PONTIFÍCIA UNIVERSIDADE CATÓLICA DO PARANÁ

CARMEN LÚCIA KUNIYOSHI REBELATTO

CÉLULAS-TRONCO MESENQUIMAIS: CARACTERIZAÇÃO E POTENCIAL DE DIFERENCIAÇÃO A CARDIOMIÓCITOS INDUZIDA POR DOADORES DE ÓXIDO NÍTRICO (SNAP E DEA/NO)

CURITIBA

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Orientador: Prof.ª Lia Sumie Nakao
Co-Orientador: Prof. Alejandro Correa

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Resumo

Introdução: A terapia celular para o tratamento de doenças cardíacas é um método terapêutico novo que pode levar a recuperação da função do miocárdio. Porém a fonte de células e o tipo celular com propriedades adequadas de regeneração do tecido cardíaco estão sendo pesquisadas. As células-tronco mesenquimais (CTMs) possuem propriedades que podem permitir uma terapia celular altamente efetiva. Entretanto a literatura apresenta resultados conflitantes em relação aos marcadores e ao seu potencial de diferenciação. **Objetivos:** Neste trabalho comparamos as CTMs obtidas da medula óssea (CTMs-MO) e do sangue de cordão umbilical (CTMs-SCU) e as células-tronco derivadas do tecido adiposo (CTDAs) e analisamos a expressão dos marcadores cardíacos após exposição com o óxido nítrico (NO). **Resultados:** As CTMs-MO, CTMs-SCU e as CTDAs apresentaram semelhanças em relação a morfologia, ao tamanho e a complexidade, porém não foram semelhantes imunofenotipicamente em relação aos marcadores CD34 e CD117 e quanto ao potencial de diferenciação. A diferenciação em osteoblastos e condrócitos foi semelhante, porém a diferenciação adipogênica demonstrou que as CTMs-SCU apresentam poucos vacúolos lipídicos, diferentemente das outras fontes. A expressão dos genes FAPB4, osteonectina e colágeno tipo II foi analisada por qPCR para confirmar a diferenciação adipogênica, osteogênica e condrogênica, respectivamente. Os resultados confirmaram que as três fontes apresentam potencial semelhante para a diferenciação osteogênica e condrogênica, mas diferem para a diferenciação em adipócitos. Após a caracterização, as CTMs-MO e as CTDAs foram expostas aos agentes NO, SNAP e DEA/NO. A expressão de marcadores musculares e musculares cardíacos foi analisada por citometria de fluxo, imunofluorescência, RT-PCR e qPCR. Em geral foi observada uma alta variabilidade entre as amostras analisadas. Após a exposição das CTMs-MO e CTDAs ao NO houve um aumento da expressão de alguns marcadores musculares como conexina-43, troponina T e actina cardíaca e do marcador CD34 nas CTMs comparativamente ao grupo de células controle. Contudo não houve diferenças significativas entre as fontes de células nem entre os agentes de NO quando baseados nos resultados da citometria de fluxo e na

imunofluorescência. A análise da expressão gênica por RT-PCR e qPCR mostrou que os agentes NO aumentaram a expressão de VEGF e conexina-43. **Conclusões:** Concluímos que o NO, ou seus metabólitos, aumentam a expressão de marcadores musculares e musculares cardíacos, VEGF e CD34 nas CTMs-MO e CTDAs.

Descritores: células-tronco mesenquimais, células-tronco derivadas do tecido adiposo, sangue de cordão umbilical, diferenciação, óxido nítrico.

Abstract

Introduction: Cell therapy for heart diseases treatment is a novel method that can lead to recovery of myocardium function. The best cell source and type for cardiac tissue regeneration, however, are not yet defined. Mesenchymal stem cells (MSCs) present properties that can allow a highly effective cell therapy, although the literature presents conflicting results regarding MSCs surface markers and their differentiation potential. **Objectives:** In this study, we compared MSCs obtained from bone marrow (BM-MSCs), umbilical cord blood (UCB-MSCs) and adipose derived stem cells (ADSCs) and analyzed the expression of cardiac markers after nitric oxide (NO) agents exposure. **Results:** BM-MSCs, UCB-MSCs and ADSCs showed similarities regarding morphology, size and complexity, but they differed in CD34 and CD117 immunophenotyping and also in their differentiation potential. Differentiation to osteoblasts and chondrocytes was similar, but adipogenic differentiation demonstrated that UCB-MSCs presented few lipid vacuoles, in contrast to the other sources. Expression of *FAPB4, osteonectin and type II collagen* was analyzed by qPCR to confirm the adipogenic, osteogenic and chondrogenic differentiation, respectively. The results confirmed that the three sources present similar potential for osteogenic and chondrogenic differentiation, but differ for adipogenic differentiation. After characterization, BM-MSCs and ADSCs were exposed to NO agents SNAP and DEA / NO. The expression of muscular and cardiac markers was analyzed by flow cytometry, immunofluorescence, RT-PCR and qPCR. In general, a high variability between samples was observed. NO exposure induced an increased expression of some muscular markers such as connexin-43, troponin T and cardiac (fetal) actin and CD34 in MSCs, compared to control group. However, no significant differences between sources of cells or NO donors was observed by flow cytometry and immunofluorescence assays. Gene expression analysis by RT-PCR and qPCR showed that the NO agents increaded the expression of VEGF and connexin-43. **Conclusion:** We concluded that NO, or its metabolites, increases the expression of muscular and cardiac markers, VEGF and CD34 in BM-MSCs and ADSCs.

Key words: Mesenchymal stem cell, adipose-derived stem cell, umbilical cord blood, differentiation, nitric oxide

SUMÁRIO

LISTA DE ABREVIATURAS

µL – microlitro
µm – micrômetro
APC – aloficocianina
CD – grupos de diferenciação celular
cAMP - adenosina 3´, 5´-monofosfato cíclico
cDNA – ácido desoxirribonucléico complementar
cGMP – guanosina monofosfato 3',5' - cíclica
cNOS – isoforma constitutiva da óxido nítrico sintase
CO_2 – dióxido de carbono
CTDAs – células-tronco derivadas do tecido adiposo
CTMs – células-tronco mesenquimais
DAPI – 4',6-diamino-2 fenilindol
DEA/NO - 2-(N,N-dietilamina)-diazenolato-2-óxido
DMEM – meio Eagle modificado por Dulbecco
eNOS - isoforma endotelial do óxido nítrico sintase
FACS - Separação de células ativadas por fluorescência
FITC – isotiocianato de fluoresceína
GAPDH – gliceraldeído-3-fosfato-desidrogenase
GSH - glutationa
GSSG – glutationa dissulfeto
IMDM – meio Dulbecco modificado por Iscove
iNOS - isoforma induzível do óxido nítrico sintase
L-NAME - N,G-nitro-L-arginina-metil-éster
MHC – complexo principal de histocompatibilidade
mL – mililitro
mM – milimolar
MO – medula óssea
mRNA – ácido ribonucléico mensageiro
NK – *natural killer*
NO – óxido nítrico
NOS – óxido nítrico sintase
PBS – solução salina tamponada com fosfato

PCR – reação em cadeia da polimerase

PE – ficoeritrina

PerCP – complexo proteína clorofila peridinina

qPCR – reação em cadeia da polimerase em tempo real quantitativa

rpm – rotações por minuto

RT- transcriptase reversa

SBF – soro bovino fetal

SCU – sangue de cordão umbilical

SNAP - S-nitroso-N-acetil-D,L-penicilamina

SSEA-4 – do inglês *stage-specific embrionic antigens-4*

TA – tecido adiposo

VEGF – fator de crescimento endotelial vascular

VMHC – cadeia pesada da miosina ventricular

1 INTRODUÇÃO

1. 1 Doenças Cardiovasculares/ Cardiomioplastia

A doença cardiovascular é a maior causa de mortalidade e morbidade em todo o mundo[1,2]. Sua incidência e prevalência têm aumentado devido ao aumento de expectativa de vida da população[3]. A insuficiência cardíaca é geralmente caracterizada pela hipertrofia cardíaca, vascularização insuficiente e perda dos cardiomiócitos funcionais[4], havendo portanto uma perda da capacidade contrátil e a substituição das células mortas pelo tecido fibroso não funcional[5]. Após o dano do miocárdio, ocorre a mobilização de células-tronco da medula óssea (MO), como células progenitoras endoteliais[6] e células progenitoras adultas multipotentes[7] para a área danificada, devido à liberação de citocinas pela região afetada[8]. O coração normal é um órgão terminalmente diferenciado[9,10], o que permite uma regeneração tecidual limitada através da autoproliferação e mobilização seletiva das células-tronco residentes e/ou circulantes[11,12]. Entretanto, devido ao recrutamento insuficiente das células-tronco cardíacas e a sua capacidade limitada de regeneração, o processo natural de regeneração é inadequado para o reparo de danos mais extensos, onde há grande perda celular como no infarto agudo do miocárdio[13].

Terapias convencionais retardam o processo da doença cardíaca, porém não são capazes de substituir a lesão por tecido contrátil funcional[2]. O transplante cardíaco é o tratamento padrão ouro, mas a escassez de doadores e as complicações do transplante cardíaco têm levado a pesquisas na área do transplante celular como alternativa para o tratamento das doenças cardiovasculares[14-24].

O transplante celular pode levar à recuperação da função do miocárdio infartado pela substituição das células musculares cardíacas perdidas durante o processo patológico ou envelhecimento[25]. Portanto, vários tipos celulares, incluindo mioblastos esqueléticos[15,16,26,27], cardiomiócitos fetais, neonatais e adultos[28,29], células musculares lisas[30] e células-tronco adultas[18,31,32], têm sido pesquisados na tentativa de serem utilizados na terapia celular. Desta forma, a identificação de uma fonte adequada de progenitores de cardiomiócitos é de grande interesse para o uso na medicina regenerativa.

Todas as técnicas de cardiomioplastia celular têm limitações, e estas dependem primariamente do tipo de célula utilizada para o transplante. O modelo ideal seria células que possuíssem propriedades eletrofisiológicas, estruturais e contráteis de cardiomiócitos e que fossem capazes de se integrar estruturalmente e funcionalmente às células miocárdicas nativas. Além disso, estas células deveriam ser de origem autóloga, pouco imunogênicas e com grande capacidade de expansão. Infelizmente, não há fontes celulares adequadas que possuam todas as propriedades mencionadas[33]. Assim, várias pesquisas para definir a fonte de células mais seguras e efetivas que possam ser utilizadas na cardiomioplastia têm sido desenvolvidas[34].

Células como mioblastos esqueléticos podem repopular o tecido danificado, resultando em espessamento da parede ventricular, elevação da fração de ejeção ventricular esquerda e aumento da contratilidade[15]. Entretanto não há um aumento significativo da função cardíaca, pois a integração eletromecânica das células transplantadas com os cardiomiócitos nativos é incompleta, não havendo uma perfeita integração anatômica e funcional ao coração do receptor.

1.2 Células-tronco

As células-tronco apresentam propriedades importantes de auto-renovação e plasticidade[35]. Os mecanismos atualmente propostos pelos quais as células-tronco reparam o miocárdio lesionado ou que levam a um aumento na função cardíaca são a diferenciação em cardiomiócitos e formação de tecido miocárdico ou a utilização destas células para direta ou indiretamente aumentar a neovascularização (vasculogênese, angiogênese e arteriogênese)[36]. Além disso, tem sido proposto que as células-tronco liberam fatores angiogênicos, protegem os cardiomiócitos da morte por apoptose, induzem a proliferação de cardiomiócitos endógenos e podem recrutar células-tronco cardíacas residentes[37-39]. Neste contexto, a terapia com células-tronco pode ser considerada como ideal para a regeneração do tecido miocárdico danificado[32,40-42], melhorando a perfusão e o desempenho contrátil[18,37,43].

Células-tronco embrionárias humanas são pluripotentes e podem se diferenciar a cardiomiócitos, sendo utilizadas como uma fonte de células para a

terapia cardíaca[44,45,46]. Estas células apresentam propriedades moleculares, estruturais e funcionais dos estágios precoces do desenvolvimento cardíaco[47-49]. Os cardiomiócitos derivados das células-tronco embrionárias humanas podem formar um sincício funcional[50] e podem integrar estruturalmente e funcionalmente com o tecido cardíaco pré-existente, tanto *in vivo* como *in vitro*[51,52]. Mas a utilização das células-tronco embrionárias na terapia celular apresenta problemas, como a tumorigenicidade, a imunogenicidade e os problemas éticos em relação ao isolamento das células de embriões humanos fertilizados *in vitro*[53]. Então outras fontes de células-tronco para a regeneração do miocárdio devem ser consideradas.

Células-tronco adultas são encontradas em muitos tecidos e participam do reparo e regeneração do tecido danificado. A utilização de células-tronco autólogas humanas para a terapia celular é clínica e éticamente justificável, devido à ausência de efeitos colaterais, especialmente a formação de teratocarcinomas[54]. Além disso, diferentemente das células-tronco embrionárias humanas, não há potencial arritmogênico e a terapia imunossupressiva é desnecessária. Portanto a vantagem terapêutica claramente prevalece e a utilização clínica tem sido realizada[55-57].

1.3 Células-tronco mesenquimais

Comparada com outros tipos de células-tronco adultas, no contexto da cardiomioplastia, as células-tronco mesenquimais (CTMs) parecem possuir propriedades que permitiriam uma terapia celular altamente efetiva[58]. As CTMs são células multipotentes, com capacidade de diferenciação a vários tipos celulares e são importantes na manutenção da integridade dos tecidos e órgãos[59-60].

A caracterização das CTMs tem sido baseada em um conjunto de características morfológicas, fenotípicas e funcionais, devido a falta de marcadores específicos para estas células. As CTMs apresentam morfologia alongada semelhante aos fibroblastos e como propriedade física, aderência ao plástico[61]. Estas células apresentam heterogeneidade fenotípica[62,63] como foi demonstrado em estudos *in vitro* e *in vivo*. São destituídas de marcadores hematopoéticos, como CD45 e CD34, e de marcadores para monócitos,

macrófagos e células B, e geralmente expressam moléculas CD105, CD90, CD73, CD29 e CD44[64]. Recentemente GANG *et al.* (2007)[65] identificaram o SSEA-4, um antígeno da *globo-series glycolipids*, como um novo marcador das CTMs. Funcionalmente, as CTMs devem ser capazes de se diferenciar em três linhagens: adipócitos[66], condrócitos[67] e osteoblastos[68]. Além destas, a literatura tem relatado que elas podem originar uma ampla variedade de linhagens celulares incluindo osteócitos, miócitos, astrócitos, neurônios, células endoteliais, hepatócitos, cardiomiócitos, entre outras[22,66,69-72].

Tem sido demonstrado que as CTMs têm propriedades imunomoduladoras[73] contra aloantígenos, podendo ser utilizadas no tratamento de rejeições no transplante de órgãos, e imunossupressivas suprimindo a função de uma ampla gama de células do sistema imunológico *in vitro*, incluindo células T[74-78], células B[79], células NK[80] e células apresentadoras de antígenos[81]. Dados clínicos e experimentais também demonstraram que a função imunorreguladora das CTMs derivadas da MO pode contribuir para a redução da incidência de doença do enxerto-*versus*-hospedeiro após o transplante de células hematopoéticas[77,82]. As CTMs são não imunogênicas (imunofenótipo MHC I^+, MHC II^-, $CD40^-$,$CD80^-$, $CD86^-$), portanto o transplante num hospedeiro alogênico não requer imunossupressão[78,83]. Por esta razão as CTMs são consideradas importantes devido a sua potencial utilização para a terapia celular e a engenharia tecidual.

As CTMs têm habilidade de mobilização para tecidos danificados, possivelmente em resposta a citocinas e fatores de crescimento, e participam ativamente no reparo tecidual[84-86].

1.4. Fontes de células-tronco mesenquimais

A MO tem representado a principal fonte de CTMs pois são de fácil obtenção, apresentam grande potencial de proliferação *in vitro*[87] e a possibilidade de serem de origem autóloga, tornando-as atrativas para o uso terapêutico[83,88]. Entretanto, além da freqüência das CTMs na MO ser muito baixa (aproximadamente 0,05% da fração celular)[89], as CTMs de MO possuem alto grau de exposição viral, apresentam uma diminuição da capacidade de proliferação/diferenciação com o aumento da idade, além do procedimento de

coleta ser invasivo e doloroso[90]. Desta forma tem sido discutida a necessidade de encontrar fontes alternativas de CTMs.

CTMs e células semelhantes às CTMs têm sido isoladas de outras fontes teciduais incluindo tecido adiposo[63,91], derme[92], sangue[93], osso trabecular[94], cordão umbilical humano[95], sangue de cordão umbilical humano[96,97], pulmões[98], polpa de dentes, ligamento periodontal[99] e parede da veia safena[100,101] sugerindo que estas células estão ampla e diversamente distribuídas *in vivo*.

As CTMs do sangue de cordão umbilical (SCU) têm despertado grande interesse para uso clínico futuro. O SCU é uma fonte rica de células-tronco hematopoéticas a qual apresenta várias vantagens éticas e práticas, podendo ser coletada sem riscos para o doador e com baixo risco de contaminação viral[100]. Contudo, a existência de CTM no cordão tem sido controversa. Alguns estudos relataram que no SCU[103,104] ou sangue periférico após mobilização de células- tronco, não há células progenitoras mesenquimais[95]. Porém ERICES *et al*. (2000)[105] mostraram que o SCU possui células aderentes com o fenótipo de células progenitoras mesenquimais fortemente expressando CD105 (SH2), SH3 e SH4 (CD73). LEE *et al*. (2004)[96] obtiveram do sangue de cordão umbilical uma população de células aderentes semelhantes às CTMs da MO em relação a morfologia, imunofenotipagem, potencial de proliferação e diferenciação. Observou-se também a expressão de genes específicos para multi-linhagens nas células derivadas do SCU, indicando que CTMs com potencial altamente proliferativo e de diferenciação estão presentes no cordão umbilical de humanos. Uma população de células-tronco CD45 e HLA classe II negativas com grande capacidade proliferativa *in vitro* sem diferenciação espontânea, mas com potencial intrínseco e direcionado para o desenvolvimento de ossos, cartilagem, células hematopoéticas, neurais, fígado e tecido cardíaco *in vivo* em diversos modelos animais (como rato, camundongo e carneiro) foi identificada, a partir de células obtidas do SCU[97]. Apesar da presença das CTMs no SCU, observou-se que a eficiência de isolamento destas células é muito baixa[72,106], e isto seria devido a fonte de CTMs e não a diferenças metodológicas nos diferentes estudos realizados[106] .

O tecido adiposo (TA) representa uma fonte de células-tronco adultas que podem se diferenciar em várias linhagens como ossos, cartilagem,

adipócitos, músculo, endotélio e células neuronais[63,91,107-110]. As vantagens das células-tronco derivadas do tecido adiposo (CTDAs) são a fácil obtenção, a freqüência mais alta (0,5%) e a presença de um grande potencial proliferativo *in vitro* comparado com as células mesenquimais obtidas da MO[111]. Assim, as CTDAs podem também apresentar potencial terapêutico para o reparo de tecidos danificados[112-114].

Estudos têm demonstrado que as CTMs-MO e CTDAs diferenciam-se em cardiomiócitos *in vitro* e *in vivo*[18,24,91,115-121] o que as tornam importantes para a cardiomioplastia celular.

1.5 Diferenciação das células-tronco mesenquimais

Os processos que regulam a diferenciação celular são complexos e as interações entre os sinais/vias ainda não são totalmente conhecidos. Entretanto, é clara a dependência do microambiente em seu desenvolvimento. Esta dependência é também observada no comissionamento de células-tronco. Estudos demonstram que as CTMs respondem a sinais do microambiente tecidual do hospedeiro e diferenciam-se em células maduras[108,122].

A diferenciação das células-tronco em uma determinada linhagem pode ser alcançada através de várias técnicas que podem ser utilizadas conjuntamente envolvendo múltiplas vias de sinalização, podendo ser mimetizadas *in vitro*[123]. Várias estratégias têm sido empregadas como o desenvolvimento de meios de cultura, de preferência quimicamente definidos, livres de soro, ou a utilização de substitutos sintéticos[124] com suplementação de citocinas recombinantes e fatores de crescimento[125].

Diversos compostos químicos sintéticos também são capazes de promover a diferenciação das células-tronco *in vitro*[72,126,127]. Substâncias químicas sintéticas são menos lábeis, com meia vida mais longa, comparada com as citocinas. A 5-azacitidina tem sido bastante considerada para a diferenciação das CTMs em cardiomiócitos em estudos experimentais[22,128-130], porém apresenta efeitos genotóxicos devido a atividade de desmetilação não específica[131] e baixa eficiência. Portanto, esta droga não se mostra atrativa do ponto de vista clínico.

Outra abordagem seria a utilização do meio condicionado da cultura de células somáticas diferenciadas, o qual poderia conter vários fatores solúveis capazes de direcionar a diferenciação das células-tronco *in vivo*[122,132]. A vantagem em se utilizar o meio condicionado sobre a co-cultura é que não há a necessidade de separar diferentes populações celulares. Além disso, filtrando-se o meio condicionado não haveria o risco de contaminação com um tipo celular diferente, porém não evitaria o risco de contaminação viral[123]. A maior limitação na utilização do meio condicionado, diferentemente da co-cultura convencional, é que não há contato físico direto e comunicação cruzada regulatória entre os diferentes tipos celulares, o que poderia limitar a transdução de sinais de diferenciação para as células-tronco[108].

O estímulo físico é outro fator que pode levar a diferenciação das células-tronco[133]. GIAMBRIONI FILHO *et al*. (2004)[134] mostraram os efeitos da eletroestimulação *in vitro* sobre as células-tronco da medula óssea humana, $CD34^+$ e mesenquimais, a fim de diferenciá-las em células miogênicas antes do transplante celular. Após a eletroestimulação sobre as células-tronco mesenquimais, observou-se uma diferenciação miogênica, com células positivas para os anticorpos anti-desmina e anti-troponina I e C. Portanto o pré-condicionamento das culturas celulares utilizando a eletroestimulação mostrou uma diferenciação miogênica *in vitro*. Porém questiona-se a expressão nestas células da conexina-43, a qual faz parte das conexões funcionais entre os cardiomiócitos. Estas conexões possibilitam um acoplamento eletromecânico entre as células diferenciadas e os cardiomiócitos já existentes.

Proteínas de matriz extracelular também podem induzir diferenciação celular, possivelmente mediada por integrinas. As integrinas são a principal classe de moléculas receptoras de proteínas da matriz extracelular, e que têm importante papel na diferenciação celular e desenvolvimento do tecido[135]. Uma das moléculas mais comuns da matriz extracelular é a fibronectina, a qual está envolvida no *homing* de células $CD34^+$ para a MO durante o transplante[133]. Células-tronco obtidas da medula óssea e cordão umbilical cultivadas na presença de fibronectina e laminina expressaram marcadores de células cardíacas[137]. Porém, foi observado que os diferentes clones de células mesenquimais obtidas apresentaram um comportamento heterogêneo em

resposta à indução da diferenciação celular com fibronectina e laminina. Além disso, somente uma pequena proporção dos clones expressam marcadores específicos cardíacos, demonstrando a baixa eficiência de diferenciação.

