a smaller reduction in tissue injury and apoptotic cells. These findings demonstrate that mesenchymal stem cells have therapeutic potential and can function as a possible future treatment for sepsis.

Keywords: Sepsis; Mesenchymal stem cells; Immunomodulator; Inflammation.

Capítulo 1

Introdução

Justificativa

Objetivos

1. INTRODUÇÃO

1.1 Definição

A sepse é uma síndrome complexa de origem infecciosa, ocasionando uma resposta inflamatória sistêmica descontrolada do indivíduo. É caracterizada por manifestações múltiplas que podem determinar disfunção ou até mesmo a falência de um ou mais órgãos, e consequentemente sua morte. Seus fatores fisiopatológicos incluem, principalmente, o local da infecção, sendo os sistemas da coagulação, fibrinolítico e inflamatório os determinantes de sua evolução[1].

O termo septicemia vem sendo usado desde a Grécia antiga para descrever casos onde havia putrefação associado com doença e morte[2]. Esta patologia foi descrita primeiramente por Tilney *et al*[3] em 1973, como "falência sistêmica seqüencial", abrangendo três pacientes que evoluíram para óbito por falência orgânica. Em 1975 Baue[4] descreveu três pacientes como "falência orgânica sistêmica progressiva, múltipla, ou seqüencial".

Devido a esta grande quantidade de termos sinônimos para designar a mesma condição clínica e a sua gravidade, em agosto de 1991, uma nova definição foi estabelecida pelo *American College of Chest Physicians* e a *Society of Critical Care Medicine*, determinando assim um consenso sobre as definições e os critérios para o diagnóstico da sepse[5]. Em 2001, a *International Sepsis Definitions Conference* (tabela 1), congregando um maior número de

pesquisadores e peritos de várias partes do mundo, optou por não modificar as definições vigentes e sim por ampliar a lista de sinais e sintomas da sepse[6].

Tabela 1- Critérios para diagnóstico na sepse

Infecção documentada ou suspeita e algum dos seguintes critérios:
– Variáveis gerais
Febre (temperatura central > 38,3º C)
Hipotermia (temperatura central < 36º C)
Freqüência cardíaca > 90 bpm ou > 2 DP acima do valor normal para a idade
Taquipnéia
Alteração de sensório
Edema significativo ou balanço hídrico positivo (> 20 ml/kg/24 horas)
Hiperglicemia na ausência de diabete (glicemia > 120 mg/dl)
– Variáveis inflamatórias
Leucocitose (contagem leucócitos totais > 12.000 / mm^3)
Leucopenia (contagem leucócitos totais < 4.000 / mm^3)
Contagem de leucócitos totais normal com > 10% de formas imaturas
Proteína C-reativa no plasma > 2 DP acima do valor normal
Procalcitonina plasmática > 2 DP acima do valor normal
– Variáveis hemodinâmicas
Hipotensão arterial (PAs < 90 mmHg, PAM < 70 mmHg, ou
Redução da PAs > 40 mmHg em adolescentes, ou PAs / PAM < 2 DP abaixo do normal para idade)
Saturação de oxigênio venoso misto > 70% (não válido para crianças)
Índice cardíaco > 3,5 L/min (não válido para crianças)
– Variáveis de disfunção de órgãos
Hipoxemia arterial (PaO_2 / FiO_2 < 300)

Oligúria aguda (diurese < 0,5 mL/kg/h)
Creatinina > 0,5 mg/dL
Alterações de coagulação (INR > 1,5 ou TPPA > 60 s)
Íleo (ausência de ruídos hidroaéreos)
Trombocitopenia (contagem de plaquetas < 100.000 / mm^3)
Hiperbilirrubinemia (Bilirrubina total > 4 mg/dL)
– Variáveis de perfusão tecidual
Hiperlactatemia (> 1 mmol/L)
Enchimento capilar reduzido ou moteamento

BPM: batidas por minuto; DP: desvio padrão; PAs: pressão arterial sistólica; PAM: pressão arterial média; PaO_2: pressão parcial de oxigênio; FiO_2: fração inspirada de oxigênio; INR: razão normalizada internacional; TTPA: tempo de tromboplastina parcial ativada.

1.2 Epidemiologia

A sepse tem representado um grave problema epidemiológico para os sistemas de saúde em todo o mundo, tanto do ponto de vista econômico como social. Atualmente a sepse acomete cerca de 18 milhões de pessoas por ano no mundo. De acordo com um estudo epidemiológico nos EUA, a incidência da sepse aumentou de 82,7 para 240,4/100 mil habitantes, bem como as mortes relacionadas a ela, ainda que a taxa de mortalidade geral entre os pacientes com sepse tenha sido reduzida nesse período[7].

Nas últimas décadas o aumento nas taxas de incidência e de morbimortalidade relacionadas à sepse está diretamente relacionado aos avanços médicos, onde cada vez mais são tratados pacientes gravemente

doentes e internados nas Unidades de Terapia Intensiva (UTIs), evoluindo para sepse secundária, decorrente do comprometimento imunológico e/ou pelas condutas e procedimentos médicos[8].

A incidência da sepse relatada na literatura pode variar de acordo com as características de cada região e local, sendo que nos EUA e Europa, a sepse é responsável por 2-11% das internações em UTI[9]. Análise retrospectiva de Jacobs *et al*[10], em mais de 2.000 admissões de uma UTI pediátrica, identificou 42,5% de pacientes com doença infecciosa, dos quais 63% destes evoluíram para o estado de choque séptico. Proulx *et al*[11], avaliando 1.058 admissões em UTI pediátrica do hospital universitário canadense, identificaram 82% de síndrome da resposta inflamatória sistêmica (SRIS), sendo 23% de etiologia infecciosa (sepse), das quais 2% com choque séptico. No Brasil a incidência da mortalidade provocada pela sepse e suas conseqüências varia de 40 a 45%, conforme dados do *Brazilian Sepsis Epidemiological Study*[12].

1.3 Resposta do Organismo à Infecção na Sepse

A inflamação é uma resposta normal do hospedeiro contra agentes infecciosos. A sepse é caracterizada pela produção excessiva de mediadores inflamatórios, assim como pela expressiva ativação de células inflamatórias, resultando em uma anarquia metabólica[13].

Quando a infecção ou bacteremia ocorre, a primeira linha de defesa do hospedeiro é realizada por células fagocitárias (macrófagos, monócitos e

granulócitos polimorfonucleares) e pela via alternativa do complemento, agindo de maneira não específica. Posteriormente, as imunoglobulinas e as células imunocompetentes iniciam à resposta imune específica[13, 14].

Os componentes da parede bacteriana, onde se destacam as endotoxinas (lipopolissacárideos) dos microorganismos gram-negativos (principalmente o lipídio A) e o ácido teicóico dos microorganismos gram-positivo são os principais ativadores da resposta do hospedeiro. Eles desencadeiam a cascata inflamatória através da indução da produção de citocinas pelos macrófagos e monócitos, que, quando ativados, produzem sequencialmente, Fator de Necrose Tumoral Alfa (TNF-α), Interleucina-1 (IL-1), Interleucina-6 (IL-6) e a Interleucina-8 (IL-8) que interagem com outras células e elementos celulares (polimorfonucleares, células endoteliais, fibroblastos, plaquetas e os próprios monócitos), induzindo a produção e liberação de mediadores secundários, contribuindo para uma resposta inflamatória tardia[14, 15].

Paralelamente à liberação das citocinas pró-inflamatórias, o organismo responde a agentes infecciosos, liberando citocinas antiinflamatórias como Interleucina 4 (IL-4), Interleucina 10 (IL-10), Interleucina 13 (IL-13), Fator de Crescimento Tecidual Beta (TGF-β), entre outras. Estes mediadores parecem tanto contrabalançar as ações dos mediadores pró-inflamatórios, através da redução da síntese e da liberação desses mediadores, quanto antagonizar seus efeitos[16, 17].

As células endoteliais possuem um importante papel na homeostasia, regulação do tônus vascular e fibrinólise[18, 19] e quando ativadas diretamente

pelas endotoxinas ou pelas citocinas, adquirem função pró-coagulante e protrombótica, provocadas pela liberação de tromboplastina, inibidor do ativador do plasminogênio e do fator ativador plaquetário (PAF), além da diminuição da produção de trombomodulina. Elas também produzem mediadores inflamatórios, tais como as Interleucinas (IL-1, IL-6 e IL-8), prostaciclina, endotelina (capaz de aumentar o tônus vascular) e o óxido nítrico[20,21]. A destruição local do endotélio pela aderência de polimorfonucleares ativos causa aumento da permeabilidade e edema tecidual, que contribui para a ampliação da reação inflamatória[19].

Alterações nas dimensões dos pequenos vasos, juntamente com alterações bioquímicas e fisiológicas sangüíneas, prejudicam a homeostasia da microcirculação durante o choque séptico, sendo esse o principal sítio de ataque, podendo, tornar-se uma área fértil para o crescimento bacteriano descontrolado[18]. Um importante fator precipitante é a diminuição da deformidade dos eritrócitos, que depende das propriedades viscoelásticas da membrana celular, viscosidade do citoplasma e da razão entre a área de superfície corpórea e o seu volume, podendo estar todos estes fatores alterados, devido à acidose, hipotermia e alterações na geometria da hemácia[18].

1.4 Apoptose Celular e Sepse

Um dos avanços mais importantes na investigação da sepse foi definir a importância da apoptose na disfunção imune característica dos pacientes

sépticos. O papel chave da apoptose em pacientes com sepse foi demonstrado pela primeira vez em um estudo em que os pacientes em unidades de terapia intensiva que morreram de sepse foram comparados com os pacientes em unidades de terapia intensiva que morreram de etiologias não-sépticas. Autópsias de pacientes com sepse que foram realizadas dentro de 30-90 minutos após a morte do paciente (evitando assim alterações celulares devido a autólise) revelou extensa apoptose de linfócitos e células epiteliais gastrointestinais[22]. Esses achados foram semelhantes aos estudos com animais que mostram morte celular generalizada de linfócitos e de células epiteliais gastrointestinais na sepse[23-26].

