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AVALIAÇÃO *IN VITRO* E *IN VIVO* DOS EFEITOS DA PROTEÍNA ÓSSEA MORFOGENÉTICA TIPO 2 RECOMBINANTE (rhBMP-2) E DE CÉLULAS-TRONCO DERIVADAS DO TECIDO ADIPOSO HUMANO NA OSTEOGÊNESE

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Orientadora: **Prof[a.] Dr[a.] Cláudia Maria Oliveira Simões**

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RESUMO

Dentre os vários fatores de crescimento que têm sido relatados na literatura para tratamento de defeitos ósseos, as proteínas ósseas morfogenéticas (BMPs) têm atraído bastante interesse, em decorrência de sua capacidade osteoindutora. As células-tronco derivadas do tecido adiposo (ASCs) também vêm se destacando na regeneração tecidual e na modulação da resposta inflamatória pelo seu efeito parácrino. O objetivo deste trabalho foi avaliar *in vitro* e *in vivo* os efeitos da BMP-2 e de ASCs na osteogênese. Os experimentos se dividiram em 4 etapas, sendo: (1) avaliação do efeito da proteína óssea morfogenética recombinante humana do tipo 2 (rhBMP-2), da BMP-4 e da BMP-7 na diferenciação osteogênica de ASCs suplementadas com arcorbato e β-glicerofosfato; (2) comparação do efeito do ácido retinóico (AR), da rhBMP-2 e da rhBMP-2+AR na diferenciação osteogênica de ASCs; (3) avaliação do efeito da implantação de ASCs incorporadas em arcabouços de poli(ácido láctico-co-glicólico) (PLGA) na inflamação causada por rhBMP-2 no tecido muscular de cães; e (4) avaliação do efeito de ASCs incorporadas em arcabouços autógenos de plasma rico em plaquetas (PRP) na regeneração óssea na tíbia de cães. Os resultados confirmaram as caracterísitcas de células-tronco mesenquimais das ASCs, através da imunofenotipagem e da diferenciação adipogência e osteogênica. Em relação à etapa 1, as ASCs expressaram BMP-4 e BMP-7 endógenas, a suplementação do meio osteogênico com rhBMP-2 não aumentou a atividade de ALP, a expressão do mRNA da osteonectina e osteocalcina, e a deposição de cálcio. No que diz respeito à etapa 2, a rhBMP-2 promoveu maior expressão de Smad 1 e BMP-7 (dia 7), maior expressão relativa do mRNA do receptor de BMP do tipo II (BMPR-II) e do mRNA da osteocalcina (dia 21). Por sua vez, o AR exibiu as maiores atividades de ALP (dias 7, 14, 21 e 28), maior expressão relativa do mRNA do receptor de BMP do tipo II (RBMP-II) (dia 14), do mRNA da Smad 1 (dia 21) e do mRNA da osteonectina (dias 14, 21 e 28). A associação rhBMP-2+AR promoveu os maiores níveis de cálcio na matriz extra-celular (dias 12 e 32), a expressão da Smad 4 considerável (dias 21 e 28), expressão da Smad 1/ 5/ 8 fosforilada similar ao MC3T3-E1 (controle positivo) (dias 7, 14, 21