A co-cultura com células somáticas diferenciadas é outro método que pode levar à diferenciação celular, pois permite o contato físico entre diferentes tipos celulares, o qual pode ativar vias diferenciativas. Além disso, a co-cultura fornece um ambiente fisiológico para diferenciação das células-tronco *in vitro* mais semelhante às condições *in vivo,* como nos tecidos e órgãos[123]. PLOTNIKOV *et al*. (2008)[138] demonstraram a formação de estruturas denominadas nanotubos, que fazem conexão com as células vizinhas, e expressão de miosina humana nas CTMs, após o co-cultivo destas células humanas com cardiomiócitos de ratos. Apesar disso, não há um consenso na literatura se esta interação célula-célula é necessária para que ocorra a diferenciação das CTMs, ou se a presença dos fatores químicos solúveis são suficientes[71,122,139].

Algumas evidências indicam que o íntimo contato físico, que ocorre também durante a co-cultura, pode levar à fusão de diferentes tipos celulares[140]. A habilidade das células-tronco adultas de se transdiferenciarem em tipos celulares que são radicalmente diferentes do seu tecido de origem, quando transplantadas *in vivo*, tem sido atribuída à fusão celular[59]. Alguns trabalhos demonstraram a capacidade dos cardiomiócitos de se fundirem com outras células somáticas ou células progenitoras, produzindo fenótipos híbridos[141,142]. A co-cultura de duas ou mais populações celulares distintas também tem o risco de transmissão de patógenos, em particular, vírus. Isto poderia constituir o maior obstáculo para aplicação clínica da co-cultura para diferenciação das células-tronco. Outro problema é a dificuldade de separação das populações celulares co-cultivadas, o que poderia ser contornado pela utilização do método de separação magnética[143] ou *sorting* por citometria de fluxo[144].

1.6 Influência de espécies reativas na diferenciação celular

O acúmulo de evidências mostrando a importância das espécies reativas no processo de diferenciação celular *in vitro* têm resgatado a teoria redox do desenvolvimento[145]. Esta teoria postula que diferentes suplementações de oxigênio nos tecidos *in vivo* resultam no estabelecimento de gradientes metabólicos e oxidativos, que têm um importante papel na diferenciação e desenvolvimento. Este conceito é sustentado pelo fato que células e tecidos em diferentes estágios de diferenciação exibem discretas mudanças em seus estados redox. Geralmente quando as células tornam-se mais diferenciadas, seu ambiente intracelular torna-se mais pró-oxidante, quando comparado a células não diferenciadas[146]. Um dos principais tampões redox utilizado pelas células para a manutenção do estado redox intracelular é o par glutationa (GSH)/glutationa dissulfeto (GSSG)[147]. Diminuições da taxa GSH/GSSG, ou seja um estado mais oxidativo, está associado a processos diferenciativos[148,149], como descrito, por exemplo, para a diferenciação de monócitos[150] e de neurônios[151,152]. Esta mudança no balanço redox é devido ao aumento de geração de espécies reativas de oxigênio e nitrogênio, os quais atuam como segundos mensageiros[153] em muitas cascatas de sinalização intracelular[154,155]. De fato, espécies reativas, principalmente superóxido e peróxido de hidrogênio, parecem ser mediadores importantes na sinalização iniciadas por fatores de crescimento[153], incluindo os fatores de crescimento hematopoéticos[156]. Recentemente, foi também demonstrado o envolvimento do radical superóxido durante a diferenciação osteogênica das CTMs[157], do peróxido de hidrogênio[158] e do óxido nítrico[159] na cardiomiogênese a partir das células-tronco embrionárias.

O óxido nítrico (NO) é um radical livre gasoso de meia-vida relativamente curta, gerado pela família de proteínas óxido nítrico sintase (NOS) e que atua como molécula sinalizadora, mediando a comunicação célula-célula[160]. Enzimaticamente, o NO é formado a partir do nitrogênio da guanidina presente na L-arginina, sob a ação catalítica das enzimas NOS, gerando concentrações equimolares de L-citrulina[161]. A síntese do NO ocorre a partir da ativação da síntese de NOS, a qual existe em três isoformas: duas isoformas constitutivas e uma induzível. As duas isoformas cNOS foram

clonadas de cérebro do rato (neuronal NOS, nNOS, ou NOS tipo I) e do endotélio vascular (endotelial NOS, eNOS, ou NOS tipo III)[162]. A ativação das cNOS é dependente da elevação de íons de Ca^{2+}. A terceira isoforma é a induzível (induzível NOS, iNOS ou NOS tipo II), que é independente de cálcio e é expressa em tecidos alvos, após estimulação com endotoxina e algumas citocinas nas respostas inflamatórias[163,164]. Ambas as vias de síntese do NO tem papel biológico no coração de mamíferos[165,166].

Os efeitos do NO podem mediar importantes processos biológicos como a proliferação[167,168], mobilização e diferenciação[169,170] de várias populações celulares precursoras derivadas de órgãos, vasorregulação, reações inflamatórias, plasticidade sináptica, contratilidade cardíaca, respiração mitocondrial, pré-condicionamento isquêmico, entre outras[171,172].

Além disso, o NO pode funcionar como um mensageiro intracelular, neurotransmissor e hormônio[160,173]. É considerado uma molécula de sinalização da diferenciação celular produzido por virtualmente todos os tipos celulares que compõem o miocárdio, sendo importante para o desenvolvimento cardíaco[166]. O papel e a regulação da produção do NO no miocárdio ocorrem principalmente através das cascatas de sinalização intracelulares iniciadas pela guanosina 3´, 5´-cíclica monofosfato (GMP cíclico, ou cGMP)[174]. Este, por sua vez, é produzido pela ativação da guanilato ciclase, após ligação do NO ao grupo heme da enzima[173]. A adenosina 3´, 5´-monofosfato cíclico (AMP cíclico ou cAMP) é outro mensageiro que também tem um importante papel na regulação da proliferação e diferenciação celular. É importante manter a razão cAMP/cGMP para fornecer o microambiente correto para sobrevivência e função celular. O aumento da regulação de cAMP é um processo chave para facilitar a diferenciação e inibir a proliferação. Além disso, cAMP poderia atuar como um indutor endógeno da produção de NO induzida por citocina pelo miocárdio em certas condições imunológicas e inflamatórias, incluindo pós transplante cardíaco, cardiomiopatia, miocardite e dano por reperfusão-isquemia.

A função cardíaca é regulada pelo NO através de efeitos dependentes e independentes da vasculatura. Os efeitos dependentes do vaso incluem a regulação do tônus do vaso coronário, trombogenicidade (NO atua como agente anti-plaquetário) e propriedades proliferativas, inflamatórias e

angiogênicas. Os efeitos diretos do NO incluem vários aspectos da contratilidade cardíaca, da regulação fina da excitação-contração e respiração mitocondrial. A atuação múltipla do NO na fisiologia cardíaca é devido a perfeita regulação molecular das três isoformas das NOS. A descompensação cardíaca, tal como a produção em excesso do NO pelas células inflamatórias, pode resultar em profundos distúrbios celulares levando a insuficiência cardíaca[175].

Estudos demonstram a importância do NO na sinalização da cardiomiogênese durante o desenvolvimento cardíaco embrionário e em células tronco embrionárias após a diferenciação em cardiomiócitos[159,174]. A expressão das isoformas iNOS e eNOS tem sido observadas no coração de camundongos durante o estágio de desenvolvimento precoce[173]. Esta expressão diminui antes do nascimento, sugerindo que o período de exposição ao NO é essencial para o desenvolvimento normal. BLOCH *et al*. (1999)[174] mostraram que a geração de NO é necessária para a cardiomiogênse, pois inibidores de NOS previnem a maturação de cardiomiócitos terminalmente diferenciados.

KANNO *et al.* (2004)[159] mostraram que a cardiomiogênese nas células-tronco embrionárias pode ser regulada pelo estado redox intracelular. O tratamento das células-tronco embrionárias com S-nitroso-N-acetyl-D,L-penicillamine (SNAP) ou sua transdução com gene iNOS aumenta o número de grupos celulares que contraem espontaneamente e a expressão da proteína cadeia leve da miosina. Estes efeitos diminuíram pelo tratamento com N,G-nitro-L-arginine-methyl-ester (L-NAME), um inibidor das iNOS, demonstrando que o NO promove a diferenciação cardíaca da células-tronco embrionárias de camundongos através de um mecanismo dual envolvendo não somente a mudança fenotípica mas também uma indução da apoptose de células não diferenciadas. Assim, este estudo indica que o NO, ou seus metabólitos, não somente regula a expressão de genes específicos cardíacos como inibe a morte celular programada nas células comissionadas a cardiomiócitos.

2. JUSTIFICATIVA E OBJETIVOS

A recuperação da função cardíaca em modelos experimentais pós-transplante têm sido parcialmente atribuída a transdiferenciação das células-tronco levando à formação de novos cardiomiócitos[18,125,177], embora os mecanismos de reparo não sejam bem conhecidos[178]. As células-tronco adultas não diferenciadas não formam teratomas, mas podem originar um tipo tecidual não desejado no local do transplante, o que poderia prejudicar a regeneração do tecido[71]. A pré-diferenciação das células-tronco para uma linhagem cardíaca *in vitro* pré-transplante pode ser mais vantajosa que o transplante de células não comissionadas. Neste contexto, o objetivo geral deste trabalho foi avaliar a capacidade do NO ou de seus metabólitos de induzir a diferenciação de CTMs a cardiomiócitos ou células semelhantes a cardiomiócitos. Particularmente, estudamos:

- a caracterização morfológica, imunofenotípica e funcional de CTMs isoladas de três fontes : MO, SCU e TA;
- os efeitos de um nitrosotiol (S-nitroso-N-acetil-D,L-penicilamina - SNAP) e de um doador NO (2-(N,N-dietilamina)-diazenolato-2-óxido - DEA/NO) em induzir a expressão de genes e proteínas musculares cardíacas e não cardíacas, bem como a expressão gênica de VEGF pelas CTMs.

Dissimilar differentiation potential of mesenchymal stem cells isolated from bone marrow, umbilical cord blood and adipose tissue

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Abstract

Mesenchymal stem cells (MSCs) have been claimed to be promising candidates for use in new cell-based therapeutic strategies, such as mesenchyme-derived tissue repair. They are easily isolated from adult tissues and are not ethically restricted. MSC-related literature, however, is conflicting in relation to MSC differentiation potential and molecular markers. Here, we compared MSCs isolated from bone marrow (BM), umbilical cord blood (UCB) and adipose tissue (AT). The isolation efficiency for both bone marrow (BM) and adipose tissue (AT) was 100%, but that from umbilical cord blood (UCB) was only 30%. MSCs from these tissues are morphologically and immunophenotypically similar even though their differentiation diverges. Differentiation to osteoblast and chondroblast was similar among MSCs from all sources, as analyzed by cytochemistry. Adipogenic differentiation showed that UCB-MSCs produced few and small lipid vacuoles in contrast to BM-MSCs and adipose tissue-derived stem cells (ADSCs) (arbitrary differentiation values of 245.57 ± 943 and 243.89 ± 145.52 μm^2/nucleus, respectively). The mean area occupied by individual lipid droplets was 7.37 μm^2 for BM-MSCs and 2.36 μm^2 for ADSCs, indicating more mature adipocytes in BM-MSCs than ADSCs treated cultures. We analyzed FAPB4, osteonectin, ALP and type II collagen gene expression by qPCR to confirm adipogenic, osteogenic and chondrogenic differentiation, respectively. Results showed that all three sources presented a similar capacity for chondrogenic and osteogenic differentiation and they differ in their adipogenic potential. Therefore, it may be crucial to pre-determine the most appropriate MSC source for future clinical applications.

KEYWORDS: mesenchymal stem cells, bone marrow, umbilical cord blood, adipose tissue, differentiation

Introduction

Mesenchymal stem cells (MSCs) comprise a population of multipotent progenitors capable of both supporting hematopoiesis and differentiating into many tissues (1). MSCs are not ethically restricted and have low immunogenicity (2). MSCs are thought to be promising candidates for novel cell-based therapeutic strategies, such as in the repair of mesenchyme-derived tissues. In fact, MSCs have already been clinically used to repair or regenerate somatic tissues (3-6), to promote engraftment, and to prevent or treat severe graft-*versus*-host disease in allogenic stem cell transplantation (7-8). In the appropriate microenvironment, MSCs differentiate into various cell types, including adipocytes, osteoblasts, chondrocytes (9-11), cardiomyocytes (12-14), and also into non-mesodermal-derived cells, including hepatocytes and neurons (15). Selective differentiation is dependent on specific environmental effectors: usually a combination of growth factors and cytokines supplied *in vitro* (1,16). They were originally isolated from bone marrow (BM) by FRIEDENSTEIN *et al*. (1970); however, similar populations have been reported in other tissues, such as peripheral blood (18), cord blood (19), trabecular bone (20), adipose tissue (1), synovium (21), skin (22), muscle and brain (23).

MSCs have been characterized by their fibroblast-like morphology, plastic-adhesive and self renewal properties, and their ability to differentiate *in vitro* into, at least, three mesodermal-derived tissues: bone, cartilage and fat (1). Immunophenotypically, MSCs have been defined as cells expressing CD29, CD44, CD90, CD105 and lacking hematopoietic lineage markers and HLA-DR (9-11). However, recent studies have demonstrated that MSCs isolated from several sources are not a homogenous population and their differentiation potential may vary depending on the source and the donor (11, 24, 25). Unfortunately, the factors affecting these differences are still unknown. BM has been considered the main MSC source because of their potential to both proliferate and differentiate (3, 7). However, other sources of similar cell populations are being investigated, as BM-derived MSC isolation requires a painful invasive procedure, their frequency is low (1), and their ability to proliferate and differentiate decline with age (26).

Human umbilical cord blood (UCB) derived MSCs are being evaluated for use in cellular therapies because they are ontogenically primitive, are less exposed to immunological challenges, are abundantly available and can be harvested without risk to the donor (27). Various reports are

conflicting in relation to the presence of MSCs in UCB (28-30); however, several groups have successfully isolated MSCs from UCB (11, 15, 24, 31-35). The frequency of mesenchymal progenitors in the UCB of full-term deliveries is extremely low (0.00003% of nucleated cells) (31); however, they have the highest expansion potential when compared with other sources (11, 25).

Adipose tissue has recently been identified as a convenient alternative source for MSC-like cells. Adipose tissue-derived stem cells (ADSCs) are available in quantities of hundreds of million cells per individual (9), have an extensive self-renewal capacity (36), are easily isolated by differential sedimentation, and can be cultured for several months *in vitro* with low levels of senescence (37). ADSCs also have the potential to differentiate into various cells, including adipocytes, osteoblasts, chondrocytes, neurons and multinucleated myocytes in response to lineage-specific induction factors (10, 11, 37-41).

The starting population for most of the trans-differentiation experiments is different; therefore, comparing results between various groups is difficult, and may also partly account for the lack of reproducibility in some of the initial reports (10). MSCs are poorly defined, and this leads separate groups to assign diverse names and phenotypes to this cell population (42). Thus, a precise characterization of MSC and its properties relating to molecular differentiation represents an absolute condition for future development and exploitation of stem cell research for clinical applications (10, 11).

In this study we characterized for the first time adult stem cells isolated from three sources (BM, UCB and AT) by flow cytometry and compared their differentiation properties to adipocytes, osteoblasts and chondrocytes by cellular (cytochemistry) and molecular (RT-PCR/qPCR) approaches.

Materials and Methods

Collection of BM, UCB and AT. Human BM stromal cells were obtained from the iliac crest of 10 dilated myocardiopathy patients, who were aged between 50 and 70 years (60.36 ± 9.86), and who had applied for a stem cell transplantation procedure. About 5 mL of BM aspirate were collected in a syringe containing 10,000 UI heparin to prevent coagulation.

UCB units from full-term deliveries (n=10) were collected from unborn placenta by a standardized procedure using syringes containing anticoagulant-citrate-dextrose (ACD), and were processed within 12 hours after collection. Donors faced no complications throughout their pregnancy.

AT were obtained from 10 donors, aged between 26 and 50 years (38.0 ± 12.55), who were undergoing elective bariatric surgery and dermolipectomy procedures. Typically, 100 mL of AT was processed.

All the samples from BM, UCB and AT were collected after informed consent following guidelines on the use of human subjects, as approved by the Ethics Committee from Pontifícia Universidade Católica of Paraná (approval number 597).

Isolation and Culture of Adherent Cells. Three sources of adherent cells were used in this work.

Bone marrow

The aspirate was diluted 1:3 with Iscove's Modified Dulbecco's Medium (IMDM) (Gibco™ Invitrogen, NY, USA) and carefully loaded onto Histopaque® (1.077 g/mL) (Sigma Chemical, St. Louis, USA) to isolate BM mononuclear cells (MNCs). MNCs were isolated by density gradient centrifugation (400*g*, 30 minutes, room temperature) and washed twice with IMDM (43). BM-derived MNCs were cultured at a density of $1x10^5$ cells/cm^2 in T75 culture flasks (TPP, Trasadingen, Switzerland) at 37ºC in a humidified atmosphere containing 5% CO_2, using IMDM supplemented with 15% of fetal calf serum (FCS) (Gibco™ Invitrogen, NY, USA), penicillin (100 units/ml) and streptomycin (100 µg/ml) (Gibco™ Invitrogen, NY, USA). The culture medium was changed to remove the remaining non-adherent cells two days after the initial plating. Thereafter, the culture medium was replaced twice-a-week.

Umbilical cord blood

UCB MNCs were isolated using two methods. In the first, each UCB unit was diluted 1:3 and processed as described for BM. The second was performed using a commercially available kit (RosetteSep® - Stem Cell Technologies, BC, Canada) according to the manufacturer's instructions, followed by a Histopaque® density separation, as described (15). UCB-derived MNCs were set in culture at a density of $6x10^5$ cells/cm^2 in T75 culture flasks in the same culture medium described above. The cultures were incubated for four days at 37ºC in a humidified atmosphere containing 5% CO_2. Non-adherent cells were then removed and fresh medium was added to the flasks. Culture medium was removed by complete exchange every 7 days.

Adipose tissue

ADSCs were isolated using enzymatic digestion. In brief, 100 mL AT was washed with sterile phosphate-buffered saline (PBS) (Gibco™ Invitrogen, NY, USA). A one-step digestion by 1 mg/mL collagenase type I (Invitrogen™, NY, USA) was performed for 30 minutes at 37℃ under permanent shaking, followed by filtration through a 100 and 40-µm mesh filter (BD FALCON ™, BD Biosciences Discovery Labware, Bedford, USA). Cell suspension was centrifuged at 800g for 10 minutes, and contaminating erythrocytes were removed by erythrocyte lysis buffer, pH 7.3. The cells were washed and then cultivated at a density of $1x10^5$ cells/cm^2 in T75 culture flasks in DMEM-F12 (Gibco™ Invitrogen, NY, USA) supplemented with 10% of FCS, penicillin (100 units/ml) and streptomycin (100 µg/ml) (44). Medium was changed two days after the initial plating. The culture medium was then replaced twice-a-week.

BM- and UCB-derived MSCs and ADSCs were subcultured after the cultures had reached 80%-90% confluence; MSCs were detached with 0.25% trypsin/EDTA (Invitrogen™, NY, USA) and were replated as passage-1 cells (the process was then continued as previously described).

Determination of the cell-surface antigen profile. BM- and UCB-derived MSCs and ADSCs, between third and fifth passage ($P_3 – P_5$), were labeled with antibodies against several human proteins to analyze cell-surface expression of typical marker proteins: non-conjugated CD105, CD90, CD44, CD31, conjugated with fluorescein isothiocyanate (FITC), CD73, CD166, CD34, conjugated with phycoerythrin (PE), CD29, CD117, CD14 conjugated with allophycocyanin (APC) (BD Pharmingen, CA, USA) and CD45 conjugated with peridinin chlorophyll protein (PerCP) (BD Pharmingen, CA, USA). Cells were detached with 0.25% trypsin/EDTA, washed with PBS, and incubated in the dark for 30 minutes at room temperature with the respective antibody. Cells were then washed with wash flow buffer and re-suspended in 500 µL of 1% formaldehyde solution. For the detection of CD105, cells were further washed and incubated for 15 minutes with secondary antibody Goat F(ab')2 anti-mouse IgG (gamma) (Caltag Laboratories, CA, USA) (45). Mouse isotype IgG1 antibodies were employed as controls (BD Pharmingen, CA, USA). Approximately twenty thousand labeled cells were passed through a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, USA) and were analyzed by FlowJo software (Flowjo, Ashland, USA).

Differentiation Procedures. BM- and UCB-derived MSCs and ADSCs were assessed for their potency by inducing their differentiation into adipocytes, osteoblasts and chondrocytes. Cells between P_3-P_5 from each source were incubated with three differentiation media.

Adipogenic Differentiation

Subconfluent (80%) MSCs cells were seeded on glass coverslips (Sarstedt, Newton, USA) in twenty-four wells plates (TPP, Trasadingen, Switzerland) and were treated with three types of media: medium 1) 0.05 µmol/L dexamethasone (Sigma Chemical, St. Louis, USA), 10 µg/ml insulin (Sigma-Aldrich, St. Louis, USA.), 60 µmol/L indomethacin (Sigma-Aldrich, St. Louis, USA) in DMEM-HG (Gibco™ Invitrogen, NY, USA) with 15% FCS (Gibco™ Invitrogen, NY, USA) (46); medium 2) 1 µmol/L dexamethasone (Sigma Chemical, St. Louis, USA), 5 µg/ml insulin (Sigma Chemical, St. Louis, USA), 60 µmol/L indomethacin (Sigma Chemical, St. Louis, USA) in IMDM (Gibco™ Invitrogen, NY, USA) with 15% FCS (Gibco™ Invitrogen, NY, USA) (46 modified); and medium 3) Poietics™ Differentiation Basal Medium Adipogenic (Cambrex Bio Science, MD, USA) supplemented with hMSC Adipogenic SingleQuots (Cambrex Bio Science, MD, USA). Adipogenic differentiation was induced by cyclic changes; the maintenance medium containing the adipogenic inducer was changed every three days during three weeks. Oil Red O (Sigma-Aldrich, St. Louis, USA.), was used to visualize lipid-rich vacuoles. Briefly, cells were treated with Bouin´s fixative (Biotec, Labmaster, Paraná, Brazil) for 10 minutes at room temperature, washed twice with 70% ethanol and once with Milliq® water, and stained with a solution of 0.5% Oil Red O (Sigma-Aldrich, St. Louis, USA) for 1 hour. Hematoxilin-Eosin (HE) (Biotec, Labmaster, Paraná, Brazil) was used for nuclear staining. Control cells were kept in IMDM medium with 15% FCS. To quantitatively analyze adipogenic differentiation, 70 fields in three biological replicates from each source of MSCs were counted using Image-Pro Plus version 4.5. We also performed RT-PCR and qPCR to estimate the level of adipocyte-specific FABP4 mRNA in induced (medium 2) and non-induced (negative control) cultures.