Há dois mecanismos principais pelos quais a apoptose contribui para imunoparalisia na sepse. O primeiro mecanismo é através da eliminação de células efetoras cruciais. A diminuição profunda no número de células T e B prejudica a resposta imunitária adaptativa. A perda de células do sistema imunitário adaptativo também diminui a resposta imune inata por causa da importância da correlação entre os sistemas de imunidade inata e adaptativa[27,28]. A apoptose induz a diminuição no número de células dendríticas (DCs), que são os apresentadores de antígenos celulares mais potentes relacionados tanto a resposta imune inata e adaptativa. O segundo mecanismo através do qual a apoptose contribui para disfunção imune é através da indução da anergia e de células T helper 2 (Th2) em respostas a células imunes sobreviventes[29-31]. Um trabalho recente demonstrou que captação de células apoptóticas por macrófagos e DCs estimula a tolerância imunológica por induzir a liberação de citocinas anti-inflamatórias, incluindo IL-10 e fator transformador de crescimento (TGF-β), e suprimir a liberação de

citocinas pró-inflamatória. Esta ligação potencial entre a liberação de IL-10 por células em apoptose e supressão imunológica na sepse é ressaltado por estudos que mostram que a concentração circulante de IL-10 é preditivo de um desfecho fatal em pacientes com sepse[32].

Para determinar se as células apoptóticas podem diminuir a resposta imune em sepse, um estudo realizado por Hotckins e colaboradores examinou o efeito da transferência de células apoptóticas ou necróticas na produção de linfócitos T helper 1 e T helper 2 e de citocinas na sobrevivência em um modelo de sepse em camundongos. A transferência adotiva de células apoptóticas aumentou muito a mortalidade, enquanto que os esplenócitos tranferidos em necrose melhorou marcadamente a sobrevivência. Os efeitos contrastantes que as células apoptóticas e necróticas exercem sobre a sobrevivência foram distintos por possuírem efeitos opostos sobre o interferon-γ (IFN-γ), induzindo diminuição ou aumento da produção, respectivamente[33]. Estas descobertas indicam que o tipo de morte celular afeta a sobrevivência em um modelo de sepse experimental clinicamente relevante e identifica um novo mecanismo para a imunossupressão, que é uma característica da sepse em humanos.

1.5 Mitocôndria e Sepse

A disfunção de um órgão é basicamente uma disfunção celular. A principal função de uma célula é manter ativos todos os processos metabólicos nacessários para funcionamento regular das células e tecidos[34].

Durante a última década, vários estudos têm demonstrado que existe uma grave disfunção mitocondrial durante a sepse, e que isso poderia estar relacionado ao desenvolvimento de falências orgânicas e um pior prognóstico[35]. De fato, diversos estudos têm demonstrado a presença de disfunção mitocondrial em os órgãos vitais como o fígado e pulmões. A patogênese da disfunção mitocondrial acredita-se ser multifatorial, com os mecanismos propostos por apresentar déficit de substratos, danos enzimáticos e de membrana, entre outros[36,37].

O aparecimento de disfunção mitocondrial pode ocorrer na presença de níveis adequados de oxigenação dos tecidos, isto é, na ausência de hipóxia tissular, indicando um mecanismo independente ao hemodinâmico e ou da microcirculação na gênese da morte celular[37]. Por esta razão, acredita-se que a disfunção orgânica da sepse esta associada à falência do metabólismo celular associado com a mitocôndria. Vários mecanismos têm sido propostos para explicar como poderia ser mediado esse fenômeno[38], entre eles podemos citar:

- A inibição da piruvato pesidrogenase provocando um aumento da atividade de proteínas quinases através de estímulos de citocinas pró-inflamatórias como TNF-α e IL-6, prejudicando o processo de fosforilação oxidativa e consequentemente levando um acúmulo de lactato no ambiente[38].

- Aumento dos níveis de iNOS (óxido nítrico sintase-induzível). Durante a sepse existe um aumento da expressão de iNOS conseqüente também há uma maior produção de óxido nítrico (NO). O NO é capaz de reagir com o ânion superóxido (O_2) para formar o composto peroxinitrito ($ONOO^-$), altamente

reativo capaz de danificar as membranas lipídicas (peroxidação lipídica) produzir fragmentação e mutação do DNA e danos proteína[39].

- Alteração da Poli (ADP-ribose) polimerase (PARP-1). A PARP-1 é um enzima que normalmente está localizado no núcleo da célula, e é responsável pela reparação de alterações no DNA. Espécies reativas de oxigênio (radicais livres) e particularmente $ONOO^-$ são capazes de ativar a PARP-1 devido ao seu efeito de fragmentação sobre o DNA. A Ativação da Poli (ADP-ribose) polimerase (PARP-1) leva ao consumo maciço de NAD, com uma queda significativa no nível celular e diminuição da taxa de glicólise, transporte de elétrons e formação de ATP. Este fenômeno pode resultar em disfunção das células ou morte celular[38].

- A morfologia celular e conteúdo mitocondrial também são afetados durante a sepse. A alteração na morfologia mitocondrial tem sido correlacionada com grau de disfunção celular. Da mesma forma, a diminuição da conteúdo das mitocôndrias durante a sepse parece não estar relacionado com um aumento da apoptose celular, mas como um aumento lisosomal[40].

1.6 Tratamento

A resposta inflamatória sistêmica da sepse pode se restringir a um fenômeno auto-limitado ou pode progredir para quadros de maior gravidade, como sepse grave, choque séptico e disfunção ou falência de um ou mais órgãos. Apesar da grande quantidade de investigações e de relatos sobre

sepse e síndromes correlatas nos últimos anos, o controle definitivo do foco infeccioso é imperativo no tratamento, sendo a primeira prioridade. Contudo, além das medidas de suporte de vida, quando indicadas, outras medidas devem ser tomadas de acordo com a gravidade de apresentação da respectiva síndrome[41].

Os antimicrobianos (AMs) são os agentes mais específicos e acessíveis para o tratamento do paciente com infecção, embora representem uma abordagem somente parcial do problema. Nas últimas quatro décadas, os estudos sobre o efeito do uso de AMs nas infecções graves por germes gram-positivos ou gram-negativos têm demonstrado uma considerável redução da morbidade e da mortalidade. Os AMs podem ser mais úteis no tratamento de estágios clínicos precoces da sepse ou bacteremia, antes que a produção seqüencial dos mediadores da inflamação do hospedeiro determine estágios mais adiantados na cascata inflamatória, com eventuais danos teciduais graves[42]. Entretanto, alguns autores sustentam a idéia de que os AMs podem exacerbar a resposta inflamatória devido à lise dos microrganismos, com liberação de material de sua parede celular e conseqüente produção de mediadores inflamatórios endógenos[43].

Atualmente, vem sendo testadas estratégias para modular a excessiva geração ou ação de mediadores na sepse. A intervenção em qualquer passo da seqüência dos eventos fisiopatológicos que caracterizam a resposta inflamatória sistêmica da sepse, no sentido de modificar (modular) essa reação do hospedeiro, parece ser a estratégia terapêutica com maiores perspectivas de mudar os resultados na terapia da sepse. Infelizmente, o uso clínico de terapias bloqueadoras de mediadores específicos tem falhado em reduzir a

mortalidade geral associada à sepse. Contudo, a interrupção da seqüência, na patogênese, em múltiplos pontos, é a melhor chance na redução da alta mortalidade atual desta patologia[41].

1.7 Células-Tronco Mesenquimais (MSCs)

As células-tronco mesenquimais foram identificadas primeiramente por Friedenstein e Petrakova (1966), que isolaram estas células progenitoras a partir da medula de ratos e observaram serem estas células capazes de se diferenciarem em linhagem de tecido conectivo, incluindo osso, tecido adiposo, cartilagem e músculo[44].

Nos últimos anos, foi descoberto que as células-tronco mesenquimais são potentes moduladoras da resposta imune. Estas células apresentam um elevado grau de quimiotaxia, baseado em citocinas pró-inflamatórias, localizando tecidos inflamados e neoplásicos[45,46,47]. Acredita-se que a capacidade proliferativa e pluripotente destas células seja independente do tecido de origem, desde que cultivadas em condições adequadas[48,49]. Morfologicamente, estas células apresentam-se fusiformes, assemelhando-se a fibroblastos.

O tecido adiposo representa uma fonte abundante e acessível de células-tronco adultas que podem se transformar em diversas linhagens celulares. As células derivadas do tecido adiposo possuem grande similaridade

com células mesenquimais encontradas na medula óssea, e seu processo de coleta é menos invasivo[50].

Em situações clínicas agudas, como a sepse, poderia ser impossível a obtenção de células-tronco autólogas em número suficiente para ter um efeito terapêutico. Entretanto, essas células parecem ter uma vantagem única em termos de transplantadas, de tal forma, que as evidências sugerem que as MSCs podem ser "imunoprivilegiadas" na medida em que estas células, mesmo alogeneticamente ou xenogênicas quando são transplantados, podem ter uma habilidade inata para evitar a detecção pelo sistema imune do destinatário. Isto levanta a possibilidade para transplante não autólogos de MSCs como uma estratégia terapêutica. Embora mais pesquisas sobre o seu uso no tratamento em diferentes patologias sejam necessárias, é possível que MSC alogênicas pudessem ser mantidas em "bancos de células" e utilizadas terapeuticamente quando indicadas, eliminando assim a necessidade de obter células autólogas e expandi-las na fase aguda[51,52,53].

As células-tronco são potentes fontes de citocinas antiinflamatórias como fator de crescimento tecidual-β (TGF- β), IL-10 e IL-13. Além disso, atenuam a inflamação, causando uma diminuição de citocinas pró-inflamatórias como TNF-α, IL-1, IL-6 [54,55,56].

Acredita-se que a sua propriedade antiinflamatória e citoprotetora aumenta para um grau ainda maior quando as células-tronco são expostas a ambientes nocivos semelhantes aos encontrados durante a sepse. Estas características das células-tronco podem ser úteis no seu uso como agentes terapêuticos da sepse. Em um modelo de lesão pulmonar induzida por LPS,

níveis reduzidos de citocinas pró-inflamatórias após o transplante de células-tronco foram associados com menor formação de edema pulmonar, redução da permeabilidade do epitélio alveolar, e um tempo de sobrevida maior. Estes benefícios são reforçados através de fatores citoprotetores e proangiogenicos secretados pelas células-tronco como VEGF (fator de crescimento vascular endotelial), fator de crescimento de hepatócitos (HGF), fator de crescimento semelhante à insulina (IGF-1), e fator de crescimento fibroblástico (FGF)[57,58].

A capacidade das células-tronco de reduzirem a apoptose celular pode conferir ainda uma outra fonte de benefício para o seu uso na sepse. As células-tronco têm demonstrado aumento da regulação de expressão de proteínas anti-apoptóticas, tais como a Bcl-2 e a diminuição da expressão de proteínas pró-apoptóticas, tais como caspases[59].

Em uma série de experimentos nocauteando ou inibindo diferentes citocinas, Németh et al.[45] mostraram que o efeito protetor das células-tronco mesenquimais foram dependentes da citocina anti-inflamatória IL 10, a qual, não foi produzida diretamente pelas MSCs injetadas, mas pelos macrófagos. Além disso, experimentos *"in vitro"* em co-culturas com os macrófagos e MSCs sugeriram que a prostaglandina E2 (produzida pela ciclooxigenase-2 das MSCs após a ativação do receptor Toll-like 4 por lipopolissacarídeo bacteriano) foi responsável pela "reprogramação" dos macrófagos. Este efeito foi reforçado pelo envolvimento do recesptor do fator de necrose tumoral (TNF-α) nas MSCs[45].

Além disso, as células-tronco mesenquimais provenientes do tecido adiposo possuem as 5 características necessárias para a utilização em algum tipo de tratamento medicinal[51]:

- Podem ser encontradas em quantidades abundantes (milhões a bilhões de células);

- Podem ser obtidas com um procedimento pouco invasivo;

- Podem ser diferenciadas por múltiplas linhagens celulares de uma maneira regulável e reprodutível;

- Podem ser seguras e eficazes se transplantadas para um hospedeiro autólogo ou alogênico;

- Podem ser produzidas em conformidade com as orientações de boas práticas laboratoriais.

2. JUSTIFICATIVA

Nos últimos 10 anos, progressos em biologia celular e molecular mostraram que a agressão bacteriana ou de seus subprodutos (endotoxinas e exotoxinas), não são os únicos responsáveis pela deterioração clínica dos pacientes em choque séptico. A resposta do hospedeiro desempenha papel importante nos diferentes tipos de agressões, quer infecciosa ou não.

A identificação de mediadores e dos mecanismos envolvidos na produção das alterações fisiológicas, metabólicas e celulares é de grande interesse, pois estão envolvidos na perda da capacidade de homeostasia celular do organismo.

A maioria dos pesquisadores concorda que melhores taxas de sobrevida em pacientes com sepse grave só poderão ser atingidas com terapias adicionais às terapias antimicrobianas convencionais. Quanto mais se conhece a complexidade e a interdependência dos mecanismos fisiopatológicos da sepse, mais se buscam estratégias terapêuticas com base em substâncias que modulem ou interrompam os efeitos dos mediadores endógenos e exógenos da sepse.

Portanto, devido ao potencial imunomodulador, a vantagem de serem encontradas em grandes quantidades e sua administração no paciente ser pouco invasiva, as células-tronco mesequimais tornam-se hábeis a serem uma possível alternativa de tratamento para a sepse, podendo levar a interrupção da sequência, na patogênese, e a redução da alta mortalidade desta patologia.

3. OBJETIVOS

3.1 Objetivo Geral

Avaliar o efeito terapêutico e imunomodulador de células-tronco mesenquimais no tratamento da sepse.

3.2 Objetivos Específicos

3.2.1 Avaliar a sobrevida dos animais sépticos tratados com células-tronco mesenquimais;

3.2.2 Determinar o comportamento de marcadores bioquímicos nos animais sépticos tratados com células-tronco;

3.2.3 Realizar análise dos mediadores inflamatórios (TNF-α, IL-12 p70, IFN-γ, IL-6 e MCP-1) e antiinflamatórios (IL-10, TGF-β1) nos animais sépticos tratados com células-tronco;

3.2.4 Avaliar a disfunção mitocondrial nos animais sépticos tratados com células-tronco;

3.2.5 Avaliar a apoptose celular em esplenócitos nos animais sépticos tratados com células-tronco.

Capítulo 2

Mesenchymal stem cells derived from adipose tissue increases survival time by decreasing apoptosis in splenocytes in experimental model of sepsis

Artigo científico submetido ao periódico científico *Critical Care Medicine.*

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Mesenchymal stem cells derived from adipose tissue increases survival time by decreasing apoptosis in splenocytes in experimental model of sepsis

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Abstract:	Objective: The aim of this research was to test the ability of mesenchymal stem cells derived from adipose tissue derived from adipose tissue, which has immunomodulatory effects, to inhibit the septic process in an experimental model of mice. Design: Prospective, controlled animal trial. Setting: Research laboratory. Subjects: Fed male C57BL/6 mices. Interventions: Three experimental groups were formed for the test: control group, untreated septic group and septic group, treated with MSCs (1 x 106 cells/animal). Measurements and Main Results: In the control group, there were no deaths; in the untreated septic group, the mortality rate was 100% within 26 hours; in the septic group treated with MSCs, the mortality rate reached 40% within 26 hrs. The group treated with MSCs was able to reduce the markers of tissue damage of the liver and pancreas. The treated group received a reduction in inflammatory markers in comparison with the untreated group septic. Furthermore, the group treated with stem cells was able to inhibit the increase of apoptosis in splenocytes observed in septic untreated group. Conclusion: MSCs reduced the mortality rate provoked by experimental sepsis, ameliorated immune response and decreased the apoptotic death.
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Mesenchymal stem cells derived from adipose tissue increase survival time by decreasing apoptosis in splenocytes in experimental model of sepsis

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Abstract

Objective: The aim of this research was to test the ability of mesenchymal stem cells derived from adipose tissue derived from adipose tissue, which has immunomodulatory effects, to inhibit the septic process in an experimental model of mice.

Design: Prospective, controlled animal trial.

Setting*: Research laboratory.*

Subjects: *Fed male C57BL/6 mices.*

Interventions*:* Three experimental groups were formed for the test: control group, untreated septic group and septic group, treated with MSCs (1 x 10^6 cells/animal).

Measurements and Main Results: In the control group, there were no deaths; in the untreated septic group, the mortality rate was 100% within 26 hours; in the septic group treated with MSCs, the mortality rate reached 40% within 26 hrs. The group treated with MSCs was able to reduce the markers of tissue damage of the liver and pancreas. The treated group received a reduction in inflammatory markers in comparison with the untreated group septic. Furthermore, the group treated with stem cells was able to inhibit the increase of apoptosis in splenocytes observed in septic untreated group.

Conclusion: MSCs reduced the mortality rate provoked by experimental sepsis, ameliorated immune response and decreased the apoptotic death.

KEY WORDS: sepsis; mesenchymal stem cells; inflammation; *Escherichia coli*; apoptosis

Introduction

Severe sepsis is currently a major cause of death in critically ill patients, with 750,000 new cases every year, and more than 200,000 fatalities (1). With the increased use rate of invasive surgical procedures and immunosuppression, the incidence is likely to increase in the next few years. Furthermore, sepsis incurs a staggering $16.7 billion cost in the US health economy (1,2).

Septic syndromes (sepsis, severe sepsis and septic shock, ranked by increased severity) are defined by the association of a systemic inflammatory response with an infection. The initial phase of the disease is dominated by an exacerbated inflammatory response (also called ‘cytokine storm’) responsible for successive organ failures and ultimately refractory hypotension leading to shock (3,4). Apoptosis is a key pathophysiological process in sepsis and leads to a striking loss of lymphocytes and dendritic cells. It also induces the decrease of immune effector cells and, combined with the immunosuppressive effect of apoptotic cells, contributes to the profound immunoparalysis that is a major cause of morbidity and mortality in this disorder (5,6). Even with appropriate antibiotic and resuscitative therapies, sepsis carries a 30% mortality rate and is significantly morbidity associated with organ failure. Thus, new therapeutic strategies are needed to improve the outcome of septic patients (7).

Mesenchymal stem cells (MSCs) are multi-potent progenitor cells that can be cultured from adult tissue and fatal tissues. They can regenerate different kind of cell lines such as tendon, cartilage, bone and adipose cells. In the last 10 years, it has been discovered that MSCs are potent modulators of immune responses (8-11). The protective role of MSCs have been also tested in early clinical trials in cardiac disease, inflammatory bowel disease, stroke and several others clinical disorders (12-16). More importantly, there is new evidence that MSCs have a beneficial effect in preclinical models of polymicrobial sepsis. In these studies, it was demonstrated that injection of MSCs into septic mice reduced the septic inflammatory response and mortality by decreasing proinflammatory cytokine expression while increasing anti-inflammatory IL-10 (17,18). However these studies do not address the response

of MSCs on cellular apoptosis that is correlated with immune dysfunction during sepsis. Consequently, the purpose of this study was to investigate the role of murine MSCs obtained from adipose tissue as a possible protector against the effects of the septic process in mouse model. We conducted a survival curve comparing animals MSCs injected with a control septic. Furthermore, we study the mechanisms involved in this protective effect as the levels of tecidual injury markers, levels cytokines pro and anti-inflammatory in addition to evaluation of apoptosis of splenocytes.

Materials and methods

Animals

The Male C57BL/6 mice (8-12 weeks old) were kept on shelves with ventilated cages that provide 60 air cycles per hour, a relative humidity ranging between 55–65%, a 12 hours light–dark cycle, a temperature of 22±2°C with free access to food and water. The animals were maintained in accordance with the Guiding Principles in the Care and Use of Animals by the Concil of the American Physiological Society. The experimental protocol was approved by the Ethics Research Committee of Pontifícia Universidade Católica do Rio Grande do Sul (protocol number 11/00252).

Cell Culture

Murine MSCs were isolated and expanded as previously described (19,20). Prior to the collection of the adipose tissue, mice were killed by cervical dislocation. Adipose tissue was obtained from the epididymal adipose tissue, cut into small pieces, collagenase-digested, filtered and then cultured using Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, USA) without ribonucleosides or deoxyribonucleosides containing 2 mM L-glutamine and 10% fetal bovine serum (FBS) (Invitrogen, USA), with 1% penicillin-streptomycin. Cells were passaged every 3-4 days by trypsinization when they reached 70-80% confluency and were used for the experiments between passages 3-4. Between each passage, viability was measured with trypan blue exclusion. MSCs were cultured in a humidified incubator at 5% CO_2 and 37°C under sterile conditions. Before each experiment, cells were trypsinized, counted, washed twice with PBS and resuspended in phosphate buffered saline (PBS) (Gibco, USA).