Osteogenic Differentiation

Cells were seeded and cultured on slides placed in twenty-four chamber plates (TPP, Trasadingen, Switzerland) to induce osteogenic differentiation. Subconfluent (80%) cultures were subjected to three types of osteogenic medium: medium 4) 0.1 µmol/L dexamethasone , 10 mmol/L ß-glycerolphosphate (Sigma-Aldrich, St. Louis, USA), 50 µmol/L ascorbate, in DMEM-HG medium with 15% FCS (16); medium 5) 0.1 µmol/L dexamethasone, 10 mmol/L ß-glycerolphosphate, 100 µmol/L

ascorbate, and IMDM medium with 15% FCS (16 modified); and medium 6) Poietics™ Differentiation Basal Medium Osteogenic (Cambrex Bio Science, MD, USA) supplemented with hMSC Osteogenic SingleQuots (Cambrex Bio Science, MD, USA). Media were replaced every three days over a three-week period. Induced monolayers were fixed for 10 minutes in Bouin's fixative (Biotec, Labmaster, Paraná, Brazil), and washed (twice with 70% ethanol and once with Milliq® water). Monolayers were then incubated for 15 minutes with Alizarin Red S at pH 7.0 and pH 4.2 (Fluka Chemie, Buchs, UK) at room temperature to evaluate calcium accumulation. Light green (Sigma-Aldrich, St. Louis, U.S.A.) was used to counterstain. Control cells were kept in IMDM medium with 15% FCS over the same period. In addition, RT-PCR and qPCR were performed to estimate the level of the osteoblast-specific osteonectin and alkaline phosphatase (ALP) mRNA from MSCs cultured in induced (medium 5), and non-induced or control medium.

Chondrogenic Differentiation

Cells were grown in a micromass culture to promote chondrogenic differentiation (47). Briefly, $2x10^5$ cells in 0.5 mL of medium were centrifuged at 300g for 10 minutes, in a 15-ml polypropylene tube to form a pellet. Without disturbing the pellet, cells were cultured for 21 days in three different chondrogenic media: medium 7) DMEM-HG supplemented with 15% FCS and 0.01 µmol/L dexamethasone (Sigma-Aldrich, St. Louis, USA), 397 µg/ml ascorbic acid-2-phosphate (Sigma-Aldrich, St. Louis, USA), 1 mmol/L sodium pyruvate (Gibco™ Invitrogen, NY, USA), 10 ng/mL TGF-ß1 (Sigma-Aldrich, St. Louis, USA) and 1% insulin-transferrin-selenium-X (Gibco™ Invitrogen, NY, USA) (27); medium 8) DMEM-HG supplemented with 1% FCS and 10 ng/ml TGFß1**,** 0.5 µg/ml of insulin (Sigma-Aldrich, St. Louis, USA), 50 µmol/L ascorbic acid (27 modified); and medium 9) Differentiation Basal Medium Chondrogenic (Cambrex Bio Science, MD, USA) supplemented with hMSC Chondrogenic SingleQuots (Cambrex Bio Science, MD, USA). Media was changed every three days. On day 21, cell aggregates were fixed in 10% formaldehyde for 1 hour at room temperature, dehydrated in serial ethanol dilutions, and embedded in paraffin blocks. Paraffin sections (4 µm thick) were stained for histology with HE, Mallory (Biotec, Labmaster, Paraná, Brazil) or Toluidine Blue solution (Sigma-Aldrich, St. Louis, USA) to demonstrate intracellular matrix mucopolysaccharides. Chondrogenic differentiation was further confirmed by RT-PCR analysis of the chondrocyte-specific protein collagen type II mRNA in induced (medium 8) and non-induced cultures.

Total RNA extraction and Reverse Transcription-Polymerase Chain Reaction. Total RNA was obtained with the RNeasy kit (QIAGEN) and treated in column with DNAse I (QIAGEN). Concentrations were determined by spectrophotometry (GeneQuant, Amersham Biosciences). Complementary DNA (c-DNA) was synthesized from 1µg of total RNA using 1 µL of 10 µM oligo dT primer (USB Corporation) and 1 µL of reverse transcriptase (IMPROM II, Promega) according to the manufacturer's instructions. Polymerase Chain Reaction (PCR) was carried out with 20 ng of c-DNA as template, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 5 pmol of primers (except for FABP4 gene, 10 pmol) (Table 1), 2.5 mM of $MgCl_2$, 0.0625 mM of dNTPs and 1U Taq polimerase (Invitrogen™, NY, USA). The oligonucleotide primer sets used for PCR and the amplicon size are depicted in Table 1. PCR included heating at 94°C for 2 minutes, followed by 30 cycles of 94°C for 15 seconds, 55 °C for 30 seconds and 72 °C for 40 seconds and, a final extension of 72 °C for 3 minutes using a Bio-Cycler II termocycler (Peltier Thermal Cycler). Ten µL of RT-PCR products were resolved by 2% agarose gel electrophoresis, visualized by ethidium bromide staining and photographed under ultra-violet light illumination (UV White Darkroom, UVP Bioimaging Systems).

Real-Time Quantitative PCR. Real-time or quantitative PCR (qPCR) was performed using the ABI PRISM 7000 sequence detection system (Applied Biosystems). Amplifications were carried out in a final reaction volume of 20 µl with the SYBR Green master mix (Applied Biosystems, CA, USA), 10 ng of c-DNA template and 5 pmol of primers (except for FABP4, 10 pmol). PCR conditions were 50°C for 2 minutes and 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds, 60°C for 30 seconds and 72 °C for 40 seconds. The melting curves were acquired after PCR amplification confirming the specificity of the amplified products. A standard curve based on cycle threshold values was used to evaluate gene expression. In brief, we used 1:5 dilutions of known concentrations of cDNA in triplicate to generate curves extending from 50 pg to 80 ng of cDNA. We generated standard curves for each gene, including the control (housekeeping) gene. The relative amount of gene expression for each sample was normalized by dividing the value obtained for the analyzed gene by the value obtained for each control gene. Results were analyzed as gene expression relative to the housekeeping gene expression. Differences in expression were reported comparing cells induced to differentiation with non-induced control samples (48).

Statistical Analysis. Continuous variables were presented as mean ± standard deviation and categorical variables were presented as frequency and percent. Comparisons between BM- and UCB-derived MSCs and ADSCs were performed by nonparametric Kruskal-Wallis exact test and values of $p<0.05$ were considered statistically significant. Analysis was performed with the SPSS V.14 software package.

Results

Isolation, Expansion, and Morphology of BM- and UCB-derived MSCs and ADSCs. The success rate for isolating BM-derived MSCs and ADCSs was 100% (10/10). By contrast, the success rate in UCB was only 30% (3/10). UCBs were processed no longer than 12 hours after umbilical cord collection. A net volume of 74.4 ± 28.7 mL and 88.1 $x10^6$ ± 48.4 $x10^6$ MNCs were obtained on average. No correlation was detected between volume, number of MNCs in the UCB after gradient separation and success in obtaining MSCs. Although evidence for the isolation of fibroblastoid cells with MSC characteristics from UCB is still under debate (11, 15, 24, 28-30, 33-35, 44), we observed that MSC-like cells can be isolated from full-term UCB units.

The commercial kit (RosetteSep®) did not significantly improve the isolation of UCB-derived MSCs. Thus, the density gradient method (Histopaque®) was used because it was less expensive and faster. Only a few cells attached to the plastic culture flasks and formed spindle-shaped adherent cells within 3-4 weeks after plating UCB-derived MNCs. By contrast, BM derived MNCs and ADSCs formed clusters of elongated, spindle-shaped (fibroblast-like) MSCs within 3 days and reached cell confluence after 1 week.

Confluent cells were trypsinized and were sub-cultured (1:2 split). Cells from BM- and UCB-derived MSCs and ADSCs after two passages were homogeneous in size (*P* value = 0.159) and granulosity (*P* value = 0.165), showing fibroblastic shape (Fig.1).

MSC cell-surface antigen profile. Cell-surface antigen expression was evaluated by flow cytometry in at least 3 samples each from BM- and UCB-derived MSCs and ADSCs, between P_3-P_5. (Fig. 2). With few exceptions, all three sources displayed similar immunophenotypes for the markers analyzed (Figure 2 and Table 2). Cells were uniformly positive for the endoglin receptor CD105, the extracellular

matrix protein CD90, the surface enzyme ecto 5´nucleotidase CD73, the activated leukocyte cell adhesion molecule CD166, the $ß_1$-integrin CD29, and the hyaluronate receptor CD44. No detectable contamination of hematopoietic cells was observed, as flow cytometry analysis was negative for markers of hematopoietic lineage, including the lipopolysaccharide receptor CD14, the leukocyte common antigen CD45, and the endothelial cell marker CD31. The percentages of expression of CD34, a hematopoietic progenitor cell marker, in MSCs isolated from BM, UCB and ADSCs were 2.16% ± 2.48, 10.52% ± 10.58 and 10.37% ± 8.37, respectively (Table 2). Statistical analysis comparing the MSCs sources regarding CD34 showed a significant difference only between BM and ADSCs (*P* value = 0.020). Flow cytometry experiments for CD117 (c-kit) were independently analyzed by three experts. The independent analyses showed that CD117 is a complex marker to evaluate. While ADSCs were clearly positive (98,11 ± 3,06), BM- and UCB-MSCs showed a dimly positive to negative staining for CD117. This became evident when observing the mean values and standard deviations of BM- and UCB-MSCs positive for CD117 (52,7 ± 46,46 and 38,84 ± 40,80 respectively; Table 2).

Differentiation assays. After cytochemical analysis, the following differentiation media were considered the most efficient to induce adipogenic (medium 2), osteogenic (medium 5) and chondrogenic (medium 8). Using these media, MSCs from the three sources, between passages P_3-P_5, were compared for their multilineage differentiation plasticity by *in vitro* assays. Differentiation to adipocytes, osteoblasts, and chondrocytes was qualitatively assessed based on cell morphology and cytochemistry.

We used the presence of lipid-rich vacuoles stained with Oil Red O to analyze adipogenic induction. BM-derived MSCs and ADSCs presented large, rounded cells with cytoplasmic lipid-rich vacuoles (Fig. 3); however, UCB-MSCs displayed few and very small intracellular lipid droplets (Fig. 4). Seventy fields in three biological replicates from each source of MSCs were analyzed to estimate the differentiation value (DV), which was calculated by dividing the lipid droplet area by the number of nuclei, so that possible differences in field cell confluences were considered. No differences in the adipogenic potential were found between BM-MSCs (DV = 245.57 ± 943 μm^2/nucleus) and ADSCs (DV = 243.89 ± 145.52 μm^2/nucleus). The impressive high variations observed in BM-MSCs DV may be a consequence of the heterogenous cell population present at the moment analyzed. However, the

mean area occupied by individual lipid droplets in BM-MSCs was 7.37 μm^2 and 2.36 μm^2 in ADSCs, indicating that adipocytes in BM-MSCs are more mature than in treated ADSCs cultures.

Osteogenic diffrerentiation was assessed by the mineralization of the extracellular matrix, visualized by Alizarin Red S staining at pH 4.2. We detected calcium carbonate and phosphate in cells from all sources after 21 days of differentiation induction (Fig. 3). No differences in the osteogenic differentiation capacity were detected among BM- and UCB-derived MSCs and ADSCs samples.

In chondrogenic differentiation assays, MSCs formed aggregates that dislodged, floating freely in the suspension culture. High-density micromass MSC cultures generated cellular nodules, which produced large amounts of cartilage-related extra-cellular matrix molecules like collagen. Paraffin sections of the aggregates stained with HE, Mallory or Toluidine Blue showed a condensed structure with cuboidal cells and chondrocyte-like lacunae. The cells stained positively for Toluidine Blue; this dye is specific for the highly sulfated proteoglycans of cartilage matrices. All samples tested, irrespective of their origin, demonstrated a cartilage-like phenotype with chondrocyte-like lacunae (Fig. 3).

Untreated control cultures, which were growing in regular medium without adipogenic, osteogenic or chondrogenic differentiation stimuli, did not exhibit spontaneous adipocyte, osteoblast or chondrocyte formation after 14 and 21 days of cultivation (Fig. 3).

Expression profile of differentiation markers by RT-PCR and q-PCR analysis. The mRNA levels of various marker genes were analyzed from total RNA isolated from induced and non-induced cultures by RT-PCR and qPCR to quantify differentiation. GAPDH was used as an internal control.

Levels of mRNA for FABP4 were analyzed as a marker for adipogenic differentiation. FABP4 expression was easily detected by RT-PCR from induced BM-MSCs and ADSCs in comparison with the non-induced control cells; importantly, control cells were cultured for the same period treated cells. The overall RT-PCR profile was very similar for replicates from the same MSC source. However, results from qPCR detected significant variability in expression among independent biological samples (Fig. 5A). No expression or low levels of expression of FABP4 were detected for induced and non-induced UCB-MSCs, in contrast to expression observed in BM-MSCs and ADSCs (Fig. 5A). Therefore, poor adipogenic potential detected in UCB-MSCs by microscopic analysis was consistent with the results observed in FABP4 expression analysis.

We analyzed osteonectin and ALP expression to evaluate osteogenic induction. Osteonectin is a glycoprotein that has been used as a differentiation marker for bone cells (49). Osteonectin expression showed no differences between induced and non-induced cells by RT-PCR (data not shown). By qPCR, we observed discordant osteonectin expression profiles among biological samples from all three MSC sources. While a considerable increase in osteonectin expression in the induced culture in comparison with the non-induced was found in one BM sample, no difference was observed in the remaining samples. Thus, we concluded osteonectin did not appear to be a suitable marker for osteogenic differentiation, at least in the culture conditions used in this study. Therefore, ALP gene mRNA levels were analyzed. We detected higher ALP mRNA levels in the induced cells than non-induced cells from all sources after performing qPCR (Fig. 5B). In all the induced UBC-MSCs replicates analyzed, ALP mRNA levels were higher than the induced samples from the other sources (Fig. 5B).

Chondrogenesis was also further studied by analyzing the mRNA level of a well-known marker, the cartilage-specific type II collagen gene. Similar to osteonectin expression, a strong band was detected in all induced and non-induced MSCs under the RT-PCR conditions used in this study. However, we detected higher type II collagen expression in induced cells than non-induced cells after performing qPCR; this increase in expression was evident for most induced cells even though individual expression levels varied (Fig. 5C). In few cases, no significant differences between induced and non-induced cells were seen (2 out of 4 ADSCs).

Discussion

The expected plasticity of human mesenchymal progenitors is paramount for upcoming therapeutic strategies for cellular therapy and tissue engineering. Functional assays are required to establish the presence of MSCs in a tissue due to a lack of specific and universal molecular markers for identifying adult MSCs. Here, we compared the biological properties and differentiation potential of MSCs isolated from the presently most important sources BM, UCB and AT.

MSCs isolation differs depending on the source. While BM-derived MSCs and ADSCs isolation efficiency was 100%, that for UCB-MSCs was only 30%.Other groups have also reported low efficacy levels for the isolation and establishment of UCB-MSCs (10, 11, 24). Sharing UCB-MSCs with

the fetus (50) and cross-contamination with monocytes and osteoclast-like cells during culture establishment (24) are some of the hypotheses to explain the low yields of MSCs from this source. Also, successes in obtaining UCB-derived MSCs are related to the time between collection and isolation, and the UCB unit blood volume (24). In this study, the storage time was less than 12 hours, and the mean volume was 74.4 ± 28.7 mL; however, the number of MNCs was low ($88.11x10^6$ ± 48.37), which might account for the extremely low frequency of UCB-derived MSCs obtained in comparison with frequencies for BM-MSCs or ADSCs. The period for establishing a BM-derived MSCs or ADSCs monolayer was shorter than that of UCB-MSCs. Growth of the latter was slower than BM-MSC and ADSC cultures, but once cultures were established, growth was maintained over multiple passages. This probably reflects the low precursor frequency for MSC in UCB (32).

No morphological differences were observed between BM- and UCB-derived MSCs and ADSCs, as has been previously reported (10, 11, 51). Also, flow cytometry measurements showed no significant differences concerning cell size and complexity in all MSC populations (data not shown). The homogeneity of MSC cultures at specific passages was apparent after assessing cell surface antigen profile. The direct comparison reported here showed that BM- and UCB-derived MSCs and ADSCs share classic MSC marker proteins (52). As expected, these cells lacked hematopoietic CD14, CD45 and the endothelial marker, CD31. However, CD34 gene expression was 2% in BM-MSCs and about 10% in UCB-MSCs and ADSCs. This observation was not unusual as freshly isolated or primary cultures of BM- and UCB-derived MSCs and ADSCs have been reported to be dimly to significantly positive for CD34 marker (15, 53-56).

CD117 was present in ADSCs and dim in BM- and UCB-MSCs. Expression of this protein by MSCs is controversial. It has been previously reported that MSCs do not express CD117 (10, 57-59), while other reports have showed that embryonic stem cells, hematopoietic stem cells and MSCs are dimly or strongly positive for this marker (60, 61); our results are consistent with the latter. Together, these data strongly suggests that BM-, UCB-derived MSCs and ADSCs are highly similar morphologically but not so immunophenotypically (54, 57, 62, 63).

In this study, we used qualitative assays to demonstrate the *in vitro* multilineage developmental potential of BM- and UCB-derived MSCs and ADSCs after exposure to specific culture conditions. BM-MSCs and ADSCs demonstrated a high *in vitro* potential to differentiate into adipocytes, osteoblasts and chondrocytes, whereas UCB-MSCs presented a more restricted, or at

least delayed, adipocyte differentiation capacity. Immaturity of these neonatal cells cannot account for their low adipocyte differentiation potential since differentiation to both osteoblasts and chondrocytes was achieved similarly to BM-MSCs and ADSCs.

BM-MSCs and ADSCs cultures had a greater propensity to differentiate into adipocytes than UCB-MSCs, under similar culture conditions. Induced BM-MSCs presented more mature adipocytes (unilocular lipid vacuoles) by morphometric assessment than induced ADSCs. KARAHUSEYINOGLU *et al*. (2007) reported that some MSCs in the BM stroma may already be committed to form mature adipocytes *in situ*. Previous studies had reported conflicting data regarding the adipogenic differentiation potential of UCB-MSCs (10, 11, 15, 24, 25, 32, 50). Here, UCB-MSCs rarely differentiated toward adipocytes under our standard differentiation protocols. Only tiny lipid vacuoles were observed in few UCB-MSCs after 21 days of induction, and FABP4 expression was poor or even absent; FABP4 is a fatty acid binding protein characteristically present in adipocytes. These tiny lipid vacuoles suggest that differentiation is at initial stages and it is highly probable that a longer culture period be necessary for UCB-derived MSC adipogenic differentiation. In fact, human umbilical cord stromal cells (HUCSCs) achieved adipogenic differentiation only after 40 days of induced culture (66); this represents a relatively longer period than with BM-MSCs and ADSCs. Also, BIEBACK *et al*. (2004) showed that adipogenic differentiation could solely be induced in MSC-like cells cultured continuously in adipogenic induction medium for at least five weeks.

In this study, human BM- and UCB-derived MSCs and ADSCs were able to proliferate and subsequently differentiate into osteoblasts. Incubation with differentiation medium induced cell aggregation and matrix production, which positively stained with the calcium-specific marker Alizarin Red S. The mRNA profiles for osteonectin were not satisfactory for the detection of osteoblast differentiation, at least under our conditions. Untreated MSCs from all three sources mostly showed no differences in osteonectin mRNA levels compared to induced MSCs. Data deposited at the GEO profile at NCBI (Accession Number GDS1288 record | GPL96 212667) show that osteonectin mRNA levels in BM-MSCs are quite high, and it has also been shown by SAGE analyses that non-induced BM- and UCB-MSCs significantly expressed this glycoprotein (67, 68); these data are consistent with our observations. Thus, we suggest that osteonectin is not appropriate hallmark genes for cultures induced to differentiate into osteoblasts during 21 days. Matrix mineralization is the latest stage of osteoblast differentiation process and osteonectin may be considered a marker for terminal

differentiation (69); terminal differentiation was not achieved in the 21 days of our induced cultures. Accordingly, PLANT and TOBIAS (2001) studied osteoblast differentiation and observed that osteocalcin, osteopontin, and osteonectin expression showed modest increases only at later times, such as 20 and 24 days after induction. Conversely, ALP showed to be a good osteogenic marker under conditions used in this study. Besides, identifying genes associated with osteoblast differentiation is a very complex task in MSCs induced to osteoblast lineage (71-74).

BM- and UCB-derived MSCs and ADSCs cultured with TGF-ß developed typical morphological features of chondrocytes and produced mucopolysaccharide, an indicator of chondrogenic differentiation. Even though the extra cellular matrix protein, collagen type II, is expressed by chondrocytes and MSCs, q-PCR assays clearly showed that its mRNA levels were higher in induced MSCs than in non-induced MSCs. A common observation for all the molecular markers analyzed was the considerable variability seen among all the biological samples. The overall profiles were similar among samples that had undergone similar treatment, but the relative mRNA levels differed enormously. It is highly probable that the variation observed was mainly due to the age, the health condition and the genetic background of the patients/donors, rather than the technical variations (75-76).

Here we have presented comparative data from human BM and UCB-derived MSCs and ADSCs. It is reasonable to conclude that MSCs can be found in these three various tissues and, although MSCs from the 3 sources analyzed here may be considered morphologically and immunophenotypically similar with the usual markers available, they clearly diverge in their differentiation capacity and/or differentiation kinetics. Presently, stem cell-based therapies are being extensively studied *in vivo*. While BM-MSCs and ADCS can produce a variety of tissues of mesodermal and non-mesodermal origins (77-82), the *in vivo* adipocyte differentiation potential of UCB-derived adherent cells seems to be reduced (27), as it has been observed in our *in vitro* assays. Therefore, further basic research is still necessary to understand the biology of MSCs obtained from different tissues and to delineate their extent and significance on clinical applications.

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Figure and Table Legends

Figure 1. Microscopic appearance of BM-MSCs (A), UCB-MSCs (B) and ADSCs (C); x400. Abbreviations: BM, bone marrow; UCB, umbilical cord blood; MSCs, mesenchymal stem cell; ADSC, adipose tissue derived stem cells. Bar indicates 20 µm.

Figure 2. Immune phenotype by flow cytometry. BM-, UCB-MSCs and ADSCs were labeled with antibodies against the indicated antigens, and analyzed by flow cytometry. Representative histograms are displayed. On the y-axis is the % of Max (the cell count in each bin divided by the cell count in the bin that contains the largest number of cells) and the x-axis is the fluorescence intensity in a log (10^0 – 10^4) scale. Isotype control is shown as a thick black line histogram. Abbreviations: BM, bone marrow; UCB, umbilical cord blood; MSCs, mesenchymal stem cell; ADSC, adipose tissue derived stem cells.

Figure 3. Differentiation of BM-, UCB-MSCs and ADSCs. Cells between P_3-P_5 from each source were incubated for 21 days in the presence of specific differentiation agents for adipocytes (medium 2), osteoblasts (medium 5) and chondrocytes (medium 8, see material and methods for media composition). Differentiation into adipocyte lineage was demonstrated by staining with Oil Red O, Alizarin Red S staining shows mineralization of the extracellular matrix, Toluidine Blue shows the deposition of proteoglycans and lacunaes; x200. Untreated control cultures without adipogenic, osteogenic or chondrogenic differentiation stimuli are shown on the bottom right corner of each photograph. Abbreviations: BM, bone marrow; UCB, umbilical cord blood; MSCs, mesenchymal stem cell; ADSC, adipose tissue derived stem cells. Bar indicates 20 µm.

Figure 4. Tiny intracytoplasmic lipid droplets (arrowheads) present in UCB-derived MSCs under standard differentiation conditions; 1000X. Bar indicates 20 µm.

Figure 5. Expression profile of differentiation markers. BM-, UCB-MSCs and ADSCs were maintained in induced or control medium for 21 days and assayed for the expression of adipogenic, osteogenic

and chondrogenic specific mRNA levels. Adipogenic differentiation marker FABP4 (A), osteogenic differentiation marker ALP (B) and chondrogenic differentiation marker Collagen type II (C) were analyzed by q-PCR. Abbreviations: BM, bone marrow; UCB, umbilical cord blood; MSCs, mesenchymal stem cell; ADSC, adipose tissue derived stem cells; I, induced cells; NI, non-induced cell (negative control); ND, non detected. Representative results of 3 independent experiments are shown. q-PCR are expressed as mean and standard deviation of the technical triplicate. GAPDH was used as an internal control. When possible, the relative amount values were normalized with the non-induced values (relative amount / N); thus, non-induced sample value = 1.