Experimental Sepsis Induction and Treatment

The animals were weighed and then anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (20 mg/kg) intraperitoneally (i.p.). The abdomen of each animal was shaved and cleansed with povidine-iodine solution. A 1 cm midline abdominal incision was made to expose the linea alba, which was lightly incised. The peritoneum was opened by using blunt dissection. All procedures were performed using sterile surgical instruments.

Sepsis was induced by introducing in the peritoneal cavity a sterile gelatin capsule size "1" containing another sterile capsule size "2" with the *Escherichia coli* (3µL, ATCC 25922) suspension and a non-sterile fecal content (20mg). *E. coli* was stored in autoclaved skimmed milk on glass beads at 70°C. Each week, a bead was inoculated onto trypticase soy agar and incubated overnight at 37°C. The culture was passed daily for use the next day. Each day, a representative colony was transferred into 10 mL of nutrient broth and incubated at 37°C with shaking for 2 hours, until the optical density at 650 nm was between 0.280 and 0.300. The culture was diluted in pyrogen-free phosphate-buffered saline to yield 4×10^8 colony forming units/mL.

The animals were then divided into three groups as follows: (i) sham (operated with introduction of empty capsule and was administered 200 µL of PBS by retro-orbital injection), (ii) sepsis (sepsis-induced and was administered 200 µL of PBS by retro-orbital injection), (iii) sepsis + MSCs (sepsis-induced and treated with MSCs 1×10^6/200 µL of PBS by retro-orbital injection at the time of induction). Blood samples were collected through cardiac puncture 12 hours after the sepsis induction.

Survival Curve

A survival curve in different experimental groups was performed. After seven days, animals that were still alive were anesthetized with and i.p. solution of ketamine (100 mg/Kg) and xylazine (50 mg/Kg) and decapitated.

Body Temperature

Body temperature was performed in animals by a rectal thermometer 12 hours after sepsis induction.

Biochemical Analysis

Biochemical analysis were performed from whole blood samples collected (12 hours after sepsis induction) in tubes without anticoagulant, centrifuged (1,000 x g for 5 minutes) after the clot retraction and the serum frozen at -70°C until analysis. Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), phosphate, glucose and amylase were evaluated using standard commercial kits (Labtest Diagnóstica, Brazil) in a semi-automated spectrophotometer (Spectronic/Genesis 8).

TGF-β1 Quantification

TGF-β1 concentration was measured in serum samples collected from mice 12hours after sepsis induction, using commercially available ELISA kit (R&D Systems, USA). The kit contained a specific monoclonal antibody immobilized on a 96-well microtiter plate that bound TGF-β1 in the aliquot and a second enzyme-conjugated specific polyclonal antibody. Following several washings in order to remove unbound substances and antibodies, a substrate solution was added to the wells. Color development was stopped by sulfuric acid, and optical density was determined at 540nm with the correction wavelength set at 570nm in an ELISA plate reader. Results were calculated according to a standard curve concentration and multiplied for the dilution factor. TGF-β1 levels were expressed as picograms per milliliter.

Cytokines Quantification

To determine cytokine levels, serum samples was collected from mice 12 hours after sepsis induction. Multiple soluble cytokines interleucine 6 (IL-6),

interleucine 12 (IL-12 p70), interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), interleucine 10 (IL-10) and monocyte chemoattractant protein 1 (MCP-1) were simultaneously measured by flow cytometry using the Cytometric Bead Array (CBA) Mouse Inflammation Kit (BD Biosciences, USA). Acquisition was performed with a FACSCanto II flow cytometer (BD Biosciences, USA). Quantitative results were generated using FCAP Array v1.0.1 software (Soft Flow Inc., Pecs, Hungary). The detection limit was 20 to 5 000 pg/mL.

Splenocytes Isolation

Single-cell suspensions of the removed spleens were prepared by passing the tissue through a 100 µm pore size mesh Cellstrainer (Falcon, BD Biosciences, Germany). The suspension was cleared from erythrocytes by treatment with Gey's solution for 5 minutes, washed twice with PBS and re-suspended in ISCOVE's medium (PAA Laboratories GmbH, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen, USA). After counting splenocytes from individual mice, cells of each group were pooled by taking equal cell numbers from individual mice and adjusted to a concentration of 10^7 cells/mL (21).

Apoptosis Quantification

Apoptosis was assessed using the FITC Annexin V Apoptosis Detection Kit I (BD Bioscience, USA). Briefly, twelve hours after sepsis induction, spleens were removed and the splenocytes isolated (21). Cells were washed twice with PBS and ressuspended in binding buffer before addition of annexin V-FITC and propidium iodide (PI). Cells were vortexed and incubated for 15 minutes in the dark at room temperature. A total of 10,000 events were acquired for each assayed sample. All data were acquired with a FACSCanto II flow cytometer (BD Biosciences). Data were analyzed using the FlowJo 7.2.5 software (Tree Star Inc., USA). Results are displayed as scatter dots allowing discrimination

between viable cells, apoptotic cells with an intact membrane and cells undergoing secondary necrosis.

Analysis of Mitochondrial Membrane Potencial (ΔΨm)

Breakdown of ΔΨm was determined by FACS analysis using the MitoScreen Kit (BD Biosciences, Germany). JC-1 (5,5,6,6-tetra-chloro-1,1,3,3-tetraethylbenzimidazol-carbocyanine iodide) dye, which is selectively incorporated into mitochondria, is a sensitive and reliable method to detect changes of the mitochondrial membrane potential (ΔΨm). Twelve hours after induction sepsis, spleens were removed and the splenocytes isolated (21). Cells were stained with 0.5 mL JC-1 solution for 15 minutes at 37°C. Stained splenocytes were washed twice in JC-1 MitoScreen wash buffer. A total of 10,000 events were acquired for each assayed sample. All data were obtained immediately after staining on a FACSCanto II flow cytometer with CellQuest PRO v4.0.2 software (BD Biosciences, Germany). Results are displayed as scatter dots allowing discrimination between polarized and depolarized cells.

Statistical Analysis

All data are expressed as mean ± SEM. The statistical analysis were made by analysis of variance (ANOVA) with the Bonferroni post hoc. Survival data is presented as Kaplan Meier curves and the statistical significance was assessed by Mantel-Cox test. A level of $p<0.05$ was considered statistically significant in all analysis. Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS, EUA) 18.0.

Results

MSCs treatment improves survival and organ injury function in experimental model of sepsis

Mice treated with MSCs had a significantly improved survival rate compared to the untreated group. The survival rate at 26 hours was nearly 54.5% in the sepsis + MSCs group, while in the sepsis group survival was 0% ($p<0.001$)(**Figure 1**). Mice from both sepsis and sepsis + MSCs groups showed a decrease in their mean body temperature after twelve hours of septic induction. MSCs administration did not attenuate the decline in temperature compared to sham group ($p<0.05$) **(Figure 2)**.

Because the lethality of sepsis is associated with organ failure, we evaluated if treatment with MSCs was able to reduce tissue damage. The concentrations of liver enzymes (aspartate aminotransferase and alanine aminotransferase) were measured in serum. The septic induction showed an increase of AST and ALT in both groups, sepsis group and sepsis + MSCs group, when compared to sham group, however ALT levels in the sepsis + MSCs group was reduced when compared to the sepsis group ($p<0.05$). This demonstrates that there was a reduction of liver damage **(Figure 3A, 3B).**

We also measured concentration of the serum amylase, which demonstrates the possible pancreatic damage. The sepsis group showed a significant increase compared with the sham group, but the sepsis + MSCs group did not have a significant increase compared with the sham group ($p<0.05$). Once again, demonstrating the ability to prevent tissue damage exerted by MSCs **(Figure 4).**

During sepsis, there is the occurrence of an increase in insulin resistance generating hyperglycemia, which causes a decrease in energy intake. Therefore, we evaluated the glucose concentration 12 hours after induction of septic in all groups. When compared with the sham group, both groups, sepsis and sepsis + MSCs, showed a significant increase ($p<0.05$). Demonstrating that

treatment with MSCs was not effective in preventing the increase of glycaemia **(Figure 5).**

Hypophosphatemia has long been reported to be associated with sepsis and has been correlated with sepsis severity. We realized the dosage of serum phosphate in all groups; however, we did not find significant difference between the tested groups when compared with the sham group **(Figure 6).**

Effect of MSCs on serum cytokine concentrations

Recent evidence suggests that MSCs may also exhibit immunosuppressive or immunomodulatory properties. Therefore, we studied multiple inflammatory cytokines (TNF-α, IL-12 p70, IFN-γ, IL-6 and MCP-1) and anti-inflammatory cytokines (IL-10 and TGF-β1). All cytokines were analyzed in serum after 12hours after induction septic and injection of MSCs.

When we measured concentration of TNF-α we observed an increase in the sepsis group compared with the sham group ($p<0.05$). In contrast, the sepsis + MSCs group did not show a significant increase compared with the sham group **(Figure 7A)**. IL-12, which is known to trigger other cytokines, have not had its bioactive form (p70) altered in sepsis and sepsis + MSCs groups, compared with the sham group **(Figure 7B)**. Consequently we had no significant alterations in the concentration of IFN-γ **(Figure 7C)**. However, IL-6 and MCP-1 concentrations had a significant increase in sepsis group compared to the sham group and the sepsis + MSCs group ($p<0.05$) **(Figure 7D, 7E)**.

We measured the concentration of IL-10 and observed a significant increase in the sepsis + MSCs group, when compared with the sepsis group and the sham group ($p<0.05$) **(Figure 7F)**. On the other hand, when we realized the dosage of another important player in the anti-inflammatory profile of MSCs, TGF-β1, we found no significant difference between the sepsis + MSCs group and the sham group, but a significant increase in the sepsis group compared to the shamgroup ($p<0.05$). The MSCs treatment prevents the increase of TGF-β1 **(Figure 8)**.

MSCs prevents the increase of apoptosis in splenocytes during sepsis

During sepsis, there is extensive apoptotic death of lymphocytes and gastrointestinal epithelial cells. The increased apoptotic death of lymphocytes is likely to be an important cause of the profound immunosuppression that is a hallmark of patients with sepsis. The potential importance of apoptosis in the pathogenesis of sepsis is also illustrated by results from animal models that demonstrate that blocking lymphocyte apoptosis by using caspase inhibitors improves survival in sepsis. The potential effect of MSCs on apoptosis was analyzed in splenocytes (isolates of the three experimental groups after 12 hours after starting the experiment) by two different methods, one detecting apoptotic cells by measuring the translocation of phosphatidylserine to the outer cell membrane surface and a second measuring the impact on mitochondrial transmembrane potential (ΔΨm).

Flow cytometric analysis of the Annexin V labeling assay detected an increase of apoptotic cells in the sepsis group compared with the sham group ($p<0.05$). The treatment with MSCs (sepsis + MSCs group) inhibited significantly the increase of apoptosis ($p<0.05$ vs. sepsis group) **(Figure 9)**.

To assess effects of treatment with MSCs on mitochondrial injury, we analyzed the ΔΨm in splenocytes. Changes of ΔΨm were determined by JC-1 staining of different experimental groups. The treatment did not provoke significant alteration of ΔΨmbetween groups **(Figure 10).**

Discussion

The results **(Figure 1)** showed that whereas the mortality rate of the untreated septic group reached 100% in 26 hours, the septic animals treated with MSCs was only 54.5%. These data show that, by some mechanism, the mesenquimal stem cells affected the septic process, reducing the mortality rate of the animals. Therefore, we evaluated several mechanisms in order to elucidate how the mesenchymal stem cells may be acting during septic improving the survival time of the animals.

After 12 hours of the start of the experiment we realized the collection of material for biochemical and immunological analysis, but first we measured the body temperature of the animals under study. Hypothermia was found in both, the sepsis group and in the sepsis + MSCs group. These data are in contrast to data found in the study by Krasnodembskaya et al. (18), where stem cells that were able to prevent hypothermia. In this study Krasnodembskaya used stem cells derived from bone marrow of humans as a treatment for peritoneal sepsis model in mice using *P. aeruginosa*. These differences in study design may have been critical to find a different result in our experiment.

In the study developed by Nemeth et al. (17), it was demonstrated that stem cells derived from bone marrow have the capacity to reduce tissue injury in a model of sepsis (cecal ligation and puncture). Furthermore, studies performed by Poll et al. (16) demonstrated that MSCs therapy has profound inhibitory effects on hepatocellular death and enhances of liver regeneration programs, and that it ultimately improves survival in rats undergoing D-galactosamine–induced fulminant hepatic failure (FHF). Corroborating these results, we observe that in our experiment that stem cells derived from adipose tissue also had a positive response in preventing tissue injury through analysis of serological markers of liver injury (ALT and AST) and pancreas (amylase). These results have effective participation in the increased survival in animals treated with stem cells.

An important marker of severity of sepsis is hyperglycemia. Several studies in animals and septic patients are joining efforts to show that glycemic control can be effective as an adjuvant during the treatment of sepsis. Certain neuroendocrine and inflammatory mediators such as interleukin-1 (IL-1), interleukin-6 and tumor necrosis factor alpha (TNF-α) are involved in this hyperglycemia process (22-24). This profile of blood glucose increase was observed in the sepsis group and also the group treated with mesenchymal stem cells, demonstrating that MSCs had no influence on energy intake during the septic process.

The therapeutic benefit of MSCs transplantation has been observed in acute tissue injuries of the lung, heart, kidney and liver. In these disease contexts, MSCs have been observed to migrate to injured sites after systemic administration. Tissue-specific engraftment is referred to as homing, and this aspect of MSCs therapy in disease may be essential for their medicinal effects (25).The homing ability of MSCs has been demonstrated in the settings of wound healing, and tissue regeneration (7,26). It is likely that increased inflammatory chemokine concentration at the site of inflammation is a major factor causing MSCs to preferentially migrate to these sites. Chemokines are released after tissue damage, and MSCs express the receptors for several chemokines (7,27,28).

Hypophosphatemia has long been reported to be associated with sepsis and gram-negative infections. Hypophosphatemia develops in the early stages of sepsis and is correlated with the severity of the patient's clinical condition (29-31).We investigated the possibility of this reduction in serum phosphate during the septic process, however we did not observe any reduction in the septic group and no change in the group treated with mesenchymal stem cells for this parameter. Probably, this effect was not found because our experimental test is very severe, therefore, after 12 hours (time of sample collection) the animals are already in a state of late sepsis, and hypophosphatemia is found only in the early stages of sepsis.

Multiple studies have demonstrated that MSCs possess potent immunosuppressive effects by inhibiting the activity of both innate and adaptive

immune cells. This immunosuppression has been shown to be mediated by cell-contact-dependent and independent mechanisms through the release of soluble factors. The list of candidate mediators released or induced by MSCs includes TGF-β, prostaglandin E2 (PGE2), IL-10 among others (15,32). In this study we investigated the immunomodulatory capacity of stem cells in response to this imbalance inflammation during sepsis.

When we observe the profile of the inflammatory cytokines evaluated between groups of study, the group sepsis + MSCs had a significant reduction in the concentration of TNF-α, IL-6 and MCP-1 when compared with sepsis group. Several studies demonstrate the effective participation of these cytokines in inflammatory response during sepsis (14,33,34). TNF-α has several functions in the human immunopathology, since it produces inflammation, cell proliferation and differentiation, tumorigenesis, viral replication and inducing cell death by apoptosis through activation of caspase 8 - known as the extrinsic apoptosis pathway in sepsis (3). In clinical models of sepsis, the administration of TNF-α cause hypotension, activation of the coagulation cascade and organ dysfunction, confirming its role as a mediator in acute phase (35,36).

The role of IL-6 in sepsis resolution is uncertain, although most evidence points show more rapid declines in serum IL-6 being associated with sepsis resolution and improved outcome (37). In the cecal ligation/perforation (CLP) sepsis model, mice genetically deficient in MCP-1 showed lower IL-10 production in peritoneal macrophages and increased mortality (38). High serum levels of MCP-1 have been demonstrated in animal models of sepsis or *systemic inflammatory response syndrome* (SIRS), as well as in sepsis patients (39-41). In a recent study profiling a large number of cytokines in the plasma of patients with severe sepsis, MCP-1 levels showed the best correlation with organ dysfunction and mortality (42). MCP-1 is primarily a chemo attractant for monocytes, memory T lymphocytes, and natural killer cells, with some recent studies also pointing to a potential role in attracting neutrophils (43).

Nemeth et al. (17) found that bone-marrow-derived MSCs, activated by LPS or TNF-α, secreted PGE2, which reprogrammed alveolar macrophages to secrete IL-10. The beneficial effect of MSCs on mortality and improved organ

function following sepsis (CLP) was eliminated by macrophage depletion or pretreatment with antibodies to IL-10 or the IL-10 receptor, suggesting an essential role for IL-10. We performed the dosage of IL-10 in all the experimental groups and significant found increase of IL-10 in the septic group treated with stem cells corroborates with previous studies demonstrating the role of this anti-inflammatory cytokine is very important in increasing survival of the animals also in our model. Nemeth et al and Gonzalez et al. (17,44) previously demonstrated in septic mice treated with MSCs that increased IL-10 is one of the factors responsible for the reduced mortality and reduction of the inflammatory response.

Interestingly when we evaluated the concentration of TGF-β1, another important anti-inflammatory mediator during sepsis. We found an increase in the sepsis group when compared to the sham group and a significant decrease concentrations in the sepsis + MSCs group when compared to the sepsis group. We believe this has occurred because our experimental model is very serious and material collection was performed after 12 hours of the start of the experiment. Therefore, these animals may be in a phase of transition from the first stage of sepsis where we have a hyper-inflammatory response and the second phase where we have an anti-inflammatory immune response that disables most immune functions by altering cytokine production, reduction of lymphocyte proliferation and increasing apoptosis (45).

Based in ours results, we decided to evaluate the effect of the MSCs in cellular apoptosis. We performed the dissection of the spleens of mice and isolated splenocytes to check if had more apoptotic cells in the septic group in contrast to the septic group treated with MSCs. We used two sets of flow cytometry, which one evaluates apoptosis through changes in mitochondrial membrane potential and the other by marking phosphatidylserine in the cell membrane.

Apoptosis is a major cause of death in lymphocytes and gastrointestinal epithelial cells in patients with sepsis and trauma. Immunohistochemical studies of spleens from patients dying of sepsis demonstrated focal regions in which 25–50% of cells were positive for markers of apoptosis (46). A study of

circulating white blood cells from patients with sepsis showed that 15–20% of circulating T and B cells were undergoing apoptosis (47). Studies by Hotchkiss et al. showed that adoptive transfer of splenocytes apoptotic worsen survival using CLP as experimental model of sepsis (48).

Stem cells possess anti-apoptotic mechanisms such as upregulating DNA-repair, down-regulating mitochondrial death pathways, increasing antioxidant activity, and altering anti- and pro-apoptotic protein expression. These mechanisms would be especially important in sepsis, where mitochondrial damage, oxidative stress, and apoptosis have clearly been implicated in pathology (7). Mei et al. (13) revealed the capacity of MSCs to prevent apoptotic cell death in the lung and kidneys of mice after cecal ligation and puncture (CLP).

After completion of the analysis it was possible to observe in the sepsis group a significant increase in the number of apoptotic cells compared with the group treated with stem cells. In contrast, the kit for checking the mitochondrial potential was an increase in the number of apoptotic cells however was not statistically significant. These findings corroborate several previous studies and show that MSCs are able to prevent this increase gives apoptosis during sepsis "delaying" the start of immunosuppression thereby helping to increase the survival time of the animals.

Conclusions

In conclusion, the treatment with mesenchymal stem cells derived from adipose tissue was able to increase the survival time of septic-induced animals. Our data showed that MSCs act modulating the immune system, inhibiting cell apoptosis and, consequently, inhibiting tissue damage during sepsis. These results corroborate other studies published showing the great potential that these cells have for treating sepsis, but more efforts are needed so that we can better understand routes by which these cells operate so that in future we can think of using them in medical clinic.