Table 1. Primer set utilized for RT-PCR and qPCR analyses.

Table 2. Comparison of the expression of surface proteins of mesenchymal stem cells derived from at least 3 samples of BM-, UCB-MSCs and ADSCs analyzed by flow cytometry.

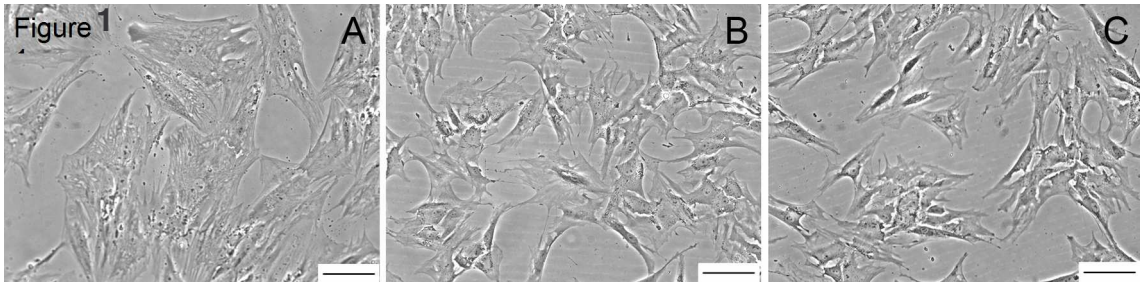
Figure 1
A
B
C

Figura 2

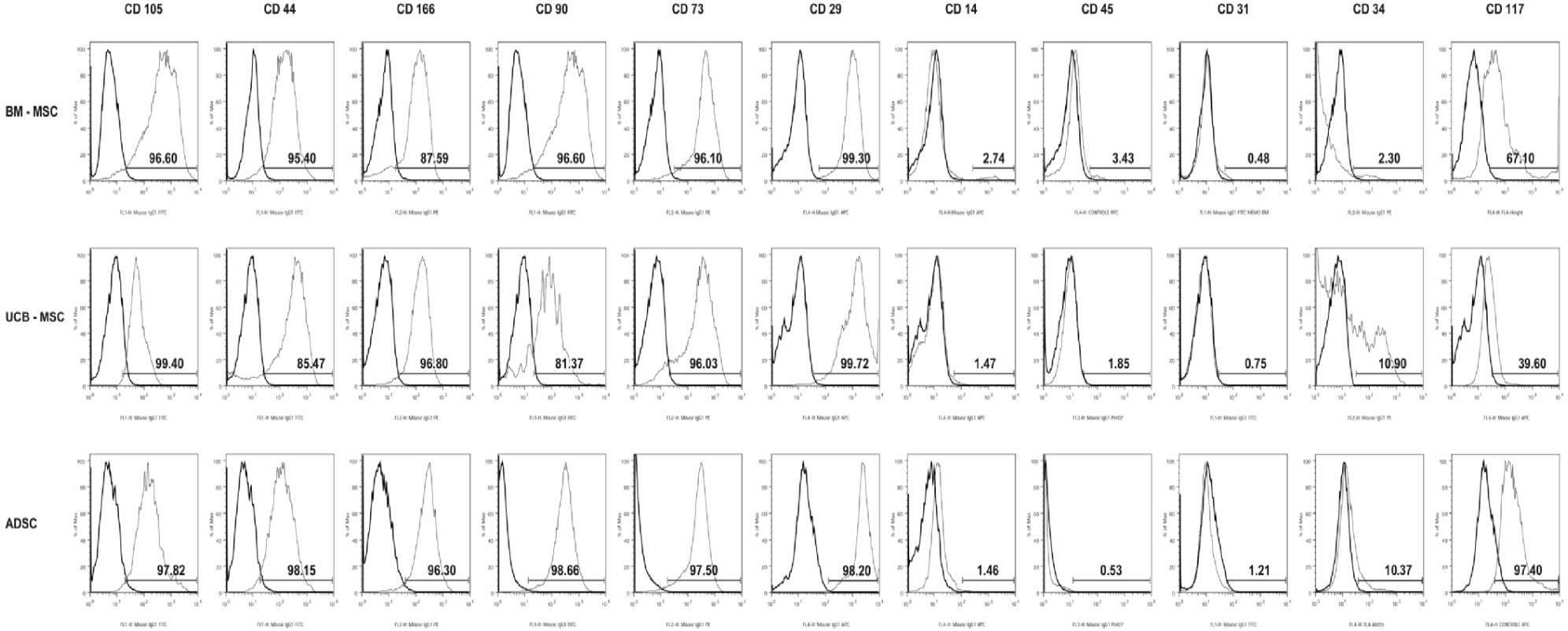
CD 105
CD 44
CD 166
CD 90
CD 73
CD 29
CD 14
CD 45
CD 31
CD 34
CD 117
BM - MSC
96.60
95.40
87.59
96.60
96.10
99.30
2.74
3.43
0.48
2.30
67.10
UCB - MSC
99.40
85.47
96.80
81.37
96.03
99.72
1.47
1.85
0.75
10.90
39.60
ADSC
97.82
98.15
96.30
98.66
97.50
98.20
1.46
0.53
1.21
10.37
97.40

Figure 3

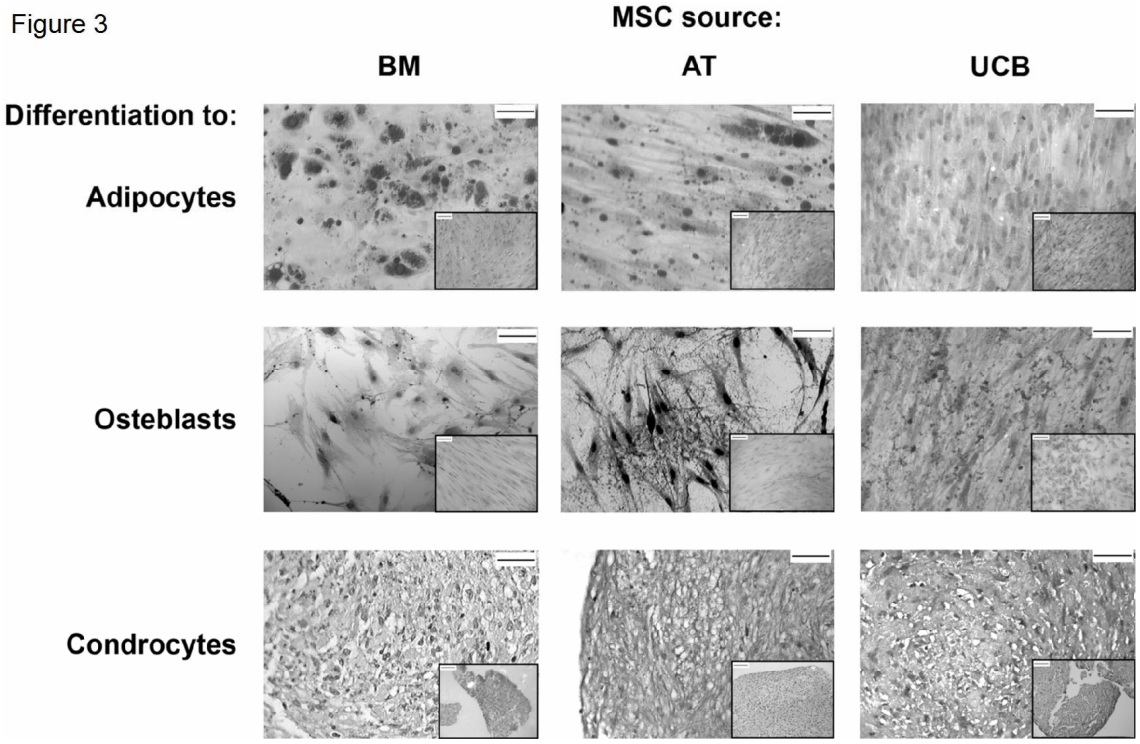

Figure 4

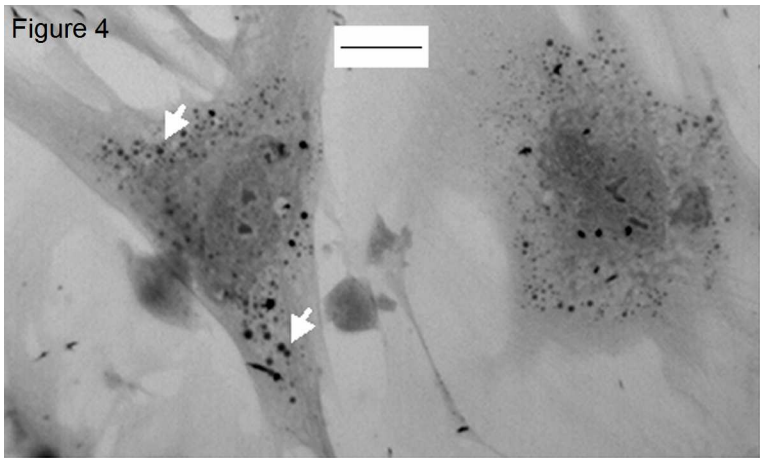

Figure 5

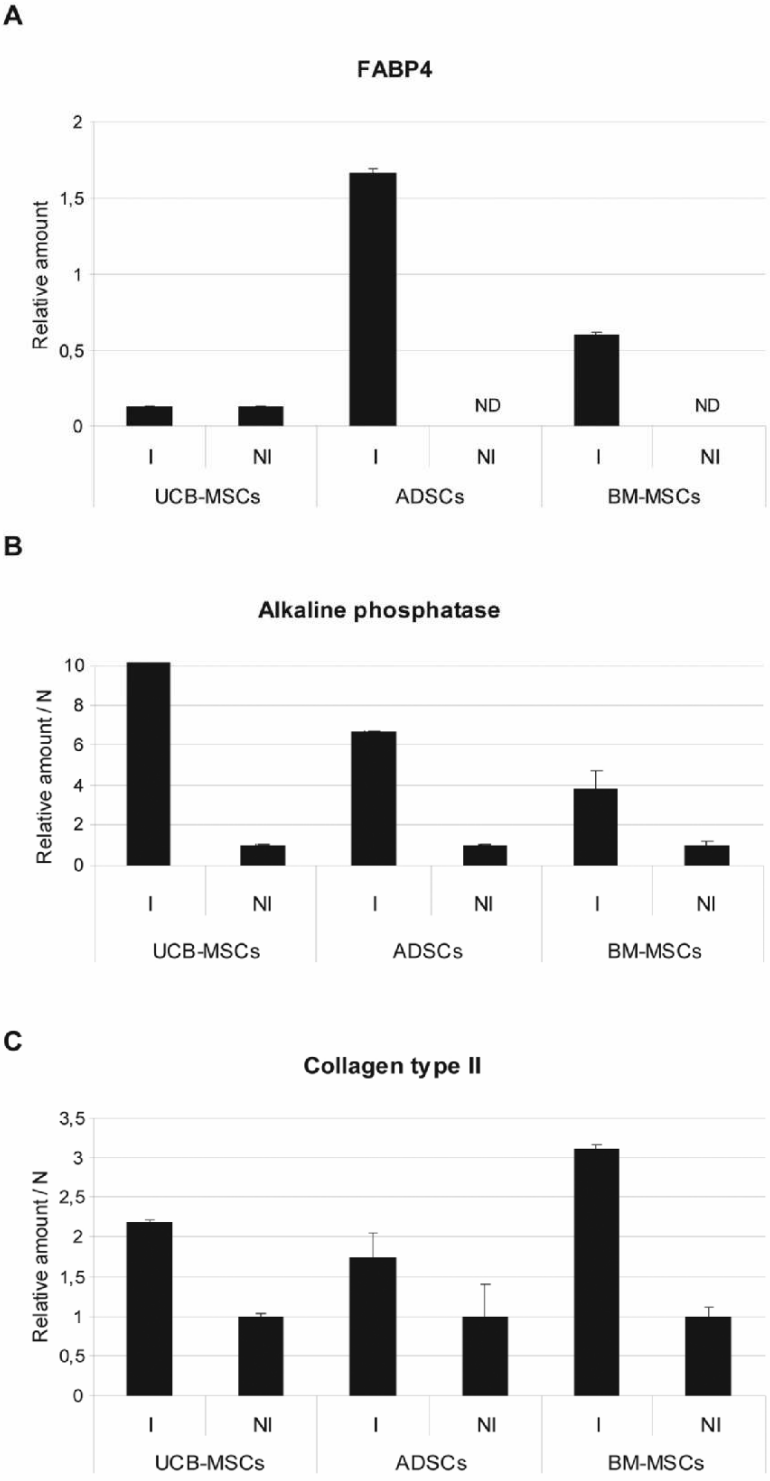
A
FABP4
Relative amount
2
1,5
1
0,5
0
ND
ND
I
NI
I
NI
I
NI
UCB-MSCs
ADSCs
BM-MSCs
B
Alkaline phosphatase
Relative amount / N
10
8
6
4
2
0
I
NI
I
NI
I
NI
UCB-MSCs
ADSCs
BM-MSCs
C
Collagen type II
Relative amount / N
3,5
3
2,5
2
1,5
1
0,5
0
I
NI
I
NI
I
NI
UCB-MSCs
ADSCs
BM-MSCs

Table 1

Gene	Sequence (5'-3')	Accession Number	Amplicon (bp)
GAPDH	Forward: GGCGATGCTGGCGCTGAGTAC Reverse: TGGTTCACACCCATGACGA	2597	150
FABP4	Forward: ATGGGATGGAAAATCAACCA Reverse: GTGGAAGTGACGCCTTTCAT	2167	97
Osteonectin	Forward: ACATCGGGCCTTGCAAATACATCC Reverse: GAAGCAGCCGGCCCACTCATC	6678	437
ALP	Forward: TACAAGGTGGTGGGCGGTGAACGA Reverse: TGGCGCAGGGGCACAGCAGAC	249	92
Collagen type II, α1	Forward: CCGGGCAGAGGGCAATAGCAGGTT Reverse: CAATGATGGGGAGGCGTGAG	1280	128

Table 2

Table

Antibody	BM	UCB	ADSC
CD105	95,75 ± 5,52[a]	96,96 ± 4,33	98,83 ± 1,01
CD90	93,16 ± 4,61	87,16 ± 5,79	96,78 ± 1,88
CD73	97,61 ± 2,83	96,84 ± 0,81	96,42 ± 2,82
CD166	91,69 ± 4,10	80,71 ± 25,31	93,79 ± 6,78
CD44	95,43 ± 4,27	92,48 ± 7,01	98,77 ± 0,62
CD29	98,72 ± 2,28	99,78 ± 0,06	97,45 ± 4,18
CD14	4,06 ± 4,35	4,32 ± 3,57	2,13 ± 1,79
CD45	1,97 ± 1,46	0,97 ± 0,88	0,45 ± 0,58
CD31	0,28 ± 0,20	0,41 ± 0,43	0,94 ± 1,54
CD34	2,16 ± 2,48	10,52 ± 10,58	10,37 ± 7,98
CD117	52,7 ± 46,46	38,84 ± 40,80	98,11 ± 3,06

[a] values indicate the mean percentage of at least three experiments ± standard deviations.

Expression of cardiac function genes is increased in bone marrow derived mesenchymal stem cells and adipose tissue derived stem cells treated with SNAP and DEA/NO

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Key words. Mesenchymal stem cells, nitric oxide, differentiation, muscular markers

Abstract

Stem cells therapy has been considered as ideal for regeneration of damaged myocardial tissue based on improved functional outcomes in injured heart, possibly due to muscle tissue regeneration and induction of neovascularization. Mesenchymal stem cells (MSCs) have received special attention in cardiomyoplasty because several studies have shown that these cells differentiate into cardiomyocyte *in vitro* and *in vivo.* Nitric oxide (NO) is a free radical signaling molecule regulates several differentiation processes including cardiomyogenesis. Here, we investigated the effects of two NO agents (SNAP and DEA/NO) able to activate both cGMP-dependent and –independent pathways, on the cardiomyogenic potential of bone marrow-derived mesenchymal stem cells (BM-MSCs) and adipose tissue-derived stem cells (ADSCs). Human bone marrow (BM) was obtained from dilated myocardiopathy patients and adipose tissue (AT) were obtained from patients undergoing elective bariatric surgery and dermolipectomy procedures. These cells were isolated, cultured and treated with NO agents. Analysis for cardiac-specific and muscular protein and gene expression was performed by indirect immunofluorescence, flow cytometry, RT-PCR and real-time PCR. We observed a considerable deal of data dispersion that might be reflecting the genetic variability among donors. Our data showed that untreated (control) ADSCs and BM-MSCs expressed some muscular markers and after exposure to NO compounds there was an increased expression of some muscular genes, VEGF and CD34. In conclusion, this work indicates that NO-derived intermediates induce an increased expression of some muscular markers, VEGF and CD34 in BM-MSCs and ADSCs. However, the low intensity of such muscular markers expression suggests that beneficial effects observed after MSC transplantation may result from an indirect contribution of MSCs, such as a paracrine signaling for angiogenesis, rather than a direct differentiation to the cardiac phenotype.

INTRODUCTION

Stem cells therapy has been considered as ideal for regeneration of damaged myocardial tissue [1-5] based on improved functional outcomes in injured heart [6-9], possibly due to muscle tissue regeneration [10-13] and induction of neovascularization [13-15].

Human embryonic stem cells (hESC) have been used as a renewable source for cardiac cell therapy [16-18], since they can efficiently acquire myocardial properties [18-21]. However, in addition to ethical issues, secondary unwanted effects are usually observed with these cells, such as tumorigenicity and immunogenicity [22].

In this context, adult stem cells appeared as a more advantageous alternative stem cell source. They are found in many tissues and participate in tissue repair and regeneration, are ethically unquestionable, present no arrhythmogenic potential, and require no immunosuppressive therapy [23-24]. Thus, therapeutical advantages clearly prevail, and clinical use has already been explored [24-25].

Among adult stem cells, mesenchymal stem cells (MSCs) have received special attention in cardiomyoplasty. Indeed, several studies have shown that MSCs differentiate into cardiomyocyte *in vivo* [26-29]. However, based on the heterogeneous populations of these cells and the differentiation capacity of stem cells, uncontrolled differentiation is a critical concern when applying these cells to humans. *In vitro* MSC differentiation to cardiomyocyte-like phenotypes has been described through 5-azacytidine treatment [29-35], coculture with cardiomyocytes [7, 36-38], a cardiomyogenic medium containing insulin, dexamethasone and ascorbic acid [39] or a conditioned medium of cardiomyocytes after hypoxia/reoxygenation [40].

Nitric oxide is a free radical signaling molecule [for a review, see 41]. NO and/or its metabolites have been shown to induce and suppress several differentiation processes [42-48], including cardiomyogenesis [49, 50]. Expression of inducible NOS (iNOS) and endothelial NOS (eNOS) isoforms has been observed in murine heart development [51]. Additionaly, eNOS gene-deficient mice present heart failure, congenital septal defects [52] and decreased cardiac maturation [53]. In agreement, NOS inhibitors prevent the maturation of terminally differentiated cardiomyocytes using ES cells [54]. Treatment of ES cells with S-nitroso-N-acetyl-D,L-penicillamine (SNAP), DEA/NO or their transduction with iNOS gene increased the number of

spontaneously contracting cell clusters and the expression of cardiac myosin light chain (MLC) protein, an effect abolished with NOS inhibitors [50].

Here, we investigated the effects of two NO agents (SNAP, a nitrosothiol and DEA/NO, NO donor) on the cardiomyogenic potential of bone marrow-derived mesenchymal stem cells (BM-MSCs) and adipose tissue-derived stem cells (ADSCs). Our data showed the induction of some muscular genes, CD34 and VEGF after exposure to those compounds. However, the low intensity of such muscular markers expression points to an indirect contribution of MSCs, such as a paracrine signaling, rather than a direct differentiation to the cardiac phenotype.

MATERIAL AND METHODS

Obtention of bone marrow and adipose tissue

Human bone marrow (BM) were obtained from the iliac crest of 10 dilated myocardiopathy patients, who were aged between 40 and 74 years (60.4 ± 9.9).

Subcutaneous abdominal adipose tissue (AT) were obtained from 10 patients, aged between 19 and 60 years (38.0 ± 12.6), undergoing elective bariatric surgery and dermolipectomy procedures.

All samples were collected after informed consent following guidelines on the use of human subjects, as approved by the Ethics Committee from Pontifícia Universidade Católica of Paraná (approval number 597).

Isolation, culture and characterization of adherent cells from BM and AT

Cultures of BM-MSCs and ADSCs were prepared and characterized as previously described by Rebelatto and colleagues [55]. Briefly, BM was loaded onto a Histopaque® solution and the mononuclear cells (MNCs) were isolated and washed twice with PBS. BM-MNCs were cultured in IMDM containing 15% of fetal bovine serum (FBS) (Gibco^{TM} Invitrogen Corporation, NY, USA), penicillin (100 units/ml) and streptomycin (100 µg/ml) (Invitrogen Corporation, NY, USA) at a density of $1x10^5$ cells/cm^2 in T75 culture flasks (TPP, Trasadingen, Switzerland) at 37ºC incubator with 5% CO_2. The culture medium was changed to remove the remaining non-adherent

cells two days after the initial plating. Thereafter, adherent cells were routinely cultured and culture medium was replaced twice-a-week.

About 100 mL of AT was minced and enzyme-digested with type I collagenase (1mg/ml) (Gibco^{TM} Invitrogen Corporation, NY, USA) for 30 min at 37ºC on a shaker. The digested tissue was passed through a 100 µm mesh filter and centrifuged at 800g for 10 min. Pelleted cells were washed twice with PBS, passed through a 40 µm mesh filter and finally ressuspended in DMEM-F12 medium (Gibco^{TM} Invitrogen Corporation, Grand Island, NY, USA) supplemented with 10% FBS, penicillin (100 units/ml) and streptomycin (100 µg/ml). The cells were plated onto T75 culture flasks at a density of $1x10^5$ cells/cm^2 [56]. When the cultures reached 80% confluence, they were detached with 0.25% trypsin/EDTA (Invitrogen^{TM}, Canada) and were replated as passage-1 cells. BM-MSCs and ADSC were subcultured approximately every 3 days.

All experiments were performed with cells from the third to fifth passages.

Cell treatment with NO agents

To explore whether NO promoted the differentiation of BM-MSCs and ADSCs into cardiomyocytes or cardiomyocyte-like cells, five BM-MSCs and seven ADSCs biological replicates were exposed to NO by exogenous supplementation of S-nitroso-N-acetyl-D,L-penicillamine (SNAP) (Calbiochem, CA, USA) and 2-(N,N-diethylamino)-diazenolate-2-oxide (DEA/NO) (Calbiochem, CA, USA). Cells were cultured until confluence and 24 h before treatment with those compounds, the culture medium was changed to 1% FBS medium.

SNAP and DEA/NO were immediately diluted before use in 5% FBS medium (IMDM for BM-MSCs and DMEM-F12 for ADSCs), and used at different final concentrations of 4, 0.4 and 0.04 mM for SNAP and 1, 0.1 and 0.01 µM for DEA/NO. They were added to cells on days 0, replaced on day 2 and maintained until day 4. After that, cultures were maintained in medium without NO agents. Medium was replenished every 2 days until day 20 when cell differentiation was analyzed [50, modified].