Aknowlodgements

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Legends

Figure 1 – Kaplan-Meier survival curve showed the mice sepsis-induced treated with MSCs had a significantly improved survival rate compared to the mice untreated ($p<0.001$). n = 15 for each group.

Figure 2 – Body temperature in different experimental groups. n = 22 for each group. * $p<0.05$ vs. sham group.

Figure 3 – Serum AST (A) and ALT (B) concentrations in different experimental groups. n = 7 for each group. * $p<0.05$ vs. sham group. $ $p<0.05$ vs. sepsis group.

Figure 4 – Serum amylase concentration in different experimental groups. * $p<0.05$ vs. sham group. n = 6 for each group. $ $p<0.05$ vs. sepsis group.

Figure 5 – Serum glucose concentration in different experimental groups. n = 6 for each group. * $p<0.05$ vs. sham group.

Figure 6 – Serum phosphate concentration in different experimental groups. n = 10 for each group.

Figure 7 – Serum TNF-α (A), IL-12 (B), IFN-γ (C), IL-6 (D), MCP-1 (E) and IL-10 (F) concentration in different experimental groups. n = 15 for each group. * $p<0.05$ vs. sham group.$ $p<0.05$ vs. sepsis group.

Figure 8 – Serum TGF-β1 concentration in different experimental groups. n = 10 for each group. * $p<0.05$ vs. sham group. $ $p<0.05$ vs. sepsis group.

Figure 9 – Apoptotic cells was assessed using the FITC Annexin V (A) in different experimental groups. n = 10 for each group. * $p<0.05$ vs. sham group. $ $p<0.05$ vs. sepsis group. Thereunder, flow cytometric scatter plot of FITC-annexin V/PI stained sham group cells (B), sepsis group cells (C) and MSCs-treated group (D). Representative experiment for each group.

Figure 10 – Mitochondrial apoptotic cells was assessed using the FACS analysis (A) in different experimental groups. n = 11 for each group. Thereunder, flow cytometric scatter plot of the MSCs impact on $\Delta\Psi m$: sham group (B), sepsis group (C) or MSCs-treated group (D). Representative experiment for each group.

Figures

Figure 1

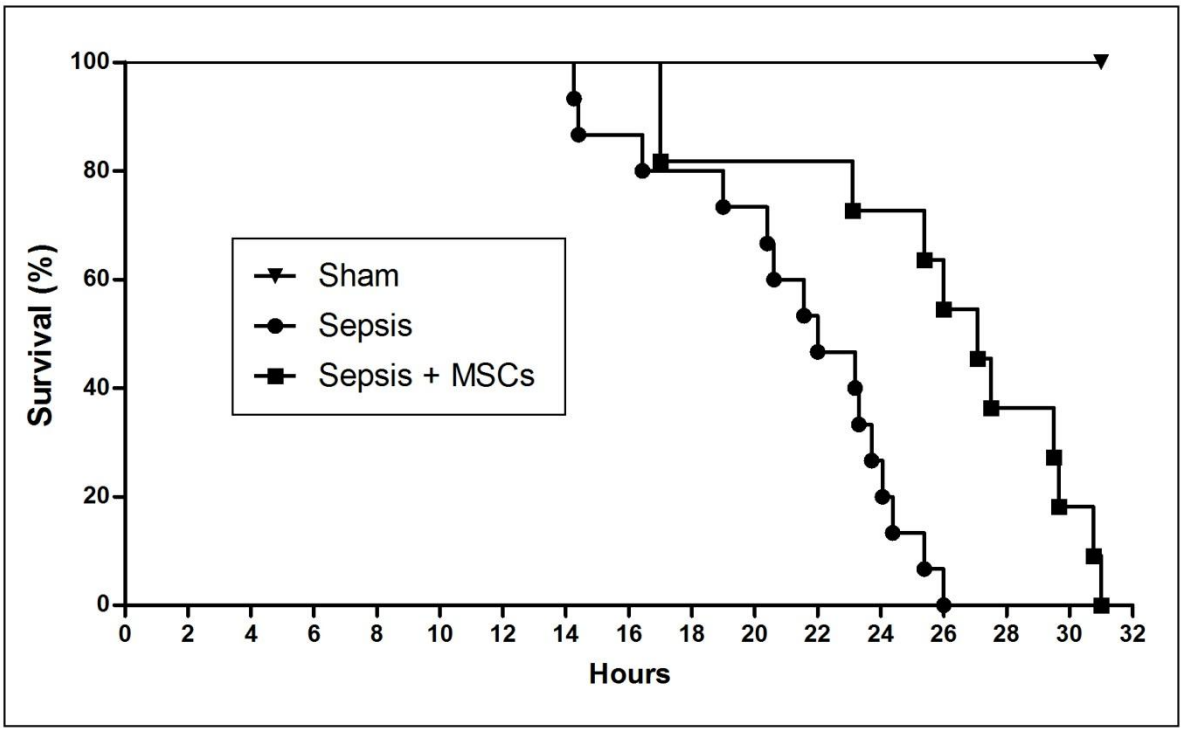
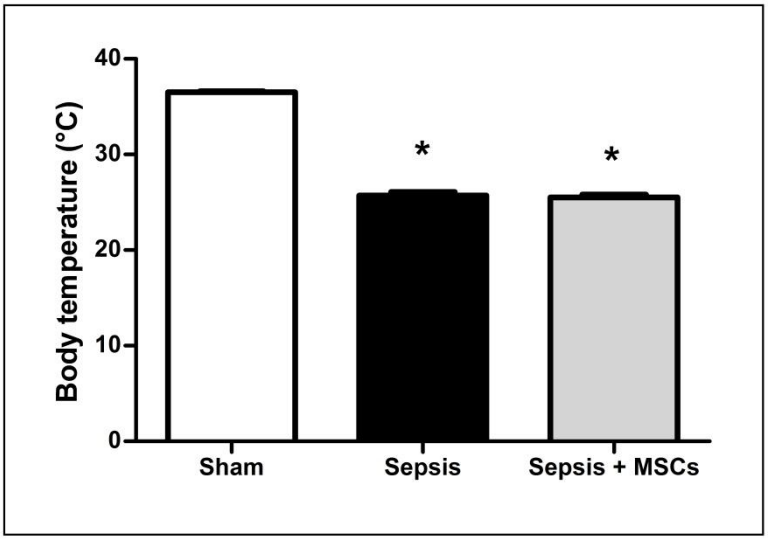


Figure 2



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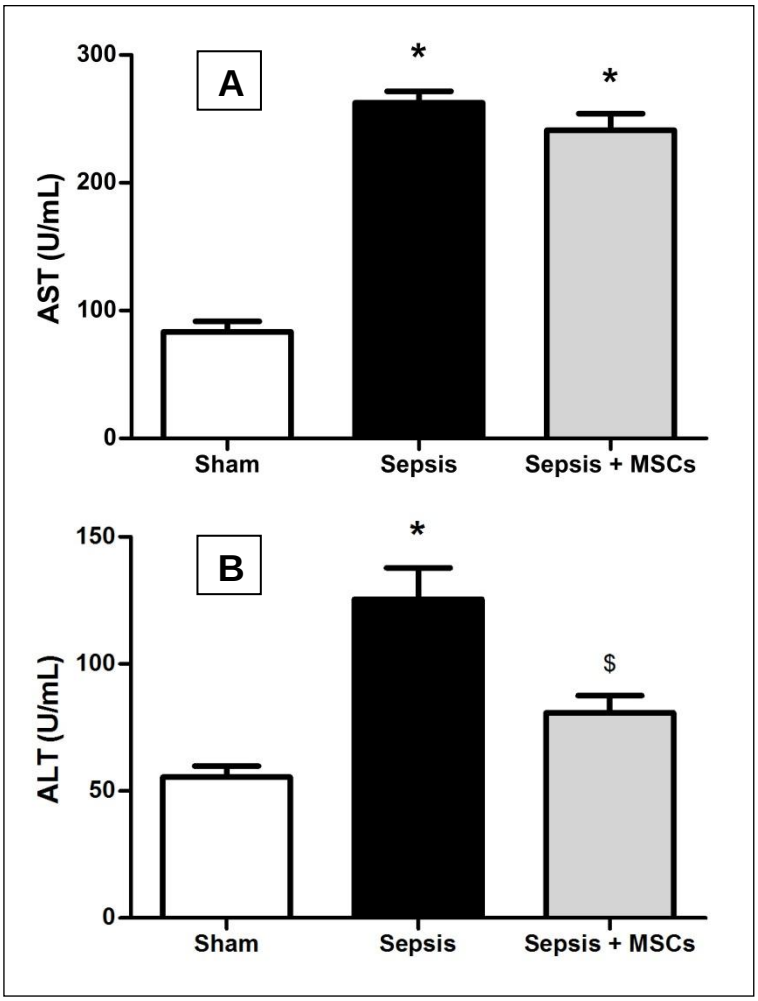

A
300
200
100
0
AST (U/mL)
*
*
Sham
Sepsis
Sepsis + MSCs
B
150
100
50
0
ALT (U/mL)
*
$
Sham
Sepsis
Sepsis + MSCs

Figure 4

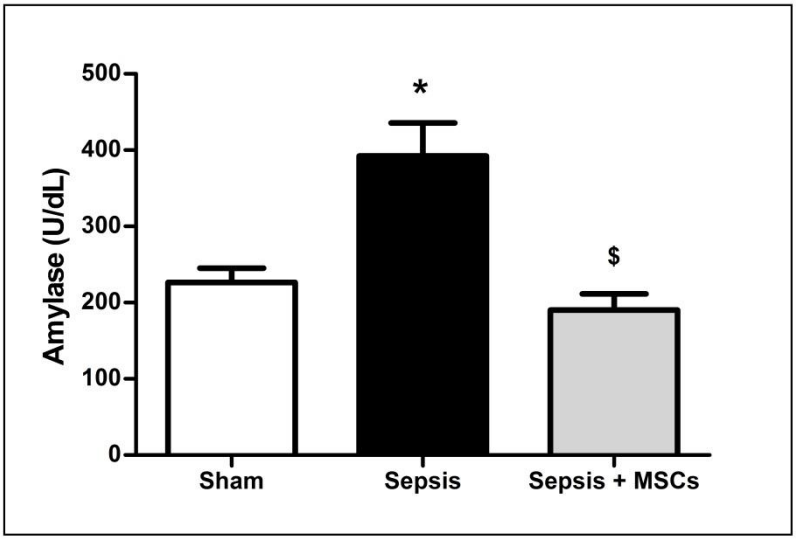
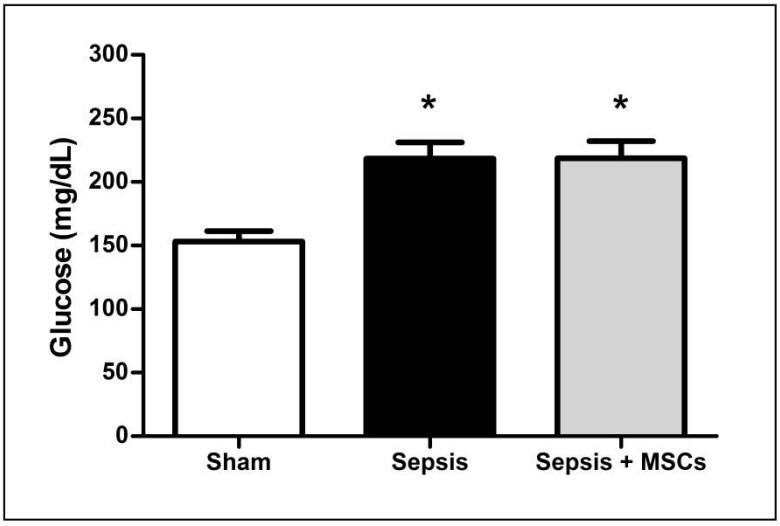


Figure 5



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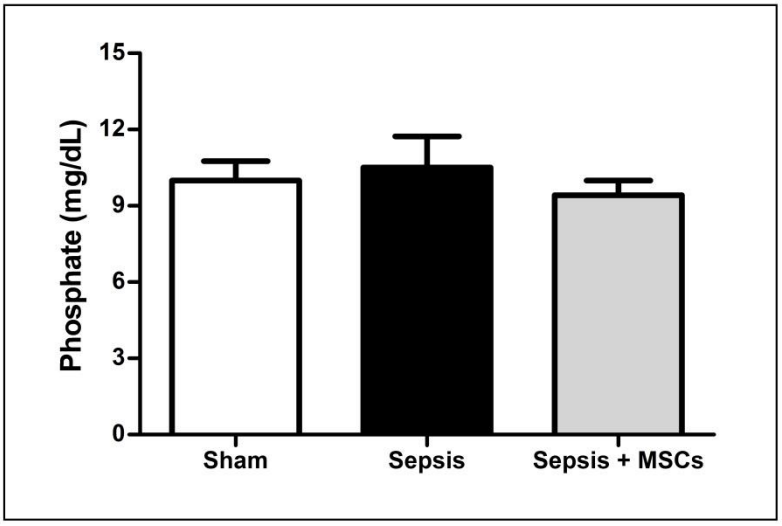


Figure 7

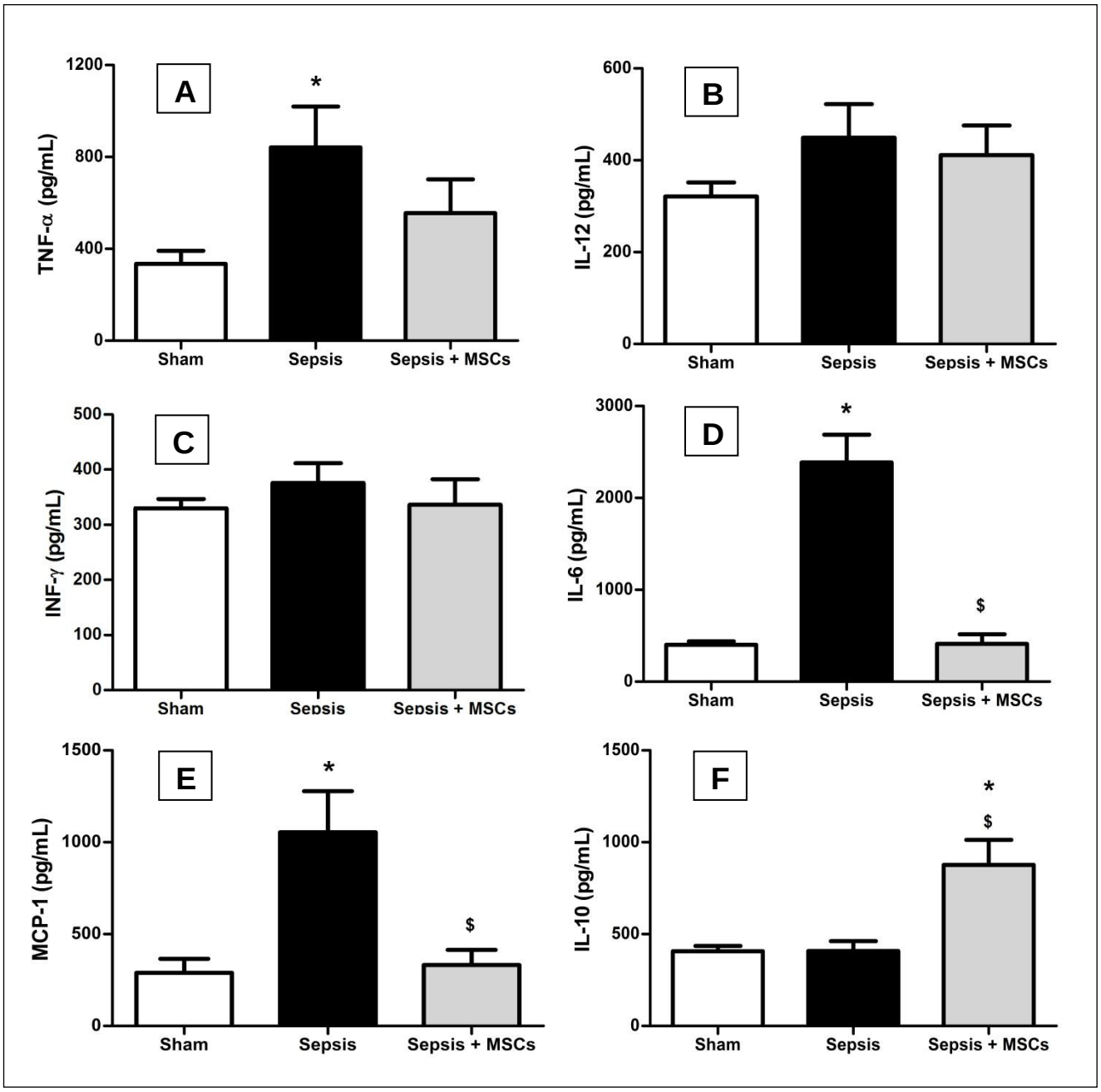

A
TNF-α (pg/mL)
1200
800
400
0
*
Sham
Sepsis
Sepsis + MSCs
B
IL-12 (pg/mL)
600
400
200
0
Sham
Sepsis
Sepsis + MSCs
C
INF-γ (pg/mL)
500
400
300
200
100
0
Sham
Sepsis
Sepsis + MSCs
D
IL-6 (pg/mL)
3000
2000
1000
0
*
$
Sham
Sepsis
Sepsis + MSCs
E
MCP-1 (pg/mL)
1500
1000
500
0
*
$
Sham
Sepsis
Sepsis + MSCs
F
IL-10 (pg/mL)
1500
1000
500
0
*
$
Sham
Sepsis
Sepsis + MSCs