To define the best concentration and time of exposure to NO, cells at days 2, 7 and 20 were analysed for expression of transcription factors and cardiac markers using RT-PCR.

After 20 days of NO exposure, BM-MSCs and ADSCs were analysed for cardiac-specific and muscular proteins by indirect immunofluorescence, flow cytometry, RT-PCR and real-time PCR.

Flow Cytometry

The cell staining procedure for the flow cytometry was performed as described by OWENS and colleagues [57]. Briefly, samples from BM-MSCs and ADSCs, before and after exposure to NO agents, were stained with antibodies to surface markers and to intracellular muscular proteins. Cells were detached from culture flasks with 0.25% trypsin-EDTA solution, washed and resuspended in PBS at a concentration of $1x10^6$ cells/mL. For surface markers staining, cells were incubated separatelly for 30 minutes at 4^0C with the following anti-human monoclonal antibodies: fluorescein isothiocyanate (FITC) conjugated anti-CD105, CD90, CD44 and CD31; phycoerythrin (PE) conjugated anti-CD73, CD166 and CD34; allophycocyanin (APC) anti-CD29, CD117 and CD14; and with peridinin chlorophyll protein (PerCP) anti-CD45. All antibodies were from BD Biosciences (San Jose, CA, USA) with the exception of anti-CD105 that came from Abcam (Cambridge, UK).

For intracellular staining, cells were first permeabilized with Fix&Perm Cell permeabilization kit (Caltag Laboratories, CA, USA) according to the manufacturer's instructions, and then incubated separatelly for 30 minutes with the following primary: anti-human cardiac troponin T (1/100), anti- ventricular myosin heavy chain (VMHC) (1/10) (both from Chemicon International, CA, USA), anti-myosin (skeletal, slow) (1/2000) (Sigma-Aldrich, MO, USA), anti-cardiac (fetal) actin (1/10) (Fitzgerald, MA, USA) and anti-connexin-43 (1/20) (BD Biosciences Pharmingen, CA, USA). Following washing, staining with a secondary Texas Red-conjugated IgG goat anti-mouse antibody (Molecular Probes, Ashland, USA) was performed for 15 minutes at room temperature.

Controls for the flow cytometry setup procedure included cells incubated with FITC, PE, PerCP and APC conjugated isotype antibodies (all from BD Bioscences Pharmingen) and a sample where the cells were first incubated with an unconjugated isotype control (BD Biosciences Pharmingen) followed by the secondary Texas Red-conjugated goat anti-mouse antibody.

After incubation, cells were washed, resuspended in PBS and flow cytometric evaluation was immediately performed in a FACSCalibur equipment (Becton Dickinson, San Jose, CA, USA). Gates were set based on side and forward scatter profiles in order to exclude debris and cell aggregates. Approximately ten thousand-gated events were acquired per sample. Five biological replicates of BM-MSCs and seven of ADSC were performed. Data were analyzed by FlowJo software (Tree Star, Ashland, OR, USA). Expression levels of surface markers and muscular intracellular proteins were expressed as a mean fluorescence intensity and percentage values ± SD.

Indirect immunofluorescence

For immunofluorescence, BM-MSCs and ADSCs were seeded on glass coverslips at 24-well culture dishes and exposed to NO agents as described. At day 20, cells were washed with PBS, fixed using 2% paraformaldehyde for 2 h and permeabilized with 0.2% Triton X-100. After blockage with 1% BSA, cells were incubated for 3 h at room temperature with primary antibodies using the manufacturer's suggested antibodies dilutions. Antibodies to cardiomyocytes markers such as anti-cardiac troponin T (1/100) (Chemicon International, CA, USA), anti-cardiac (fetal) actin (1/10) (Fitzgerald, MA, USA), and anti-VMHC (1/10) (Chemicon International, CA, USA), and to muscular proteins such as anti-troponin I (1/50) (Santa Cruz Biotechnology, CA, USA), anti-α-actinin (sarcomeric) (1/250) (Sigma-Aldrich, Missouri, USA), anti-connexin-43 (1/20) (BD Biosciences Pharmingen, CA, USA), anti-myosin (skeletal, slow) (1/2000) (Sigma-Aldrich, Missouri, USA), and anti-desmin (1/10) (Sigma-Aldrich, Missouri, USA) were used. Cells were then incubated with secondary antibodies rabbit anti-goat IgG Texas Red (Santa Cruz Biotechnology, CA, USA) at dilution 1:100, goat anti-rabbit IgG (Molecular Probes, Oregon, USA) at dilution 1:100 and goat anti-mouse IgG Texas (Molecular Probes, Oregon, USA) at dilution 1:1,500 for 1 h at room temperature. Nuclei were counterstained by 4´,6-diamidine-2´-phenylindole dihydrochloride (DAPI). Non-specific binding of secondary antibodies was assessed by omitting the primary antibody in a control test. Slides were examined on a Nikon E-600 microscope. Digital images were captured using CoolSNAP-PRO*cf* (Media Cybernetics) camera controlled by Image Pro-Plus software system from Diagnostic Instruments.

Total RNA extraction and Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated with the RNeasy kit (QIAGEN) and treated column with DNAse I (QIAGEN). Concentrations were determined by spectrophotometry (GeneQuant, Amersham Biosciences). Complementary DNA (c-DNA) was synthesized from 1µg of total RNA using 1 µL of 10 µM oligo dT primer (USB Corporation) and 1 µL of reverse transcriptase (IMPROM II, Promega) according to the manufacturer's instructions. Polymerase Chain Reaction (PCR) was carried out with 20 ng of c-DNA as template, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 5 pmol of primers, 2.5 mM of $MgCl_2$, 0.0625 mM of dNTPs and 1U Taq polimerase (Invitrogen™, NY, USA) in a final volume of 20 µL. The oligonucleotide primer sets used for PCR, the amplicon size and the anneling temperature are depicted in Table 3. PCR included heating at 94°C for 2 minutes, followed by 30 cycles of 94°C for 15 seconds, annealing temperature as table 3 for 30 seconds and 72°C for 40 seconds and, a final extension of 72°C for 3 minutes using a Bio-Cycler II termocycler (Peltier Thermal Cycler). Ten µL of RT-PCR products were resolved by 2% agarose gel electrophoresis, visualized by ethidium bromide fluorescence and photographed under ultra-violet light illumination (UV White Darkroom, UVP Bioimaging Systems).

Real-Time Quantitative PCR

Real-time or quantitative PCR (qPCR) was performed using the ABI PRISM 7300 sequence detection system (Applied Biosystems). Amplifications were carried out in a final reaction volume of 20 µl with the SYBR Green master mix (Applied Biosystems, CA, USA), 10 ng of cDNA template and 5 pmol of primers. PCR conditions were: 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds, annealing temperature as table 1 for 30 seconds and 72 °C for 40 seconds. The melting curves were acquired after PCR amplification confirming the specificity of the amplified products. A standard curve based on cycle threshold values was used to evaluate gene expression. In brief, we used 1:5 dilutions of known concentrations of cDNA in triplicate to generate curves extending from 50 pg to 80 ng of cDNA. We generated standard curves for each gene, including the control (housekeeping) gene. The relative amount of gene expression for each sample was normalized by dividing the value obtained for the analyzed gene by the value obtained for each control gene. Results were analyzed as gene expression relative to the

housekeeping gene expression. Differences in expression were reported comparing cells induced to differentiation with non-induced control samples [55, 58].

Statistical Analysis

Continuous variables were presented as mean ± standard deviation and categorical variables were presented as frequency and percent. Comparisons between control cells and treated cells and between NO agents (SNAP and DEA/NO), were performed by nonparametric Wilcoxon test. P-values<0.05 were considered statistically significant. Analysis was performed with the SPSS V.14 software package.

RESULTS

Characterization of BM-MSCs and ADSCs was demonstrated in a previous work by our group [55] and showed that these cells are similar in morphology, immunophenotype and differentiation capacity. Here, we investigated if exposure of human BM-MSCs and ADCS to the NO agents SNAP and DEA/NO induces the expression of muscular genes and proteins, as determined by flow cytometry, cellular immunofluorescence, RT-PCR and real time PCR analysis.

In order to define an efficient, but non-lethal concentration of the NO agents, cells were treated with 4, 0.4 and 0.04 mM SNAP and 1, 0.1 and 0.01 μM DEA/NO. Cells were exposed to the NO agents for 4 days and left in culture in the absence of the compounds for up to 20 days. The time points analyzed were 2 (i.e. during the NO agents treatment), 7 and 20 days. Expression of cardiac markers α-myosin heavy chain (α-MHC), β-myosin heavy chain (β-MHC), atrial natriuretic peptide (ANP), α-cardiac actin, myosin light chain 2A (MLC-2A) specific for the atrium, myosin light chain 2V (MLC-2V) specific for the ventricle, cardiac troponin I, cardiac troponin T, connexin-43 and desmin and transcription factors GATA-4 and Nkx2.5 were analyzed using RT-PCR in two biological samples of BM-MSCs and ADSCs. Results showed that the highest concentration of SNAP was cytotoxic. Overall, at day 20, 0.4 mM of SNAP and 0.1 μM DEA/NO-treated cells expressed α- cardiac actin, cardiac troponin T, connexin-43 and desmin in both BM-MSCs and ADSC more consistently than with the other tested conditions (data not shown). Thus, we chose these concentrations and time of treatment for further analyses.

Quantification of the percentage of immunopositive cells and of the mean fluorescence intensity of muscular markers was obtained by flow cytometry (Table 1, Figure 1). Because spontaneous (i.e. in the absence of treatment) expression of some markers could take place, results were expressed as a ratio of immunopositive treated/ immunopositive non-treated cells at day 20. With the exception of CD34, all hematopoetic or mesenchymal cell markers analyzed did not significantly changed their expression pattern throughout the 20 days in both cell types exposed to both NO agents. Percentage of CD34-positive cells increased (Table 2) in BM-MSCs treated with SNAP and DEA-NO (ratios of 9.16 ± 14.79 and 6.34 ± 12.35 respectively) and in ADSCs treated with SNAP (ratio of 4.00 ± 6.86). Therefore, these data suggest that NO agents increase the number of CD34-positive cells in a BM-MSCs and ADSCs population.

Muscular markers analysis, on the other hand, was not straightforward, since inter-individual samples presented a high variability (Figure 1). The more efficient donor or adult stem cell source could not be significantly determined by flow cytometry analysis. Some markers showed a clear tendency to be increasingly expressed after NO agents exposition. For instance, expression of connexin-43 and myosin appears higher in treated ADSCs. In the case of BM-MSCs, expression of troponin T e VMHC, myosin and cardiac (fetal) actin seems also to increase after NO agents treatment (Figure 1). However, more samples would be required to allow more powerfull statistical tests that could reveal significant differences in both BM-MSCs and ADSCs exposed to SNAP and DEA/NO with control samples. Regarding data homogeneity, ADSCs samples were homogeneous for connexin-43, myosin and cardiac (fetal) actin markers, but were quite dispersed for troponin T and VMHC, when percentage of immunopositive-cells was analyzed. When mean fluorescence intensity was assessed, sample homogeneity was observed for connexin-43, troponin T and cardiac (fetal) actin, in contrast to VMHC and myosin markers. Analyzing the percentages of immunopositive- BM-MSCs, it was observed that most of the samples were homogeneous for muscular markers used in this study, with the exception of connexin-43 and cardiac (fetal) actin. When mean fluorescence intensity was analyzed, all samples were homogeneous for muscular markers; for example, three out of four and four out of five samples showed an increase in VMHC expression in comparison with control samples, after cells exposure to SNAP and DEA/NO respectively.

Indirect immunofluorescence microscopy was employed to study expression intensity and pattern of connexin-43, troponin T, VMHC, myosin, cardiac (fetal) actin, troponin I and α-actinin (sarcomeric). Results showed that 0.4 mM SNAP and 0.1 µM DEA/NO induced the expression of some cardiac and muscular markers by ADSCs (Fig. 2A) and BM-MSCs (Fig. 2B) at day 20. Such expression was observed either in some isolated cells, like troponin T and VMHC, or in most of the cells, like connexin-43, cardiac (fetal) actin and α-actinin (sarcomeric). Interestingly, some muscular markers were detected even in non-treated cells, suggesting that MSC spontaneously express connexin-43, cardiac (fetal) actin and α-actinin (sarcomeric). In many cases, connexin-43 and cardiac (fetal) actin expression seemed to increase after exposure to NO agents, by visual analysis. These results were similar for BM-MSCs and ADSCs exposed to SNAP and DEA/NO, although it could be observed a high variability in staining intensity for each marker used.

Finally, gene expression of some muscular markers, as well as of VEGF, was also assessed by RT-PCR and qPCR (Table 3). RT-PCR results corroborated the data high dispersion from inter-individual samples, independently on the source. On one end of the spectrum, MLCV expression was detected in only one ADSCs sample treated with SNAP and, on the end, markers like connexin-43, VEGF and troponin T were detected in most of the samples analyzed. In agreement with the immunofluorescence data, in some cases control cells from both sources were also expressing several markers (Figure 3). Because some of these genes were expressed in most of the samples and treatments, qPCR was performed to better quantify the expression of two of them, connexin-43 and VEGF (a gap junction component and angiogenic factor, respectively). Interestingly, the mRNA levels of both markers were considerably increased in all BM-MSCs samples analyzed, after treatment with either SNAP or DEA/NO. However, only one ADSCs sample was in agreement with these data and, the other two showed equal or even lower mRNA levels between control and NO agents-treated samples (Figure 4).

DISCUSSION

Based on studies showing that MSCs are multipotent cells able to differentiate into cardiomyocytes [59-62], on the importance of NO in heart development [51, 54] and on

embryonic stem cells differentiation into cardiomyocytes after exposure to NO agents or endogenously produced NO [50], our focus was to study the effects of NO agents on cardiomyogenic differentiation of MSCs. In addition, it was recently demonstrated that eNOS induces neonatal cardiomyocyte proliferation [63]. Nitric oxide and/or its metabolites interfere in signaling pathways by activating guanylate cyclase and/or inducing post-translational modifications, especially nitros(yl)ations [64]. Indeed, cysteine nitrosation has been considered a switch on/off of signaling proteins, similar to serine, tyrosine and threonine residues phosphorylation [65]. Here, we used SNAP and DEA/NO, which produces NO^+ and free NO gas, respectively. While the former species nitrosates cysteine, NO radical activates GC [41, 66] Thus, we employed NO agents able to activate both cGMP-dependent and –independent pathways.

Our results evidenced a trend to increase the expression of some muscular markers in MSC treated with the NO agents. Connexin-43, troponin T, VMHC, myosin and cardiac (fetal) actin expressions seem to be higher after NO-treatment compared with control cells, as determined by flow cytometry. However, the percentage of cells expressing muscle markers was low, both in BM-MSCs and ADSCs. Differently to ES cells [50], MSCs exposed to the NO agents did not fully differentiated to cardiomyocyte-like cells. That group has shown that NO may influence cardiac differentiation of ES cells both by inducing a phenotype switch, with expression of cardiac troponin T, α- or ß-MHC and myosin filaments in a high percentage of treated cells, and inducing apoptosis in cells not committed to cardiac differentiation. Therefore, in line with the current thought, regarding cardiomiogenic differentiation capacity, MSC are more restricted than ES cells.

Immunofluorescence microscopy also demonstrated that some muscular markers were expressed after NO agents exposure. However, even untreated cells were highly immunostained, which contrasted with the low frequency of staining obtained by flow cytometry. This apparent discrepancy may be due to the fact that epitopes can be more or less hidden or even modified depending on the sample preparations. This might affect their recognition by the antibody. However, considering the relative increase in expression, *i.e*, comparing NO-treated cells with untreated cells after 20 days in culture, flow cytometry and immunofluorescence results are consistent.

Due to a considerable deal of data dispersion, it was only possible to conclude that overall our results demonstrate a tendency of increased expression of the cardiac/muscular markers analyzed. This fact might be reflecting the genetic variability among donors and also the different diseases they may present, other than the informed/diagnosed dilated cardiomyopathy in BM samples. Data dispersion was also observed by Bernardo and colleagues [67] that noted a huge variability in terms of proliferative capacity and BM-MSCs life span among different BM donors. Tsai and colleagues [68] showed that gene expression profiles within each group of MSCs from the same origin show a smaller variability than that between MSCs derived from different origins.

An interesting finding was that untreated (control) ADSCs and BM-MSCs expressed some muscular markers like connexin-43, α-cardiac actin, troponin I and α-sarcomeric actin, detected by immunofluorescence and RT-PCR. Also, it was observed at BM-MSCs and ADSCs (between second and fifth passage) that were not cultivated for 20 days. In a preliminary test, we have observed a lower expression of muscular markers compared to untreated cells that were cultured for 20 days in parallel with NO exposed cells (data not shown). Spontaneous expression of muscular markers by MSCs at the molecular level has also been observed by other groups. Antonitsis and colleagues [34] described the expression of α-cardiac actin, beta-myosin heavy chain and cardiac troponin-T in both uninduced and induced cells with 5–azacytidine using RT-PCR analysis. After single-cell microarrays analysis, Seshi and colleagues [69] showed that isolated single MSCs simultaneously express transcripts associated with osteoblast, fibroblast, muscle and adipocyte differentiation. Expression of connexin-43 in undifferentiated cells (controls) was observed in human fetal bone marrow mesenchymal stem cells from the second trimester [70]. The expression of this protein was up-regulated in ADSCs in long-term cultures, independent of 5-azacytidine exposure, but dependent on the cell density, and cytoeskeletal protein α-SMA was present in control and treated ADSCs [71]. Indeed, Tondreau and colleagues [72] suggested that expression of some proteins by MSCs depend on time culture, rather than on specific induction factors. These studies, combined with the findings reported by the Verfaille's group, support the theory that MSCs are "pluridifferentiated" cells at the molecular level [25]. Even though the mRNAs for these proteins are present in untreated ADSCs and BM-MSCs, they might have non-canonical functions [73] or might be functionally inactive.

Comparing the two MSCs sources, in this study BM-MSCs samples seem to be more promising compared with ADSCs, as determined by qPCR analysis. This fact may result from the pre-commitment of these cells for cardiomyogenic lineage, since all BM donors were dilated cardiomyopathy patients. It is recognized that local or systemic production of inflammatory mediators might influence not only MSC migration to injured tissues [74, 75], but also MSC proliferation, differentiation [76] and engraftment [77]. Indeed, it was recently shown that nitric oxide produced by MSC inhibits T-cell proliferation [78].

Recent evidences suggest that mesenchymal progenitor cells do not result in the formation of functional syncytia and that the reported beneficial effects in post-infarcted myocardium scarring might be mediated more by paracrine factors-stimulated angiogenesis than by cardiomyogenic differentiation itself [15, 62, 79, 80]. An increased expression of connexin-43 in cardiomyocytes was attributed to secretion of paracrine factors by transplanted mesenchymal progenitor cells from BM [81]. Through this indirect mechanism, MSCs transplantation could lead to a recovery of cardiac performance and to induction of neovascularization [15, 62], although some degree of differentiation into cardiomyocytes could also take place. Our qPCR results point to this direction. We have shown that untreated BM-MSCs and ADSCs express connexin-43 and VEGF mRNAs and, more importantly, their expressions were greatly increased after NO-agent exposure, mostly with DEA/NO. VEGF is a critical angiogenic factor which could not only contribute to endothelial lineage cell survival through VEGF-mediated phosphorylation of protein kinase B and endothelial nitric oxide synthase [82-84] and also accelerate development of microvessels and enhance regional blood flow in ischemic tissue [85]. It was demonstrated that neovascularization induced in ischemic myocardium is directly related to the paracrine action of engrafted MSCs in ischemic myocardium [80]. The cardioprotective effects of MSC are known to be mediated not only by their differentiation into vascular cells and cardiomyocytes, but also by their ability to supply large amounts of angiogenic, anti-apoptotic and mitogenic factors [14, 62, 86, 87]. It would be interesting to determine if expression of other factors like basic fibroblast growth factor (bFGF), hepatocyte growth factor, insulin-like growth factor 1, monocyte chemoattractant protein (MCP)-2 and MCP-3 [14, 15, 83, 86] are as well increased in MSCs after NO-agents treatment.

Quantification of the percentage of immunopositive cells by flow cytometry reveals an increase of CD34 expression in BM-MSCs exposed to both NO agents and in ADSCs exposed to SNAP. CD34 has been considered a marker of activated stem cells [1, 88]. Beauchamp and colleagues [89] speculated that CD34 plays a fundamental role in regulating progenitor cell differentiation in a range of adult tissues, including blood and muscle. Lee and colleagues [90] showed that a population of muscle-derived cells expresses both early myogenic markers, including desmin, and stem cell markers like CD34. Leone and colleagues [91] demonstrated that $CD34^+$ cells levels were significantly correlated to VEGF levels in health controls.

In conclusion, this work indicates that NO-derived intermediates increase the expression of some muscular markers, VEGF and CD34 in treated BM-MSCs and ADSCs. Upregulation of such genes may contribute to the beneficial effects observed after MSCs transplantation in several cardiac diseases. Our data corroborate the role of MSCs in angiogenic paracrine signaling rather than in MSCs differentiation to cardiomyocytes.

FIGURE AND TABLE LEGENDS

Figure 1. Quantification of the percentage of immunopositive cells and of the mean fluorescence intensity of muscular markers by flow cytometry. Mesenchymal stem cells from bone marrow (A, B) and adipose derived stem cells (C, D) were induced for 4 days with NO agents SNAP (■) and DEA/NO (♦) and cultivated for more 16 days without the agents. Y-axis shows the ratio of immuno positive cells treated/control cells and X-axis shows muscular and cardiac markers. Abbreviations: BM-MSCs, mesenchymal stem cells derived from bone marrow; ADSCs, adipose derived stem cells; NO, nitric oxide.

Figure 2. Indirect immunofluorescence showing the expression of muscular and cardiac markers. Mesenchymal stem cells from bone marrow (A) and adipose derived stem cells (B) were induced for 4 days with 0.4 mM SNAP and 0.1 µM DEA/NO and cultivated for more 16 days without the agents. Control samples were non-induced cell (negative control). Abbreviations: BM-MSCs, mesenchymal stem cells derived from bone marrow; ADSCs, adipose derived stem cells.

Figure 3. Expression profile of VEGF and cardiac markers. Mesenchymal stem cells from adipose tissue and bone marrow were induced for 4 days with NO agents SNAP and DEA/NO and cultivated for more 16 days without the agents as described in materials and methods. Expression profile of VEGF and cardiac markers in three independent samples of AT (A). Expression profile of VEGF and cardiac markers in three independent samples of BM (B). Control samples were non-induced cell (negative control). Representative results of 3 independent experiments are shown. GAPDH was used as an internal control. Abbreviations: BM, bone marrow; AT, adipose tissue; NO, nitric oxide; S, SNAP; D, DEA/NO; ND, Non detected.

Figure 4. q-PCR of Connexin-43 and VEGF. Mesenchymal stem cells from bone marrow and adipose tissue were induced for 4 days with NO agents SNAP and DEA/NO and cultivated for more 16 days without the agents. The expression of Connexin-43 and VEGF was assayed by q-

PCR. Expression of Connexin-43 in BM (A). Expression of VEGF in BM (B). Expression of Connexin-43 in AT (C). Expression of VEGF in AT (D). Representative results of 3 independent experiments are shown. q-PCR are expressed as mean ± SD of the technical triplicate. GAPDH was used as an internal control and for normalize gene expression. Relative levels of mRNA expression represents the ratio between NO-induced cells and non induced control cells (C). Abbreviations: BM, bone marrow; AT, adipose tissue; NO, nitric oxide; S, SNAP; D, DEA/NO.

Table 1. Flow cytometry analysis of BM-MSC and ADSC muscular markers expression after exposure to NO.

Table 2. Flow cytometry analysis of CD34 expression in BM-MSC and ADSC after exposure to NO.

Table 3. Primer set utilized for RT-PCR and qPCR analyses.

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Figure 1

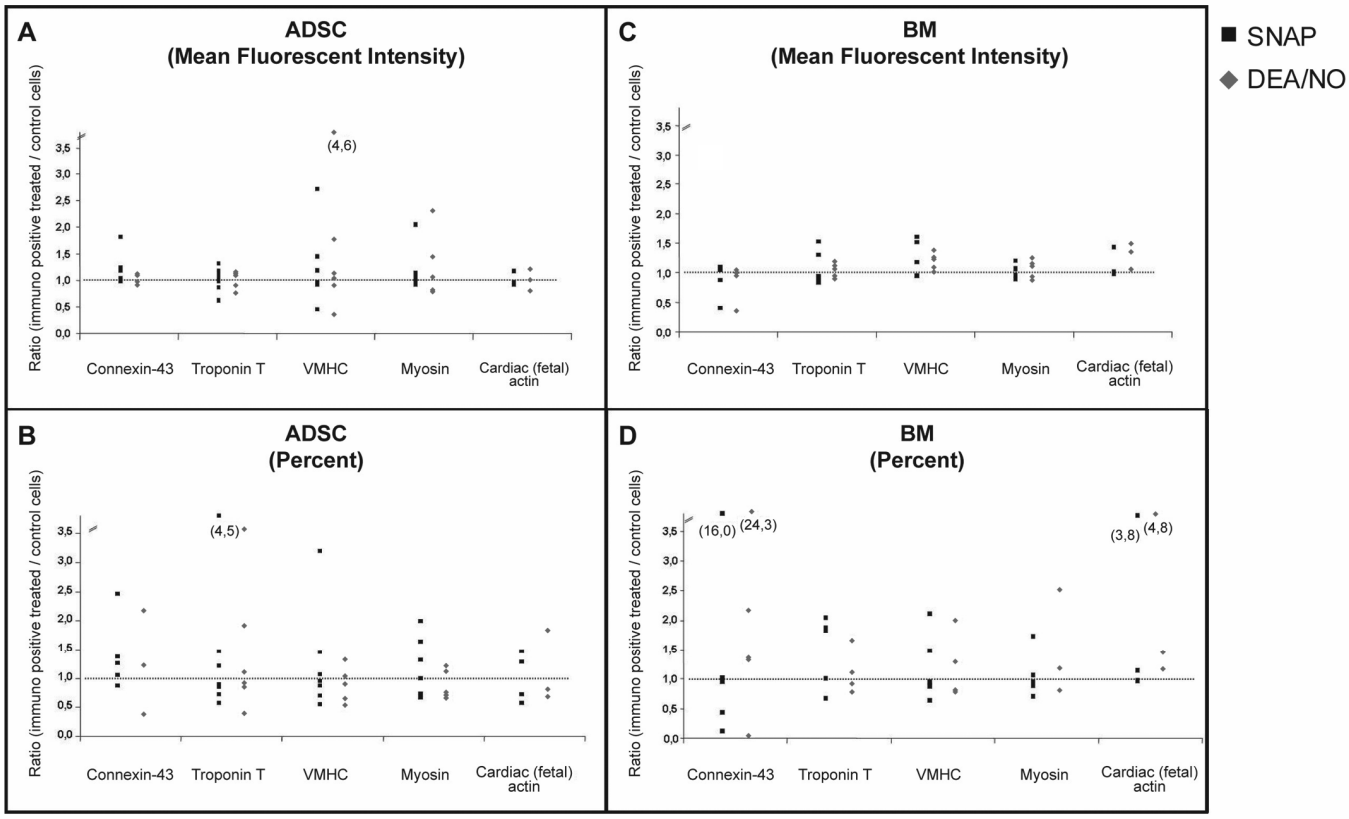
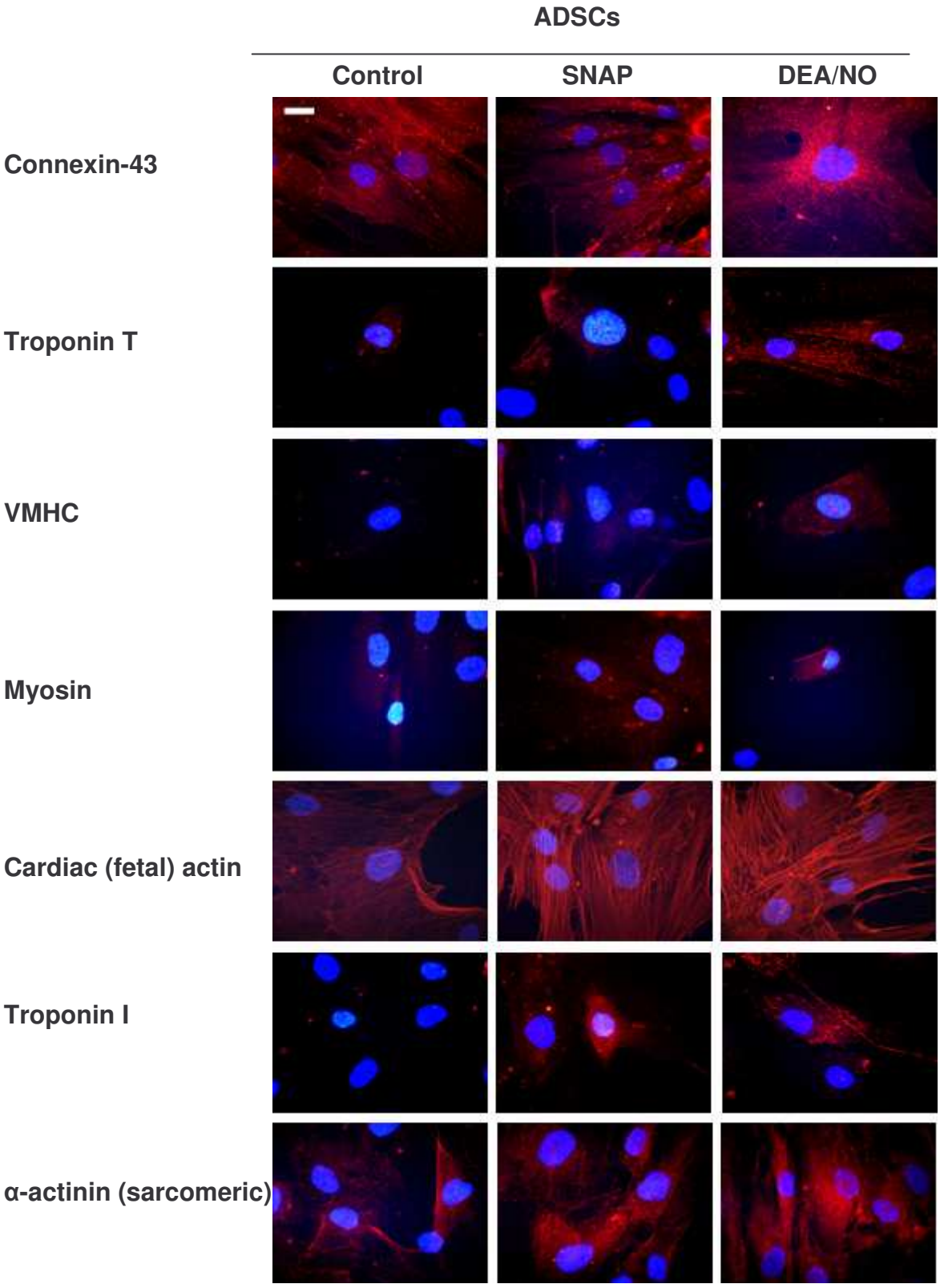

A
ADSC
(Mean Fluorescent Intensity)
C
BM
(Mean Fluorescent Intensity)
B
ADSC
(Percent)
D
BM
(Percent)
Ratio (immuno positive treated / control cells)
(4,6)
(4,5)
(16,0)
(24,3)
(3,8)
(4,8)
Connexin-43
Troponin T
VMHC
Myosin
Cardiac (fetal) actin
SNAP
DEA/NO

Figure 2


ADSCs
Control
SNAP
DEA/NO
Connexin-43
Troponin T
VMHC
Myosin
Cardiac (fetal) actin
Troponin I
α-actinin (sarcomeric)

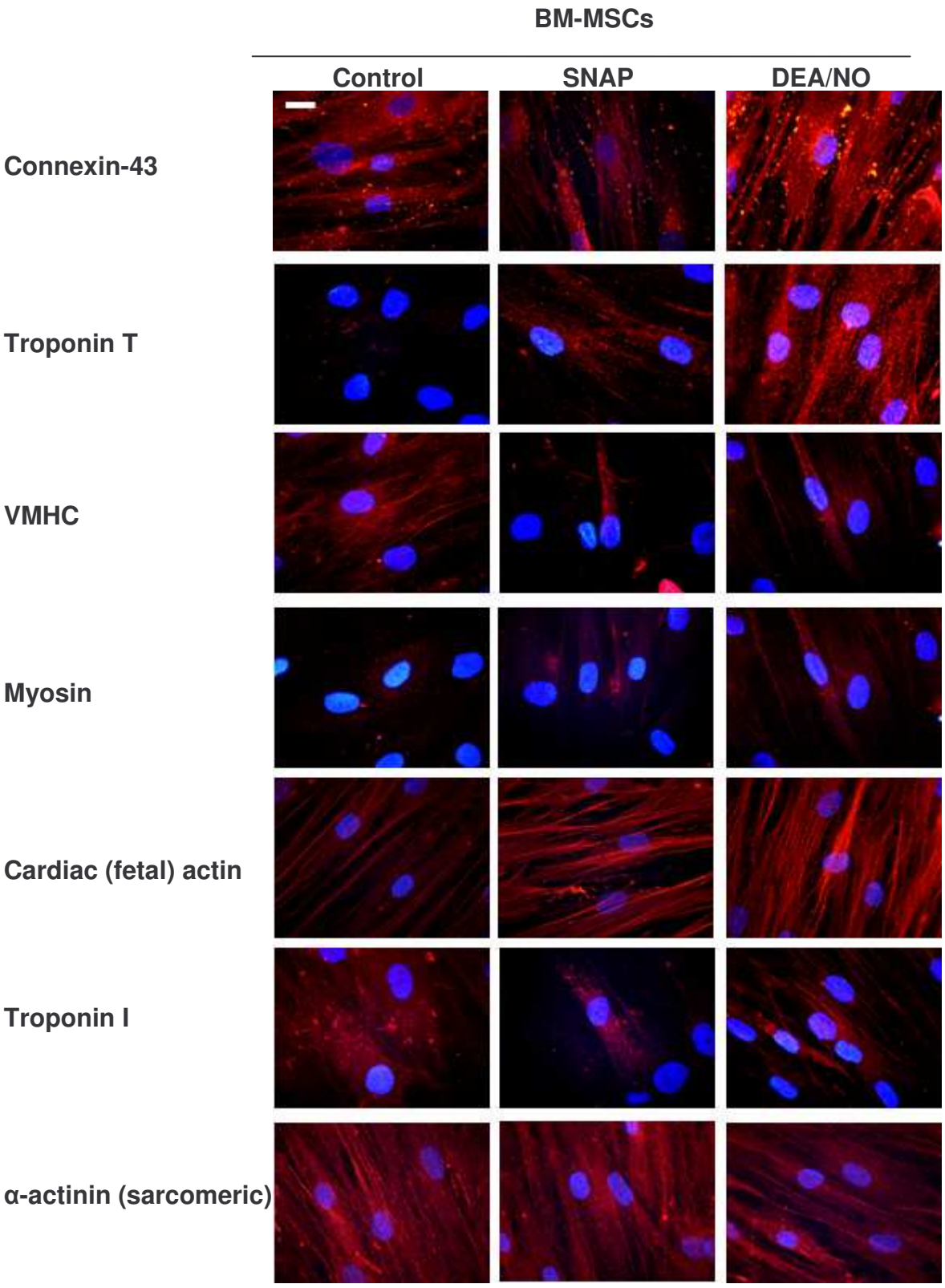
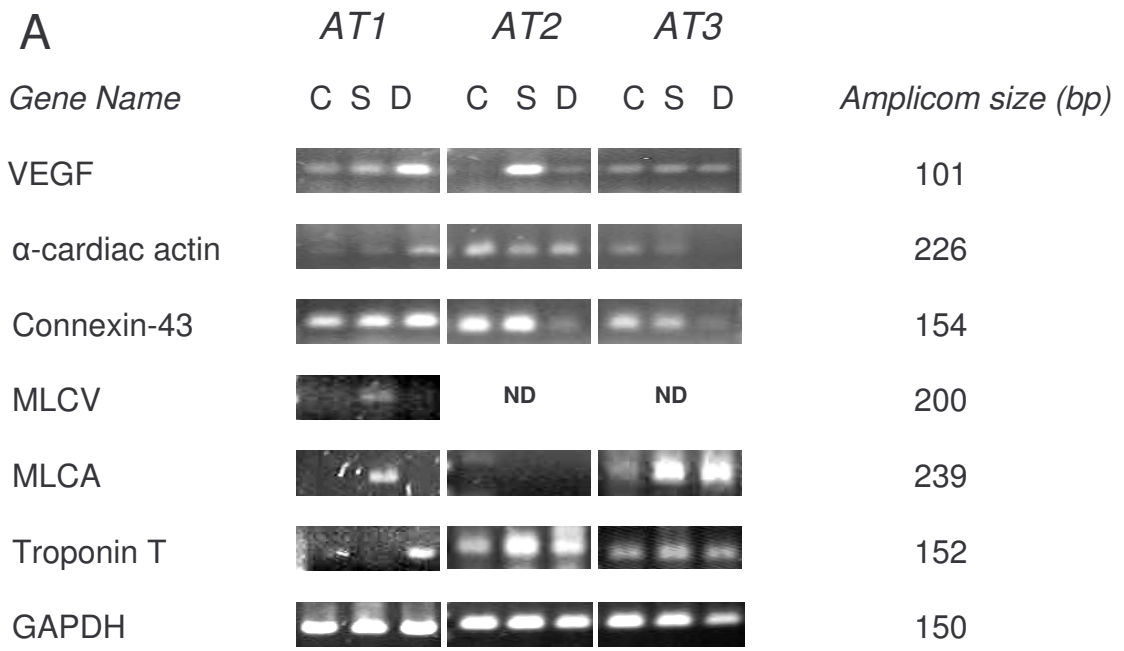
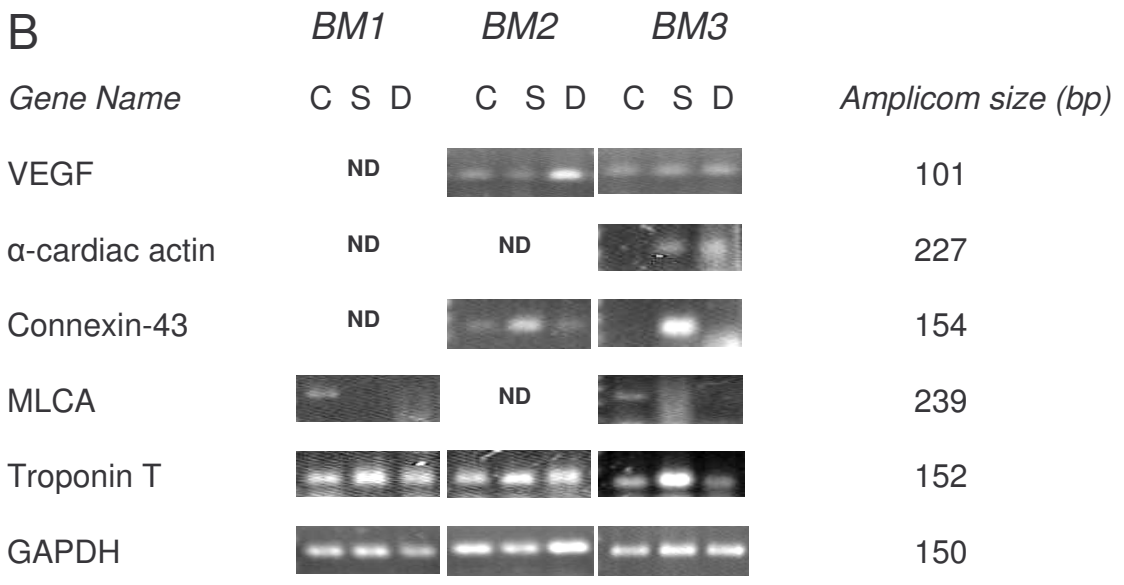

BM-MSCs
Control
SNAP
DEA/NO
Connexin-43
Troponin T
VMHC
Myosin
Cardiac (fetal) actin
Troponin I
α-actinin (sarcomeric)

Figure 3





BM- Bone marrow
AT- Adipose tissue
C - Control (untreated culture)
S - SNAP
D - DEA/NO
ND- Non-detected

Figure 4

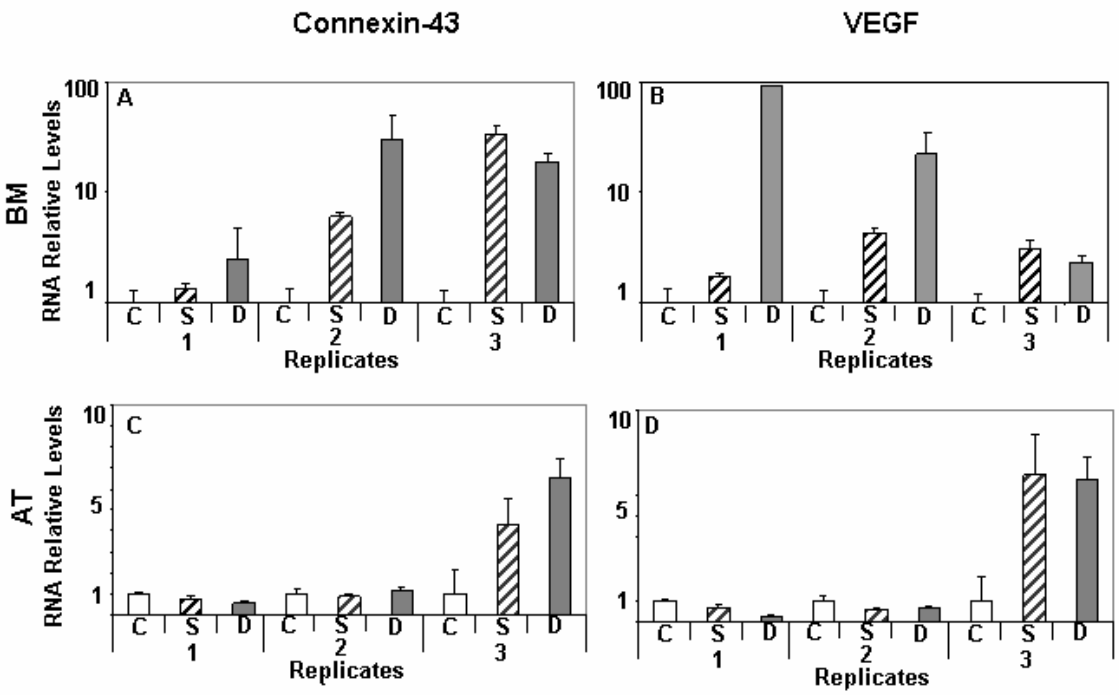

Connexin-43
VEGF
BM
AT
RNA Relative Levels
A
B
C
D
100
10
5
1
C
S
D
1
2
3
Replicates

Table 1. Flow cytometry analysis of BM-MSC and ADSC muscular markers expression after exposure to NO.

MARKER	POSITIVE CELLS	SOURCE	SNAP/CONTROL	DEA/NO/CONTROL
Connexin-43	Mean fluorescent intensity	ADSC	1.24 ± 0.33	1.01 ± 0.09
		BM	0.85 ± 0.27	0.86 ± 0.29
	Percentage	ADSC	1.4 ± 0.62	1.15 ± 0.78
		BM	3.7 ± 6.88	5.83 ± 10.32
Troponin T	Mean fluorescent intensity	ADSC	1 ± 0.22	0.96 ± 0.2
		BM	1.09 ± 0.3	1.04 ± 0.12
	Percentage	ADSC	1.46 ± 1.38	1.37 ± 1.07
		BM	1.48 ± 0.61	1.15 ± 0.34
VMHC	Mean fluorescent intensity	ADSC	1.23 ± 0.72	1.53 ± 1.41
		BM	1.31 ± 0.3	1.2 ± 0.15
	Percentage	ADSC	1.25 ± 0.9	0.84 ± 0.28
		BM	1.21 ± 0.59	1.21 ± 0.49
Myosin	Mean fluorescent intensity	ADSC	1.18 ± 0.39	1.2 ± 0.53
		BM	1.03 ± 0.12	1.06 ± 0.16
	Percentage	ADSC	1.15 ± 0.51	0.83 ± 0.23
		BM	1.06 ± 0.39	1.25 ± 0.73
Cardiac (fetal) actin	Mean fluorescent intensity	ADSC	0.99 ± 0.12	0.99 ± 0.17
		BM	1.14 ± 0.25	1.3 ± 0.22
	Percentage	ADSC	1.01 ± 0.43	1.1 ± 0.52
		BM	1.96 ± 1.57	2.48 ± 2.02

ADSC, adipose derived stem-cell; BM, bone marrow, (n = 5-7/group)

Table 2. Flow cytometry analysis of CD34 expression in BM-MSC and ADSC after exposure to NO.

POSITIVE CELLS	SOURCE	CONTROL	SNAP	DEA/NO	SNAP/CONTROL	DEA/NO/CONTROL
Mean fluorescent intensity	ADSC	11.61 ± 6.4	14.58 ± 9.55	12.12 ± 6.06	1.4 ± 0.87	1.07 ± 0.27
	BM	5.39 ± 3.09	5.8 ± 2.67	5.57 ± 2.52	1.34 ± 0.86	1.24 ± 0.66
Percentage	ADSC	3.94 ± 4.8	3.95 ± 3.99	3.89 ± 4.45	4 ± 6.86	1.25 ± 0.51
	BM	0.44 ± 0.43	2.37 ± 4.55	0.59 ± 0.67	9.16 ± 14.79	6.34 ± 12.35

ADSC, adipose derived stem-cell; BM, bone marrow, (n = 5-7/group)

Table 3. Primer set utilized for RT-PCR and qPCR analyses.