Figure 8

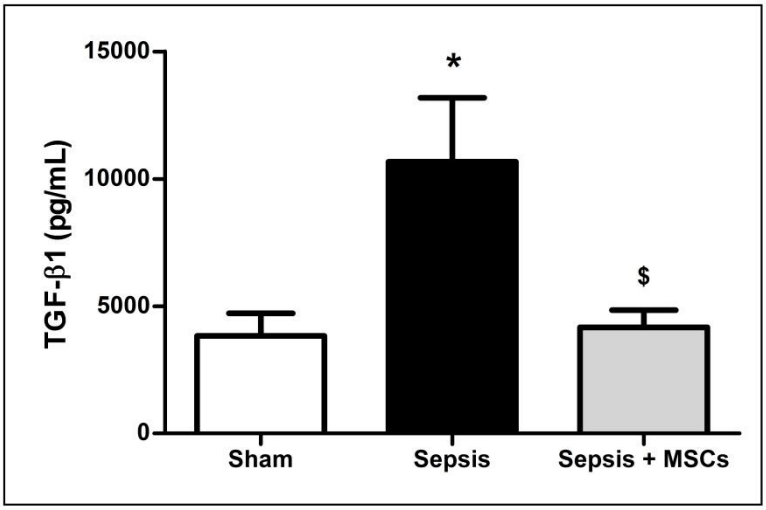


Figure 9

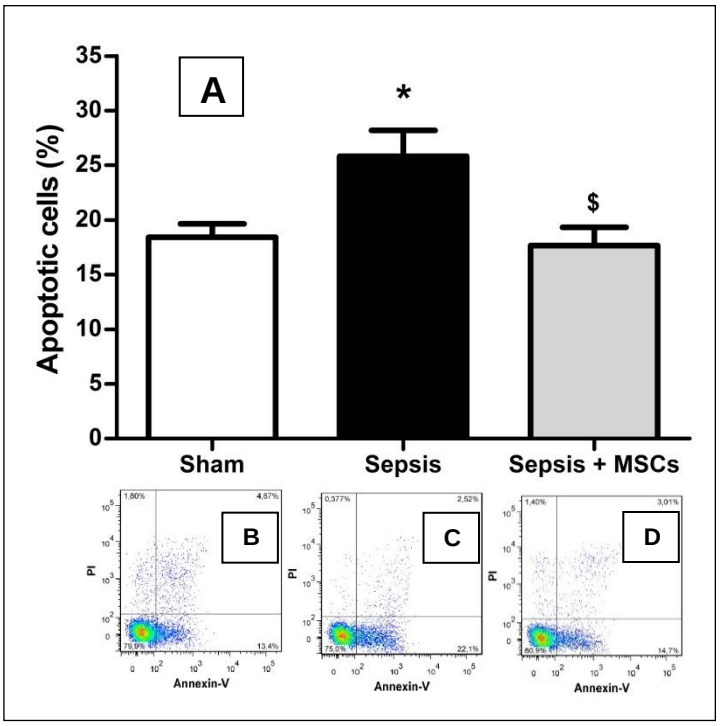
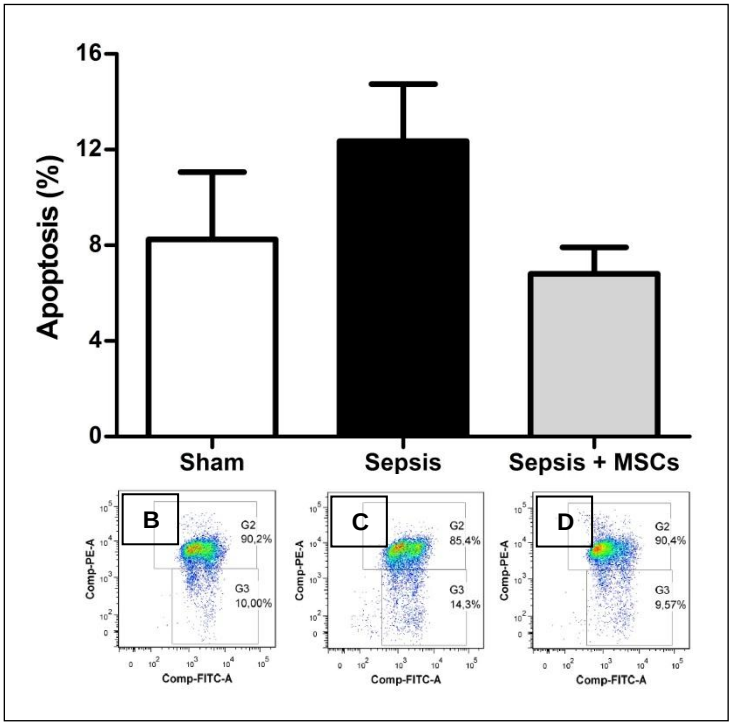

A
*
$
Apoptotic cells (%)
35
30
25
20
15
10
5
0
Sham
Sepsis
Sepsis + MSCs
B
C
D
PI
Annexin-V
1,80%
4,87%
79,9%
13,4%
0,377%
2,52%
75,0%
22,1%
1,40%
3,01%
80,9%
14,7%

Figure 10


Apoptosis (%)
16
12
8
4
0
Sham
Sepsis
Sepsis + MSCs
B
G2
90,2%
G3
10,00%
C
G2
85,4%
G3
14,3%
D
G2
90,4%
G3
9,57%
Comp-PE-A
Comp-FITC-A

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Capítulo 3

Considerações Finais

CONSIDERAÇÕES FINAIS

A sepse, uma doença considerada como uma resposta exacerbada sistêmica à infecção, é a principal causa de morte em unidades de cuidados intensivos. Apenas nos Estados Unidos a cada ano ocorrem 750.000 novos casos sendo que 210.000 pessoas morrem anualmente devido a esta síndrome. Além disso, tem sido sugerido que este número é maior em países menos desenvolvidos tais como os da América Latina. No Brasil,relatórios epidemiológicos demonstram taxas de mortalidade que chegam a 56%[64].

Alertado nos últimos anos pela morbidade e mortalidade decorrentes da sepse, principalmente por deficiências dos atuais regimes terapêuticos, pesquisadores se voltaram à terapia baseada em células como uma nova abordagem para o tratamento da sepse. Embora inicialmente acreditava-se que o maior potencial de células-tronco residia na sua capacidade de enxertar e se diferenciar em tipos de células de órgãos lesados, elas também exibem uma gama de habilidades benéficas, incluindo migração para locais lesionados, modulação da cascata inflamatória, prevenção de apoptose celular nos tecidos, promoção da neoangiogênese, ativação de células-tronco residentes e a modulação da atividade de vários tipos celulares do sistema imunológico[65].

Considerando esse potencial terapêutico exercido pelas células-tronco, este trabalho teve como objetivo verificar a capacidade destas células de funcionarem como um possível tratamento para sepse, utilizando um modelo de sepse experimental em camundongos. Os objetivos principais foram: avaliar a capacidade do tratamento com células-tronco mesenquimais em aumentar a

sobrevida dos animais, a capacidade de dimimuirem a lesão tecidual, avaliar o potencial imumodulador destas células durante a sepse e a capacidade de alterarem os níveis de apoptose celular.

Para verificar o possível efeito benéfico destas células para o tratamento da sepse nós realizamos uma curva de sobrevida dividindo os animais em três grupos: controle (sham), animais sépticos (sepse) e animais sépticos tratados com células-tronco mesenquimais (sepse + MSCs). Após 16 horas de experimento evidenciamos que no grupo sepse + MSCs 60% dos animais encontravam-se vivos enquanto que no grupo sepse apenas 20% dos animais estavam com vida. Este resultado nos levou a buscar possíveis explicações para esta diminuição de mortalidade no grupo tratado com células-tronco.

Nós realizamos a dosagem de dois marcadores que se correlacionam com a severidade séptica, glicose e fosfato, entretanto não encontramos nenhuma diferença significativa quando comparados os grupo sepse e sepse + MSCs. Entretanto, quando nós realizamos a dosagem de marcadores de lesão tecidual do fígado (ALT e AST) e do pâncreas (amilase) nós observamos que o grupo sepse + MSCs teve uma diminuição destes marcadores quando comparados com o grupo sepse. Estes resultados corroboram com estudos anteriores, demonstrando o efeito protetor das células MSCs e uma das possíveis ações benéficas do tratamento durante a sepse[45].

Para verificar se as células MSCs possuíam efeito importante sobre o quadro inflamatório, nós avaliamos diversos marcadores inflamatórios e anti-inflamatórios envolvidos durante a sepse. O tratamento com as células-tronco mesenquimais foi efetivo na redução de três marcadores inflamatórios TNF-α,

IL-6 e MCP-1. Diversos estudos demonstram papel fundamental de destas citocinas na inflamação sistêmica, na correlação de níveis elevados com piores prognósticos e no envolvimento na apoptose celular[66,67].

Corroborando com o potencial imunomodulador observado, os animais tratados com células-tronco (sepse + MSCs) tiveram um aumento de IL-10, descrita como fundamental nos efeitos benéficos das células-tronco mesenquimais durante o curso da sepse experimental[45]. Entretanto, quando observamos a dosagem de TGF-β, citocina anti-inflamatória, nós observamos um aumento significativo no grupo sepse quando comparado com o grupo sham e uma reversão desse aumento no grupo sepse + MSCs. Nós acreditamos que, devido à severidade do modelo empregado, estes animais podem estar em uma fase de transição entre a primeira etapa de sepse, onde temos uma resposta hiper-inflamatória e a segunda fase, onde temos uma resposta imune anti-inflamatória onde ocorrem alterações das funções imunitárias através da alteração da produção de citocinas, redução da proliferação de linfócitos e aumento da apoptose.

Para avaliar se realmente poderíamos ter um aumento de células apoptóticas maior nos animais do grupo sepse, nós realizamos através de citometria de fluxo a verificação do número de esplenócitos em apoptose em todos os grupos em experimentação. Nossos achados demonstraram que o grupo sepse teve uma maior quantidade de células em apoptose quando comparados com o grupo sham e que os animais tratados com células-troncos mesenquimais (grupo sepse + MSCs) diminuíram a quantidade de células apoptóticas. Este resultado demonstra mais um efeito benéfico do tratamento com estas células e evidencia a diversidade de ação das mesmas. Estudos

anteriores realizados por Hotchkiss e colaboradores demonstraram que a injeção de esplenócitos em apoptose diminuiu o tempo de sobrevida de camundongos em um modelo de sepse experimental o que corrobora com nossos resultados[68].

Podemos sugerir com este estudo que as células-tronco mesenquimais podem funcionar futuramente como uma possível alternativa para o tratamento da sepse. Ficou evidenciado claramente que o tratamento aumentou a sobrevida dos animais através da modulação do sistema imune e por esta razão reduziu o dano tecidual e o número de células apoptóticas. Cabe salientar que mais estudos devem ser realizados para aprofundar o conhecimento dos mecanismos pelos quais estas células agem. Isso se torna pertinente, pois fica claro o grande potencial que estas células têm em exercerem diferentes efeitos benéficos no tratamento da sepse.

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ANEXO 1

Guia para autores

Critical Care Medicine

Instructions for Authors

Critical Care Medicine is an international, peer-reviewed journal that is interested in publishing the highest quality scientific studies in the field of critical care medicine. Last year, approximately 25% of the original manuscripts submitted to the journal for publication were accepted.

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Human and Animal Subjects. All studies of human subjects must contain a statement within the Materials and Methods section indicating approval of the study by the Institutional Review Board (or institutional review body) that subjects have signed written informed consent, or that the Institutional Review Board waived the need for informed consent. *Before* your submission can be sent out for peer review, it is necessary that you address this issue of institutional review approval. This is in accordance with the International Committee of Journal Editors uniform requirements for manuscripts submitted to biomedical journals. Please see http://www.icmje.org for more details. All animal studies must contain a statement within the Materials and Methods section confirming approval by the Institutional Animal Care and Use Committee and that the care and handling of the animals were in accord with National Institutes of Health guidelines or other internationally recognized guideline for ethical animal treatment.

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••For standard American units, do not use values that are more significant than your analysis is capable of accurately measuring (e.g., Pao2 84 torr [11.2 kPa], not 83.7 torr).

••Hemodynamic measurements for pressure (e.g., MAP) should appear in mm Hg and gas tension measurements (e.g., Po2) should appear in torr with SI units in parentheses. The units of vascular resistance are dyne.sec/cm5.
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Review Articles. These consist of critical assessment of literature and data pertaining to clinical topics. In these review articles, emphasis shouldbe placed on cause, diagnosis,

therapy, prognosis, and prevention. Information concerning the type of study or analysis, population, intervention, and outcome should be included for all data used. The selection process used for all data should be described. Meta-analyses will be considered as review papers.The recommended length of review articles is 2000 to 3000 words (8 to 12 typed, double-spaced pages).

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