Target	NCBI Seq	Amplicon (pb)	TM (°C)	Primer Sequence	Reference
GAPDH	2597	150	55	F 5' GGCGATGCTGGCGCTGAGTAC 3' R 5' TGGTTCACACCCATGACGA 3'	Yoon et al. Differentiation 74 (4), 149-159.
RNA POL II	NM00937	187	55	F 5' TACCACGTCATCTCCTTTGATGGCTCCTAT 3' R 5' GTGCGGCTGCTTCCATAA 3'	Radonie et al. Biochemical and Biophysical Research Communications. 313: 856-862. 2004
GATA-4	NM_002052	422	62	F 5' CTCCCCTGGCAAAACAAGAG 3' R 5' TGCCGTGTCTTAGCAGTCGT 3'	Yoon et al. Differentiation 74 (4). 149-159. 2006.
Nkx2.5	AB021133	322	62	F 5' ATAGGCGGGGTAGGCGTTAT 3' R 5' GCTCTTTGGGGAGAAGCTCA 3'	Yoon et al. Differentiation 74 (4), 149-159.
α-MHC	NM_002471	413	62	F 5' GTCATTGCTGAAACCGAGAATG 3' R 5' GCAAAGTACTGGATGACACGCT 3'	Yoon et al. Differentiation 74 (4), 149-159.
β-MHC	X06976	396	62	F 5' AGATGGATGCTGACCTGTCC 3' R 5' GGTTTTTCCTGTCCTCCTCC 3'	Yoon et al. Differentiation 74 (4), 149-159.
ANP	NM_006172	406	62	F 5' GAACCAGAGGGGAGAGACAGAG 3' R 5' CCCTCAGCTTGCTTTTTAGGAG 3'	Yoon et al. Differentiation 74 (4), 149-159.
Alpha Cardiac Actin	NM_005159	226	55	F 5' GCAAGGACCTGTATGCCAACAATG 3' R 5' GCCTCATCGTACTCTTGCTTGCTA 3'	Zaehres et al. Stem Cells 23: 299-305.2005.
Conexina 43	M65188	154	60	F 5' CCTTCTTGCTGATCCAGTGGTAC 3' R 5' ACCAAGGACACCACCAGCAT 3'	Chen et al. Stem Cells, 24: 1265-1273. 2006.
MLC-A	BC027915	239	62	F 5' GCTCTTTGGGGAGAAGCTCA 3' R 5' CGTCTCCATGGGTGATGATG 3'	Yoon et al. Differentiation 74 (4), 149-159.
MLC-V	BC031006	200	62	F 5' GGCGCGTGAACGTGAAAAAT 3' R 5' CAGCATTTCCCGAACGTAAT 3'	Yoon et al. Differentiation 74 (4), 149-159.
Cardiac troponin	BC002653	152	62	F 5' GGCAGCGGAAGAGGATGCTGAA 3' R 5' GAGGCACCAAGTTGGGCATGAACGA 3'	Yoon et al. Differentiation 74 (4), 149-159.
VEGF	M27281	101	60	F5'CTACCTCCACCATGCCAAGTG 3' R5'TGCGCTGATAGACATCCATGA 3'	Yoon et al. Differentiation 74 (4), 149-159.
Desmin	nm_001927	408	60	F 5' CCAACAAGAACAACGACG 3' R 5' TGGTATGGACCTCAGAACC 3'	Xu, W. et al. Exp Biol Med 229:623–631, 2004
Troponin I	X54163	211	62	F 5' CCTGCGGAGAGTGAGGATCT 3' R 5' TAGGCAGGAAGGCTCAGCTC 3'	Yoon et al. Differentiation 74 (4), 149-159.

4. CONSIDERAÇÕES FINAIS

O transplante celular na área da cardiomioplastia é um método terapêutico novo. O objetivo deste método é o tratamento de doenças cardíacas degenerativas, pois pode levar a recuperação da função do miocárdio[179-181]. Porém a fonte de células e o tipo celular com propriedades mais adequadas de regeneração do tecido cardíaco estão sendo pesquisados[34]. As CTMs possuem propriedades que podem permitir uma terapia celular altamente efetiva. São facilmente isoladas e cultivadas[58] e estão presentes em muitos tecidos adultos[63,91-99]. A MO, o SCU e o TA têm sido extensivamente estudados como fontes de CTMs. No primeiro artigo comparamos as CTMs derivadas da MO e do SCU e as CTDA. Nossos resultados mostraram que estas três fontes fornecem CTMs com propriedades morfológicas e imunofenotípicas semelhantes, mas não idênticas. A porcentagem de células $CD34^+$ na população aderente, por exemplo, foi semelhante entre as CTMs derivadas do SCU e as CTDAs, porém diferiu das CTMs derivadas da MO. O potencial de diferenciação em adipócitos também diferiu de acordo com a fonte. Empregando técnicas de coloração histológica e moleculares, mostramos que as CTMs derivadas do SCU são mais resistentes a diferenciação adipocítica. Entretanto, as CTMs derivadas da MO e as CTDAs diferenciam bem a adipócitos. Os vacúolos lipídicos encontrados nos adipócitos derivados das CTMs da MO apresentaram tamanho médio maior comparativamente aos vacúolos observados nas CTDAs, indicando que a MO permite um maior grau de diferenciação. Neste trabalho também relatamos que a osteonectina não foi um bom marcador de diferenciação osteogênica, pelo menos nas condições utilizadas. Outros estudos mostram que as CTMs da MO e do SCU expressam a osteonectina constitutivamente[182,183], tornando-a um marcador inapropriado para a avaliação da diferenciação osteogênica.

Após a definição que as células isoladas das três fontes eram CTMs, iniciamos a segunda etapa do nosso trabalho. Como a literatura relata que as CTMs apresentam um potencial de diferenciação em cardiomiócitos *in vitro* e *in vivo* [24,91,115,117,120,121], o objetivo foi diferenciar as CTMs a cardiomiócitos ou a um fenótipo semelhante a cardiomiócito, para um possível uso em transplante

celular. Pela dificuldade de obtenção das CTMs do SCU, optamos por trabalhar somente com as células-tronco derivadas da MO e as CTDAs.

O óxido nítrico (NO) parece ser um fator importante para o desenvolvimento cardíaco[159,176]. De fato, diversos artigos mostraram que as isoformas iNOS e eNOS são expressas no coração de embriões e que camundongos $NOS^{-/-}$ apresentam defeitos cardíacos[174,184,185]. Também, foi recentemente demonstrado que o NO facilita a diferenciação das células-tronco embrionárias para o fenótipo cardíaco[159]. Devido a necessidade de definir o melhor tipo de célula-tronco adulta com potencial cardiomiogênico e um estímulo adequado para esta diferenciação, consideramos importante avaliar o potencial de diferenciação das CTMs a cardiomiócitos utilizando o NO.

Os resultados obtidos mostraram que a exposição das CTMs a um nitrosotiol e um doador de NO, SNAP e DEA/NO respectivamente, induziu a expressão de genes e/ou proteínas musculares. Apesar da clara tendência ao aumento da expressão, a alta variabilidade inter-indivíduos e o pequeno número de amostras não permitiu que as diferenças observadas fossem estatisticamente significativas.

Outro resultado interessante deste estudo foi o aumento da expressão tanto do gene VEGF como de células $CD34^{+}$ nas CTMs tratadas. O marcador CD34 é uma glicoproteína de superfície celular presente em células-tronco ativadas[183] e tem sido utilizada como marcador de células-tronco hematopoéticas. Porém, ele também está expresso nas células progenitoras endoteliais e em progenitores estromais fetais e adultos[187]. Especula-se que este marcador tenha um papel importante na regulação da diferenciação das células progenitoras em tecidos adultos como sangue e músculo[188]. Além disso, LEONE *et al*. (2006)[189] demonstraram que o número de células CD34 estava correlacionado com os níveis de VEGF nos controles sadios. Embora nossos dados não tenham permitido a avaliação da correlação entre níveis de expressão gênica do VEGF e CD34, eles corroboram os dados destes autores, sugerindo uma associação ou até mesmo que a células $CD34^{+}$ secretem VEGF. A secreção de VEGF poderia assim estar envolvida na função das CTMs diferenciadas. De fato atualmente acredita-se que os efeitos benéficos do transplante de CTMs na lesão do miocárdio pós-infarto possa ser mediado

mais pelos efeitos parácrinos que levam a angiogênese do que pela sua diferenciação em cardiomiócitos[32,190-192]. SUURONEN *et al.* (2007)[193], por exemplo, relatam o aumento de expressão da conexina-43 nos cardiomiócitos atribuída a secreção de fatores parácrinos pelas CTMs derivadas da MO transplantadas em ratos. Também foi demonstrado que a neovascularização induzida no miocárdio isquêmico está diretamente relacionada com a ação parácrina das CTMs no miocárdio isquêmico[191]. Assim os efeitos cardioprotetores das CTMs podem ser mediados não somente pela diferenciação em células vasculares e cardiomiócitos, mas pela sua habilidade de suprir grandes quantidades de fatores angiogênicos, anti-apoptóticos e mitogênicos[32,125,117,194,195].

HAHN *et al*., (2008)[125] demonstraram que o pré-tratamento das CTMs de ratos com uma combinação de fatores de crescimento, levou a um aumento da expressão de fatores de transcrição cardíacos, e após a co-cultura com cardiomiócitos, ocorreu o aumento da expressão de genes específicos cardíacos incluindo a conexina-43 e uma melhor formação das junções *gap* funcionais com os cardiomiócitos comparativamente com as CTMs não tratadas. O aumento da formação das junções *gap* e da sobrevida da célula no miocárdio infartado, redução do tamanho do infarto e melhora da função do ventrículo esquerdo, foram observados após o transplante das CTMs tratadas com fatores de crescimento, em ratos com infarto do miocárdio.

Apesar da degradação em produtos diferentes, não observamos diferenças significativas entre os dois agentes de NO empregados. Portanto concluímos que o NO ou seus metabólitos induziram um aumento da expressão de alguns marcadores musculares, CD34 e VEGF nas CTMs derivadas da MO e nas CTDAs. O aumento da expressão destes genes poderá contribuir para os efeitos benéficos observados após o transplante destas células em várias doenças cardiovasculares. Nossos dados corroboram o papel das CTMs preferencialmente na sinalização parácrina para angiogênese à diferenciação em cardiomiócitos.

Em conjunto, os dados obtidos nestes trabalhos explicitam ainda dois aspectos importantes da biologia das CTMs e que são relevantes no contexto da cardiomioplastia. Um deles está relacionado a variabilidade genética

dependente de cada indivíduo e das condições fisiopatológicas de cada doador como a idade e a presença de doenças.

Ainda não há um consenso na literatura se realmente há uma diminuição do número e função das CTMs derivadas da MO com o aumento da idade do doador[196-198]. A redução do número das CTMs relacionada com o aumento da idade, pode ser acompanhada pela perda da funcionalidade de certas CTMs, por exemplo, devido ao acúmulo dos danos oxidativos[199], aumento da freqüência das aberrações cromossômicas[200], encurtamento do telômero[201] ou a defeitos no microambiente ósseo[202]. Alguns estudos com ratos[198] e humanos[197], relataram que o potencial de diferenciação das CTMs *in vitro* não depende da idade dos doadores, porém ZHENG *et al*. (2007)[203] demonstram que as CTMs de ratos mais velhos apresentam capacidade reduzida em gerar uma matriz condrogênica *in vitro*. SETHE *et al*. (2006)[196], mostraram que há mudanças das CTMs humanas com o aumento da idade como a perda do potencial de diferenciação, de proliferação, aumento no número de células senescentes e perda da formação óssea *in vivo*. STOLZING *et al*. (2008)[204] estudando doadores humanos de MO de várias idades e utilizando marcadores de senescência celular, demonstraram haver uma redução no número de CTMs, reduzida capacidade de proliferação e capacidade de diferenciação osteogênica e condrogênica.

A idade também pode influenciar a capacidade regenerativa do tecido, a qual diminui com a idade. Este declínio tem sido atribuído a uma diminuição na resposta das células-tronco específicas de cada tecido[205,206]. CONBOY *et al*. (2005)[202], sugerem que há fatores sistêmicos que podem modular vias de sinalização molecular críticas para a ativação de células progenitoras tecido específicas. Foi demonstrado que o declínio do potencial regenerativo com o aumento da idade pode ser revertido através da modulação de tais fatores sistêmicos (como ativação da via *Notch* para as células satélites musculares), sugerindo que células-tronco tecido específicas, mesmo com o aumento da idade, retêm muito do seu potencial proliferativo intrínseco e que mudanças no microambiente sistêmico no qual ocorra ativação das células-tronco, pode levar a uma efetiva regeneração tecidual.

Com base nestes trabalhos sugerimos que parte da grande dispersão observada nos nossos dados pode ser conseqüência da variabilidade na idade dos indivíduos que participaram deste estudo.

A presença de doenças nos doadores de medula óssea e tecido adiposo que pudessem influenciar na expressão de marcadores musculares e angiogênicos nas CTMs expostas ao NO é outro fator que poderia estar contribuindo para a variabilidade observada entre as diferentes amostras. A obesidade, fator presente em todos os doadores de TA, é considerada um fator de risco cardiovascular, contribuindo para o desenvolvimento da aterosclerose. O tecido adiposo subcutâneo, o qual é menos ativo metabolicamente comparativamente ao tecido adiposo visceral, secreta citocinas e quimiocinas, como interleucinas 6 e 8, fator de necrose tumoral α (TNF- α), quimiocinas pro-aterogênicas e peptídeos pro-angiogênicos, os quais parecem contribuir diretamente para inflamação crônica, alterações do tônus vascular, proliferação de células musculares lisas, neo-angiogênese e o desenvolvimento de complicações cardiovasculares[207]. Assim, estes fatores poderiam influenciar o pré-comissionamento das CTDAs em diferentes graus, resultando na dispersão de dados observada para esta fonte.

No nosso estudo após as análises por citometria de fluxo, imunofluorescência RT-PCR e qPCR, observamos que as amostras derivadas da MO mostraram-se mais comissionadas para cardiomiócitos em comparação às CTDAs. De fato os doadores de MO eram todos portadores da cardiomiopatia dilatada. A produção local ou sistêmica de mediadores inflamatórios pode influenciar não somente a migração das CTMs para os tecidos danificados[208,209], mas também a proliferação, diferenciação[210] e pega do enxerto[211]. Um estudo realizado por LARGHERO *et al*. (2007)[212] comparando as CTMs derivadas da MO de indivíduos saudáveis e pacientes com esclerose múltipla, demonstrou não haver diferenças fenotípicas, proliferativas, no potencial de diferenciação e nas propriedades imunossupressoras destas células. Porém ainda não sabemos se outras doenças poderiam alterar o comportamento das CTMs.

O segundo aspecto importante a ser discutido seria o potencial de diferenciação das CTMs a cardimiócitos. Nossos dados confirmam outros trabalhos demonstrando que a porcentagem de células que se diferenciam em

cardiomiócitos é baixa[22,71,132,213-215] e que estas células atuam *in vivo* estimulando a angiogênese pela liberação de fatores parácrinos. A injeção de células-tronco derivadas do músculo esquelético no miocárdio infartado de camundongos induz uma melhora funcional[216,217]. Tal melhora foi atribuída à secreção de VEGF pelas CTMs, o qual induziu uma neoangiogênese nas células hospedeiras. Como poucas CTMs do doador diferenciaram em cardiomiócitos ou fusionaram-se em estruturas semelhantes a vasos sangüíneos, concluiu-se que o VEGF poderia ser o primeiro fator no ambiente isquêmico que seria responsável pela indução da angiogênese nesta região[216]. AL-KHALDI *et al*. (2003)[70] também demonstraram que as CTMs de camundongos C57Bl/6 induzem a vasculogênese, e a neovascularização dependente de VEGF. A neovascularização é um processo fisiológico de formação de novos vasos sanguíneos e é extremamente importante para a melhoria da função do coração após um estresse isquêmico. Este processo inclui a formação de capilares (angiogênese) e artérias colaterais. A isquemia ou a oclusão da artéria coronária induz a expressão de VEGF no modelo de infarto do miocárdio em ratos, e esta molécula é responsável pela circulação colateral coronária e retenção do suprimento sanguíneo para a área isquêmica. A recuperação do suprimento sanguíneo para a área isquêmica previne a morte dos cardiomiócitos e o remodelamento cardíaco[217]. PAYNE *et al*. (2007)[219] demonstraram que o VEGF estimula e é essencial para a angiogênese e melhora funcional observada após o transplante de células-tronco derivadas do músculo esquelético para o reparo do infarto do miocárdio. Estas células também aumentam a secreção de VEGF quando expostas à hipóxia, condição que ocorre após o transplante no miocárdio infartado. Portanto, o meio isquêmico do miocárdio infartado é um importante estimulador da produção de VEGF pelas células transplantadas.

Durante a expansão *in vitro* das CTMs ocorre a diminuição da expressão do marcador CD34. Ainda não está definido se isto é um artefato idiossincrático devido a manipulação *in vitro* das CTMs ou resultado do comissionamento destas células para uma linhagem celular específica, como ocorre com a diferenciação das células-tronco hematopoéticas[220,221]. No nosso trabalho observamos um aumento da expressão do marcador CD34 após o tratamento das células-tronco com NO, o que poderia estar correlacionada com o aumento

da capacidade de angiogênese destas células. COPLAND *et al*. (2008)[222] compararam o perfil do RNAm das células $CD34^{pos}$ e $CD34^{null}$ nas CTMs em camundongos e observaram a expressão de diferentes genes de proteínas secretadas. Nas células $CD34^{pos}$ foram identificados fatores de crescimento que podem influenciar o processo de angiogênese e também foi mostrado o aumento da expressão da proteína de matrix extracelular tenascina C[223] e glipicano[224], as quais influenciam a angiogênese através do VEGF. Portanto sugere-se que CD34 possa modular a composição do meio extracelular e aumentar a resposta vascular derivada do hospedeiro. Além disso, observou-se que a expressão de CD34 pelas CTMs está correlacionada com o aumento do potencial de vasculogênese e angiogênese *in vivo*[222]. LIU *et al*. (2007)[225] cultivando CTMs humanas $CD34^{neg}$ em meio de crescimento endotelial, observou o aumento da expressão de CD34 e outros marcadores de células endoteliais, confirmando que a expressão desta molécula esteja relacionada com a plasticidade das CTMs. Assim, o envolvimento da proteína CD34 na angiogênese tem sido cada vez mais considerado, e nossos dados confirmam esta relação.

As espécies reativas de nitrogênio, como o NO e seus derivados, são produtos do metabolismo celular normal. O NO pode ter um papel dual tanto deletério como benéfico nas funções fisiológicas normais. O NO é uma substância vasoativa, com propriedades anti-plaquetárias[161] e angiogênicas[226]. É considerado uma molécula de sinalização da diferenciação, produzida por todas as células que compõem o miocárdio. A expressão de NOS durante o desenvolvimento cardíaco, assim como os defeitos congênitos resultantes da ausência de eNOS[227,228] demonstram sua relevância na cardiomiogênese. ZHU *et al*. (2006)[229] demonstraram que a pequena exposição *in vitro* a hipóxia/reoxigenação, direta ou indiretamente, produz espécies reativas de oxigênio em células endoteliais arteriolares coronárias humanas induzindo estresse oxidativo, o qual está associado com o aumento da expressão de VEGF e angiogênese. Portanto a adição *in vitro* de agentes de NO nas CTMs e CTDAs neste estudo, poderia estar estimulando o aumento da expressão do VEGF por estas células.

Entretanto, considerando uma intervenção terapêutica, a utilização de tais drogas requer atenção. Uma produção excessiva do NO pode

comprometer a função cardíaca. HEBA *et al.* (2001)[230] relataram que a expressão de TNFα, iNOS e VEGF na região do miocárdio infartado tem um papel importante na disfunção do miocárdio e remodelamento após o infarto do miocárdio e este processo está relacionado com o desenvolvimento progressivo da insuficiência cardíaca. A expressão aumentada do TNFα e iNOS é responsável pela deterioração cardíaca causando um efeito inotrópico negativo e apoptose[231]. A disponibilidade local do TNFα é atribuída a indução de iNOS e conseqüente alta produção de NO[232]. O excesso de NO pode ser citotóxico para os cardiomiócitos e para as células endoteliais[233]. Os efeitos da isquemia e hipertrofia podem ser melhorados com a indução da proteína VEGF, o qual serve como um sinal para a angiogênese compensatória. Porém esta é prejudicada durante o processo de remodelamento e desenvolvimento da insuficiência cardíaca, devido à presença do TNFα em altas concentrações inibindo a angiogênese induzida pelo VEGF[234].

Finalmente, a pré-diferenciação das CTMs para cardiomiócitos ou para células que induzam a angiogênese no local do transplante é uma condição necessária para que estas células não se diferenciem em um tecido não apropriado. BREITBACH *et al*. (2007)[235], por exemplo, observaram que após o transplante intra-miocárdio de CTMs após infarto do miocárdio em camundongos, houve a formação de estruturas encapsuladas contendo calcificações e/ou ossificações, sugerindo que estas células se diferenciaram em osso *in vivo*. Neste trabalho portanto, concluíram que a diferenciação das CTMs não está restrita ao meio ambiente no qual ela foi transplantada. Desta forma ressaltamos a importância dos estudos da pré-diferenciação das CTMs *in vitro*.

Como continuidade dos nossos trabalhos faremos a quantificação da proteína VEGF liberada pelas CTMs e CTDAs pré e pós-tratamento com ON, para sabermos se o VEGF está sendo secretado pelas células tratadas com NO. Estudos *in vivo* com animais poderão ser realizados utilizando o transplante das CTMs transfectadas com o gene *VEGF* e analisando a capacidade de angiogênese e miogênese destas células. Outra linha de pesquisa poderia ser a co-cultura das CTMs com os cardiomiócitos já que existem diversos trabalhos[122,125,138] que demonstram que o contato célula-célula estimula a diferenciação em cardiomiócitos.

5. CONCLUSÕES

Deste trabalho concluímos que:

- A eficiência de obtenção depende da fonte de CTMs;
- O período de estabelecimento da monocamada das culturas das CTMs obtidas da MO e das CTDAs é menor comparativamente às CTMs obtidas do SCU;
- As células-tronco derivadas da MO, SCU e TA apresentam semelhanças em relação a morfologia, ao tamanho e a complexidade, porém não são semelhantes imunofenotipicamente em relação aos marcadores CD34 e CD117;
- As CTMs-MO e CTDAs apresentam o mesmo potencial de diferenciação em adipócitos, osteoblastos e condrócitos, mas diferem das CTMs-SCU na diferenciação para adipócitos;
- As CTMs-MO apresentam adipócitos mais maduros em relação às CTDAs, portanto a MO demonstra um maior grau de diferenciação para esta linhagem;
- O tratamento das CTMs-MO e CTDAs com SNAP e o DEA/NO aumentam a expressão de marcadores musculares e musculares cardíacos, VEGF e CD34;
- Há uma grande variabilidade nos resultados entre os diferentes doadores de MO e TA;
- A baixa indução dos marcadores musculares e cardíacos associada a uma indução consistente de VEGF, indicam que o tratamento com SNAP e DEA/NO não leva as CTMs para um fenótipo cardíaco altamente diferenciado, mas para um fenótipo associado à angiogênese.

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7. ANEXOS

Pontifícia Universidade Católica do Paraná
Pró-Reitoria de Pesquisa e Pós-Graduação

Curitiba, 05 de maio de 2005
Of. 134/05/CEP-PUCPR

Ref. "Estudo da plasticidade das células Tronco Mesenquimais Obtidas da Medula e Cordão Umbilical".

Prezado (a) Pesquisador (es),

Venho por meio deste informar a Vossa Senhoria que o Comitê de Ética em Pesquisa da PUCPR, no dia 04 de maio do corrente ano aprovou o Projeto Intitulado "Estudo da plasticidade das células Tronco Mesenquimais Obtidas da Medula e Cordão Umbilical", pertencente ao Grupo III, sob o registro no CEP nº 597, e será encaminhado a CONEP para o devido cadastro. Lembro ao senhor (a) pesquisador (a) que é obrigatório encaminhar relatório anual parcial e relatório final a este CEP.

Atenciosamente,

Profª M. Sc Ana Cristina Miguez Ribeiro
Coordenadora do Comitê de Ética em Pesquisa - PUCPR

Ilma Sra.
Carmem Lucia K. Rebelatto

Rua Imaculada Conceição, 1155 - Prado Velho - CEP 80215-901 - Caixa Postal 16210
http://www.pucpr.br - Telefone: (41) 271-1543 - Telefax: (41) 332-6886 - Curitiba - Paraná - Brasil

Pontifícia Universidade Católica do Paraná
Pró-Reitoria de Pesquisa e Pós-Graduação

Curitiba, 11 de novembro de 2005.
Of. 515/05/CEP-PUCPR

Ilma Sra.
Carmem Lucia K. Rebelatto

Ref. "Estudo da plasticidade das células Tronco Mesenquimais Obtidas da Medula e Cordão Umbilical"

Prezado Pesquisador

O referido CEP vem através deste notificar o recebimento e aprovação dos seguintes documentos:

- Emenda referente a adição de Tecido Adiposo no estudo de 18 de setembro de 2005;
- Alteração do título para "Estudo da plasticidade das células tronco mesenquimais obtidas da medula óssea, cordão umbilical e Tecido Adiposo".

Atenciosamente,

Profª M. Sc. Ana Cristina Miguez Ribeiro
Coordenadora do Comitê de Ética em Pesquisa - PUCPR

Rua Imaculada Conceição, 1155 - Prado Velho - CEP 80215-901 - Caixa Postal 16210 - CEP 81611-970
Telefone: (41) 3271-1521 - Telefax: (41) 3271-1661 - Curitiba - Paraná - Brasil

Pontifícia Universidade Católica do Paraná
Pró-Reitoria Acadêmica e de Pesquisa
Núcleo de Bioética

Curitiba, 31 de agosto de 2006.
Of. 473/06/CEP-PUCPR

Ref. "Estudo da plasticidade das células Tronco Mesenquimais Obtidas da Medula e Cordão Umbilical "

Prezado (a) Pesquisador (es),

Venho por meio deste informar a Vossa Senhoria que o Comitê de Ética em Pesquisa da PUCPR, no dia 30 de agosto do corrente ano **aprovou** a emenda ao projeto, que solicita a mudança do local de coleta do tecido adiposo para o Instituto de Medicina e Cirurgia do Paraná.
Salientamos que os métodos aprovados no projeto original devem ser mantidos, inclusive a assinatura do Termo de Consentimento Livre Esclarecido.

Lembro ao senhor (a) pesquisador (a) que é obrigatório encaminhar relatório anual parcial e relatório final a este CEP.

Atenciosamente,

Profª M. Sc Ana Cristina Miguez Ribeiro
Coordenadora do Comitê de Ética em Pesquisa - PUCPR

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Citometria de Fluxo

TA	PERCENTUAL		Teste não-paramétrico de Wilcoxon, p<0,05								
		Valid N	Mean	Median	Minimum	Maximum	Std.Dev.	Controle pré x controle pós	Controle pós x SNAP	Controle pós x DEA	SNAP x DEA
CD44	Controle pré	4	95,20	95,95	89,80	99,10	4,54	Pares completos:1	Pares completos:5	Pares completos:5	Pares completos:5
	Controle pós	5	91,95	93,90	83,10	97,10	5,76	---	0,686	0,079	0,043
	SNAP	5	92,80	93,20	88,10	97,40	3,76				
	DEA	5	94,88	95,10	92,00	97,80	2,60				
CD90	Controle pré	2	99,25	99,25	98,80	99,70	0,64	Pares completos:2	Pares completos:5	Pares completos:	Pares completos:
	Controle pós	5	94,34	95,95	88,20	96,72	3,59	---	0,500	0,500	0,500
	SNAP	5	94,78	96,73	91,10	97,45	3,19				
	DEA	5	94,64	97,55	87,50	98,30	4,69				
CD105	Controle pré	2	95,70	95,70	93,60	97,80	2,97	Pares completos:0	Pares completos:3	Pares completos:3	Pares completos:3
	Controle pós	3	79,40	78,80	78,70	80,70	1,13	---	---	---	---
	SNAP	3	86,90	88,10	82,10	90,50	4,33				
	DEA	3	82,77	89,40	66,40	92,50	14,26				
CD34	Controle pré	4	5,72	6,53	1,94	7,90	2,73	Pares completos:3	Pares completos:7	Pares completos:7	Pares completos:7
	Controle pós	7	3,94	2,63	0,10	13,90	4,80	---	0,866	1,000	1,000
	SNAP	7	3,95	2,67	0,90	12,60	3,99				
	DEA	7	3,89	2,39	0,12	12,90	4,45				
CD73	Controle pré	2	98,45	98,45	98,30	98,60	0,21	Pares completos:2	Pares completos:5	Pares completos:5	Pares completos:5
	Controle pós	5	88,86	92,10	79,60	96,80	8,27	---	0,225	0,686	1,000
	SNAP	5	97,04	97,00	94,70	98,70	1,65				
	DEA	5	91,76	95,80	69,20	99,20	12,71				
CD45	Controle pré	4	0,73	0,47	0,21	1,77	0,71	Pares completos:3	Pares completos:7	Pares completos:7	Pares completos:7
	Controle pós	7	0,34	0,15	0,07	1,06	0,36	---	0,866	0,916	0,735
	SNAP	7	0,44	0,32	0,07	1,26	0,44				
	DEA	7	0,84	0,23	0,10	4,72	1,71				
CD29	Controle pré	4	97,58	98,80	93,30	99,40	2,87	Pares completos:3	Pares completos:4	Pares completos:4	Pares completos:4
	Controle pós	4	97,25	97,80	95,10	98,30	1,49	---	0,715	0,465	0,109
	SNAP	4	97,08	97,20	95,90	98,00	0,90				
	DEA	4	97,70	97,95	96,40	98,50	0,91				

CD31	Controle pré	2	0,52	0,52	0,21	0,83	0,44	Pares completos:2	Pares completos:2	Pares completos:2	Pares completos:2
	Controle pós	2	0,14	0,14	0,14	0,14	0,00	---	---	---	---
	SNAP	2	0,12	0,12	0,09	0,15	0,04				
	DEA	2	0,14	0,14	0,09	0,19	0,07				
CONE	Controle pré	4	0,19	0,19	0,13	0,26	0,05	Pares completos:1	Pares completos:5	Pares completos:5	Pares completos:5
	Controle pós	5	0,75	0,78	0,42	0,94	0,21	---	0,138	0,893	0,225
	SNAP	5	1,03	0,98	0,53	1,65	0,45				
	DEA	5	0,79	0,66	0,30	1,46	0,51				
TROP	Controle pré	4	0,17	0,17	0,08	0,25	0,08	Pares completos:3	Pares completos:7	Pares completos:7	Pares completos:7
	Controle pós	7	0,59	0,19	0,10	1,59	0,68	---	1,000	0,735	0,499
	SNAP	7	0,66	0,23	0,07	1,67	0,66				
	DEA	7	0,58	0,25	0,08	1,44	0,57				
MHC	Controle pré	4	0,20	0,21	0,10	0,30	0,09	Pares completos:3	Pares completos:7	Pares completos:7	Pares completos:7
	Controle pós	7	0,51	0,43	0,09	1,28	0,42	---	1,000	0,310	0,176
	SNAP	7	0,71	0,30	0,09	2,01	0,73				
	DEA	7	0,46	0,23	0,09	1,16	0,45				
MIOS	Controle pré	4	0,21	0,20	0,10	0,33	0,09	Pares completos:3	Pares completos:7	Pares completos:7	Pares completos:7
	Controle pós	7	0,34	0,21	0,09	0,73	0,26	---	0,600	0,237	0,063
	SNAP	7	0,49	0,24	0,06	1,45	0,55				
	DEA	7	0,29	0,22	0,07	0,76	0,25				
ACTI	Controle pré	4	0,26	0,24	0,11	0,47	0,17	Pares completos:3	Pares completos:4	Pares completos:4	Pares completos:4
	Controle pós	4	0,34	0,21	0,11	0,81	0,32	---	1,000	0,715	0,273
	SNAP	4	0,28	0,29	0,08	0,46	0,16				
	DEA	4	0,32	0,32	0,09	0,56	0,22				

MO	PERCENTUAL		Teste não-paramétrico de Wilcoxon, p<0,05								
		Valid N	Mean	Median	Minimum	Maximum	Std.Dev.				
CD44	Controle pré	3	89,73	92,70	77,50	99,00	11,05	Pares completos:2	Pares completos:3	Pares completos:3	Pares completos:3
	Controle pós	3	95,24	95,50	91,82	98,40	3,30	---	---	---	---
	SNAP	3	92,47	98,10	80,40	98,90	10,46				
	DEA	3	97,40	98,00	95,20	99,00	1,97				
CD90	Controle pré	1	97,00		97,00	97,00		Pares completos:1	Pares completos:3	Pares completos:3	Pares completos:3
	Controle pós	3	93,10	94,05	88,80	96,45	3,91	---	---	---	---
	SNAP	3	95,63	96,23	92,60	98,05	2,77				
	DEA	3	95,11	95,77	91,20	98,35	3,62				
CD105	Controle pré	1	97,60		97,60	97,60		Pares completos:0	Pares completos:1	Pares completos:1	Pares completos:1
	Controle pós	1	79,10		79,10	79,10		---	---	---	---
	SNAP	1	74,60		74,60	74,60					
	DEA	1	78,80		78,80	78,80					
CD34	Controle pré	3	2,34	2,72	1,49	2,81	0,74	Pares completos:3	Pares completos:5	Pares completos:5	Pares completos:5
	Controle pós	5	0,44	0,29	0,02	1,13	0,43	---	0,686	0,686	0,109
	SNAP	5	2,37	0,50	0,02	10,50	4,55				
	DEA	5	0,59	0,50	0,01	1,72	0,67				
CD73	Controle pré	1	90,00		90,00	90,00		Pares completos:0	Pares completos:2	Pares completos:2	Pares completos:2
	Controle pós	2	84,30	84,30	72,70	95,90	16,40	---	---	---	---
	SNAP	2	94,00	94,00	93,30	94,70	0,99				
	DEA	2	95,70	95,70	95,00	96,40	0,99				
CD45	Controle pré	3	0,72	0,76	0,62	0,77	0,08	Pares completos:3	Pares completos:5	Pares completos:5	Pares completos:5
	Controle pós	5	0,08	0,09	0,01	0,17	0,06	---	0,500	0,079	0,686
	SNAP	5	0,47	0,14	0,06	1,90	0,80				
	DEA	5	0,46	0,16	0,05	1,71	0,70				
CD29	Controle pré	3	90,43	90,50	84,00	96,80	6,40	Pares completos:2	Pares completos:3	Pares completos:3	Pares completos:3
	Controle pós	3	97,67	98,60	95,50	98,90	1,88	---	---	---	---
	SNAP	3	98,43	98,70	97,80	98,80	0,55				
	DEA	3	98,87	98,80	98,70	99,10	0,21				

CD31	Controle pré	1	0,26		0,26	0,26		Pares completos:0	Pares completos:1	Pares completos:1	Pares completos:1
	Controle pós	1	0,12		0,12	0,12		---	---	---	---
	SNAP	1	0,11		0,11	0,11					
	DEA	1	0,25		0,25	0,25					
CONE	Controle pré	3	0,37	0,28	0,16	0,67	0,27	Pares completos:3	Pares completos:5	Pares completos:5	Pares completos:5
	Controle pós	5	1,10	0,22	0,04	4,32	1,83	---	0,500	0,500	0,225
	SNAP	5	0,35	0,36	0,06	0,64	0,23				
	DEA	5	0,57	0,48	0,08	1,12	0,46				
TROP	Controle pré	3	0,35	0,40	0,14	0,50	0,19	Pares completos:3	Pares completos:5	Pares completos:5	Pares completos:5
	Controle pós	5	0,49	0,16	0,06	1,76	0,72	---	0,273	0,686	0,686
	SNAP	5	0,87	0,23	0,11	3,59	1,52				
	DEA	5	0,49	0,20	0,05	1,63	0,65				
MHC	Controle pré	3	0,28	0,19	0,13	0,53	0,22	Pares completos:3	Pares completos:5	Pares completos:5	Pares completos:5
	Controle pós	5	0,45	0,19	0,09	1,55	0,62	---	0,686	0,345	0,686
	SNAP	5	0,62	0,22	0,07	2,29	0,94				
	DEA	5	0,58	0,22	0,07	2,01	0,81				
MIOS	Controle pré	3	0,28	0,16	0,13	0,56	0,24	Pares completos:3	Pares completos:5	Pares completos:5	Pares completos:5
	Controle pós	5	0,45	0,23	0,06	1,44	0,56	---	0,893	1,000	0,500
	SNAP	5	0,37	0,22	0,10	1,02	0,38				
	DEA	5	0,46	0,32	0,05	1,14	0,43				
ACTI	Controle pré	3	0,33	0,40	0,14	0,46	0,17	Pares completos:3	Pares completos:3	Pares completos:3	Pares completos:3
	Controle pós	3	0,17	0,21	0,07	0,24	0,09	---	---	---	---
	SNAP	3	0,37	0,23	0,08	0,79	0,37				
	DEA	3	0,48	0,35	0,08	1,01	0,48				

TA	Média Intensidade fluorescência	Teste não-paramétrico de Wilcoxon, p<0,05									
		Valid N	Mean	Median	Minimum	Maximum	Std.Dev.	Controle pré x controle pós	Controle pós x SNAP	Controle pós x DEA	SNAP x DEA
CD44	Controle pré	4	37,45	36,10	13,10	64,50	27,35	Pares completos:1	Pares completos:5	Pares completos:5	Pares completos:5
	Controle pós	5	144,96	136,60	16,80	268,00	106,77	---	0,686	0,893	0,500
	SNAP	5	134,34	151,50	42,60	249,00	82,77				
	DEA	5	144,86	170,50	50,30	232,00	84,24				
CD90	Controle pré	2	467,00	467,00	401,00	533,00	93,34	Pares completos:2	Pares completos:5	Pares completos:	Pares completos:
	Controle pós	5	131,43	134,82	11,35	268,00	119,42	---	0,893	0,686	0,893
	SNAP	5	128,02	193,00	11,55	213,67	102,38				
	DEA	5	138,35	174,00	9,52	278,00	122,40				
CD105	Controle pré	2	71,85	71,85	57,00	86,70	21,00	Pares completos:0	Pares completos:3	Pares completos:3	Pares completos:3
	Controle pós	3	50,00	46,90	43,50	59,60	8,49	---	---	---	---
	SNAP	3	40,10	27,40	23,70	69,20	25,27				
	DEA	3	36,33	32,20	28,60	48,20	10,43				
CD34	Controle pré	4	14,14	12,92	6,01	24,70	7,76	Pares completos:3	Pares completos:7	Pares completos:7	Pares completos:7
	Controle pós	7	11,61	10,29	4,78	24,67	6,40	---	0,612	0,735	1,000
	SNAP	7	14,58	9,80	7,99	32,30	9,55				
	DEA	7	12,12	10,85	4,14	23,33	6,06				
CD73	Controle pré	2	318,00	318,00	305,00	331,00	18,38	Pares completos:2	Pares completos:5	Pares completos:5	Pares completos:5
	Controle pós	5	254,80	251,00	106,00	412,00	145,98	---	0,225	0,465	0,345
	SNAP	5	346,80	334,00	308,00	411,00	38,67				
	DEA	5	301,40	339,00	106,00	381,00	111,80				
CD45	Controle pré	4	2,89	2,90	2,17	3,60	0,60	Pares completos:3	Pares completos:7	Pares completos:7	Pares completos:7
	Controle pós	7	4,65	4,53	2,78	6,28	1,40	---	0,866	1,000	0,237
	SNAP	7	4,88	4,25	2,78	9,14	2,20				
	DEA	7	4,97	5,31	2,50	8,92	2,23				
CD29	Controle pré	4	268,88	265,00	221,00	324,50	42,52	Pares completos:3	Pares completos:4	Pares completos:4	Pares completos:4
	Controle pós	4	392,75	399,00	307,00	466,00	78,35	---	0,465	0,715	0,715
	SNAP	4	371,25	384,50	309,00	407,00	43,39				
	DEA	4	377,25	406,00	280,00	417,00	65,17				

CD31	Controle pré	2	1,60	1,60	1,46	1,73	0,19	Pares completos:2	Pares completos:2	Pares completos:2	Pares completos:2
	Controle pós	2	3,18	3,18	2,67	3,68	0,71	---	---	---	---
	SNAP	2	3,29	3,29	2,60	3,98	0,98				
	DEA	2	3,06	3,06	2,58	3,54	0,68				
CONE	Controle pré	4	4,73	4,46	1,34	8,64	3,84	Pares completos:1	Pares completos:5	Pares completos:5	Pares completos:5
	Controle pós	5	6,57	2,89	2,22	12,70	5,42	---	0,079	0,686	0,225
	SNAP	5	7,67	4,01	2,64	15,60	6,02				
	DEA	5	7,00	2,77	2,15	14,20	6,22				
TROP	Controle pré	4	4,65	3,98	1,12	9,54	4,21	Pares completos:3	Pares completos:7	Pares completos:7	Pares completos:7
	Controle pós	7	3,93	2,41	1,24	8,81	3,26	---	0,866	0,866	0,612
	SNAP	7	3,94	2,61	1,21	9,84	3,40				
	DEA	7	3,80	2,29	1,23	9,34	3,36				
MHC	Controle pré	4	4,14	4,12	1,14	7,19	3,38	Pares completos:3	Pares completos:7	Pares completos:7	Pares completos:7
	Controle pós	7	4,18	1,76	1,21	9,52	3,74	---	0,735	0,612	0,735
	SNAP	7	4,10	2,41	1,16	8,93	3,27				
	DEA	7	4,45	3,11	1,15	9,22	3,43				
MIOS	Controle pré	4	4,23	3,96	1,14	7,85	3,54	Pares completos:3	Pares completos:7	Pares completos:7	Pares completos:7
	Controle pós	7	3,69	1,79	1,20	9,59	3,73	---	0,612	0,612	0,612
	SNAP	7	3,92	2,03	1,24	9,18	3,36				
	DEA	7	3,83	2,82	1,15	9,16	3,31				
ACTI	Controle pré	4	4,70	4,21	1,12	9,28	4,20	Pares completos:3	Pares completos:4	Pares completos:4	Pares completos:4
	Controle pós	4	6,23	5,61	1,30	12,40	5,74	---	0,715	0,715	0,715
	SNAP	4	6,42	6,34	1,19	11,80	5,99				
	DEA	4	6,10	5,70	1,20	11,80	5,59				

MO	Média Intens. fluorescência	Teste não-paramétrico de Wilcoxon, p<0,05									
		Valid N	Mean	Median	Minimum	Maximum	Std.Dev.				
CD44	Controle pré	3	45,17	50,30	19,50	65,70	23,52	Pares completos:2	Pares completos:3	Pares completos:3	Pares completos:3
	Controle pós	4	56,78	58,28	5,58	105,00	49,35	---	---	---	---
	SNAP	3	71,00	86,90	21,10	105,00	44,15				
	DEA	3	82,63	97,80	30,10	120,00	46,83				
CD90	Controle pré	1	228,00		228,00	228,00		Pares completos:1	Pares completos:3	Pares completos:3	Pares completos:3
	Controle pós	3	144,63	127,00	109,70	197,20	46,34	---	---	---	---
	SNAP	3	141,08	112,50	103,50	207,25	57,48				
	DEA	3	137,75	111,00	106,65	195,60	50,15				
CD105	Controle pré	1	16,65		16,65	16,65		Pares completos:0	Pares completos:1	Pares completos:1	Pares completos:1
	Controle pós	1	29,70		29,70	29,70		---	---	---	---
	SNAP	1	36,70		36,70	36,70					
	DEA	1	20,30		20,30	20,30					
CD34	Controle pré	3	3,80	3,68	2,30	5,42	1,56	Pares completos:3	Pares completos:5	Pares completos:5	Pares completos:5
	Controle pós	5	5,39	5,62	1,36	9,89	3,09	---	0,345	0,893	0,500
	SNAP	5	5,80	5,94	3,18	10,04	2,67				
	DEA	5	5,57	5,92	3,25	9,37	2,52				
CD73	Controle pré	1	143,00		143,00	143,00		Pares completos:0	Pares completos:2	Pares completos:2	Pares completos:2
	Controle pós	2	248,00	248,00	200,00	296,00	67,88	---	---	---	---
	SNAP	2	215,00	215,00	208,00	222,00	9,90				
	DEA	2	236,50	236,50	231,00	242,00	7,78				
CD45	Controle pré	3	2,54	2,56	1,84	3,23	0,70	Pares completos:3	Pares completos:5	Pares completos:5	Pares completos:5
	Controle pós	5	4,23	4,05	1,83	6,69	1,79	---	0,225	0,225	0,225
	SNAP	5	4,44	4,00	1,81	7,32	2,03				
	DEA	5	4,56	3,98	1,73	7,54	2,16				
CD29	Controle pré	3	209,00	190,00	165,00	272,00	55,97	Pares completos:2	Pares completos:3	Pares completos:3	Pares completos:3
	Controle pós	3	473,33	501,00	313,00	606,00	148,45	---	---	---	---
	SNAP	3	449,33	426,00	341,00	581,00	121,69				
	DEA	3	508,67	475,00	327,00	724,00	200,63				

CD31	Controle pré	1	2,26		2,26	2,26		Pares completos:0	Pares completos:1	Pares completos:1	Pares completos:1
	Controle pós	1	3,56		3,56	3,56		---	---	---	---
	SNAP	1	3,60		3,60	3,60					
	DEA	1	3,69		3,69	3,69					
CONE	Controle pré	3	5,97	7,47	1,43	9,02	4,01	Pares completos:3	Pares completos:5	Pares completos:5	Pares completos:5
	Controle pós	5	8,31	11,40	1,38	15,60	6,49	---	0,345	0,345	0,686
	SNAP	5	6,57	4,65	1,30	13,50	5,78				
	DEA	5	6,88	4,13	1,36	16,20	6,60				
TROP	Controle pré	3	5,76	7,71	1,37	8,21	3,81	Pares completos:3	Pares completos:5	Pares completos:5	Pares completos:5
	Controle pós	5	4,35	3,80	1,47	8,08	3,01	---	0,893	0,225	0,893
	SNAP	5	4,90	3,38	1,30	10,40	3,92				
	DEA	5	4,78	4,05	1,38	9,02	3,62				
MHC	Controle pré	3	6,04	7,12	1,42	9,57	4,18	Pares completos:3	Pares completos:5	Pares completos:5	Pares completos:4
	Controle pós	5	4,04	2,77	1,32	8,22	3,08	---	0,273	0,043	0,273
	SNAP	4	5,90	5,98	1,55	10,10	3,79				
	DEA	5	4,79	3,50	1,43	8,77	3,52				
MIOS	Controle pré	3	4,98	5,90	1,37	7,68	3,25	Pares completos:3	Pares completos:5	Pares completos:5	Pares completos:5
	Controle pós	5	4,54	2,73	1,39	8,67	3,70	---	0,893	0,225	0,500
	SNAP	5	4,49	2,60	1,55	8,95	3,54				
	DEA	5	4,97	2,38	1,37	9,72	4,31				
ACTI	Controle pré	3	5,83	7,66	1,39	8,43	3,86	Pares completos:3	Pares completos:3	Pares completos:3	Pares completos:3
	Controle pós	3	6,76	8,42	1,47	10,40	4,69	---	---	---	---
	SNAP	3	7,87	10,10	1,50	12,00	5,59				
	DEA	3	8,46	10,90	1,99	12,50	5,66				