e 28), maior expressão relativa do mRNA da Smad 1, mRNA da osteocalcina e mRNA da osteonectina (dias 14, 7 e 7, respectivamente). Em relação à etapa 3, a agregação da rhBMP-2 aos arcabouços foi de aproximadamente 78, 74 e 61% para 1, 2,5 e 5 µg de rhBMP-2.mL^{-1}, respectivamente. A liberação acumulada da rhBMP-2, em 21 dias, foi de aproximadamente 90, 64 e 64% para 1, 2,5 e 5 µg de rhBMP-2.mL^{-1}, respectivamente. Os arcabouços de PLGA foram quase que totalmente degradados em 6 semanas de pós-operatório. As ASCs foram capazes de modular a resposta inflamatória causada por baixa dose de rhBMP-2 (2,5µg) no tecido muscular de cães, diminuindo o número de focos inflamatórios, células gigantes e aumentando a neovascularização. Os resultados da etapa 4 mostraram que PRP+ASCs apresentou menor quantidade de tecido de granulação e promoveu maior quantidade (osso primário e secundário) e maturação (osso secundário) de tecido ósseo neoformado nos defeitos ósseos, comparado com osso autógeno e PRP, isoladamente. Resumidamente, pode-se concluir que a suplementação da rhBMP-2 ao meio osteogênico não melhorou a osteogênese das ASCs; a associação de rhBMP-2+AR se mostrou mais eficiente na osteodiferenciação das ASCs, comparado com rhBMP-2 ou AR isoladamente; as ASCs reduziram o processo inflamatório e aumentaram a neovascularização nos sítios musculares que receberam baixa dose de rhBMP-2; e as ASCs associadas com PRP apresentaram menor quantidade de tecido de granulação e promoveram maior quantidade e maturação de tecido ósseo neoformado nos defeitos ósseos, comparado com osso autógeno e PRP.

Palavras-chave: Proteína óssea morfogenética 2. rhBMP-2. Células-tronco mesenquimais. Osteogênese. Ácido retinóico. Diferenciação celular. Arcabouços.

ABSTRACT

Recombinant human bone morphogenetic protein type 2 (rhBMP-2) is a potent osteogenic growth factor, which has been used for treatment of bone defects in maxillofacial, oral, orthopedic, and spine surgeries. The adipose-derived stem cells (ASCs) have been draun attention in tissue regeneration and modulation of inflamatory disease because of their paracrine effect. This study evaluated the *in vitro* and *in vivo* effects of rhBMP-2 and ASCs in osteogenesis. The experiments were divided into 4 parts: (1) evaluated the effect of recombinant human bone morphogenetic protein type 2 (rhBMP-2), BMP-4, and BMP-7 in osteogenic differentiation of ASCs suplemented with arcorbate and β-glycerophosphate; (2) compared the effect of retinoic acid (RA), rhBMP-2, and rhBMP-2+AR in osteogenic differentiation of ASCs; (3) evaluated the effect of the implantation of ASCs incorporated into scaffolds of poly lactic-co-glycolic (PLGA) in canine muscular tissue; and (4) evaluated the effect of ASCs incorporated into scaffolds of platelet-rich plasm (PRP) in bone regeneration in canine tibia. The results confirmed the mesenchymal stem cells characteristics of ASCs. According to part 1, ASCs expressed endogenous BMP-4 and BMP-7. The supplementation of osteogenic medium with rhBMP-2 did not increase ALP activity, mRNA expression of osteonectin and osteocalcin, and the calcium deposition. According to the results of part 2, rhBMP-2 promoted the highest expression of Smad1 and BMP-7 (day 7), the highest relative expression of BMP receptor type II (BMPR-II) mRNA and osteocalcin mRNA (day 21). RA exhibited the highest phosphatase alkaline activity (ALP) (days 7, 14, 21 and 28), highest expression of BMPR-II mRNA (day 14), Smad 1 mRNA (day 21) and osteonectin mRNA (days 14, 21, and 28). The association rhBMP-2+RA promoted the highest levels of calcium in extracellular matrix (days 12 and 32), a considerable expression of Smad 4 (days 21 and 28), expression of phosphorylated Smad 1/ 5/ 8 similar to MC3T3-E1 (positive control) (days 7, 14, 21, and 28), the highest expression of Smad 1 mRNA, osteocalcin mRNA, and osteonectin mRNA (days 14, 7, and 7, respectivamente). According to part 3, the rhBMP-2 loading into the scaffolds was approximately 78, 74, and 61% for 1, 2.5, and 5 μg of rhBMP-2.mL^{-1}, respectively. Cumulative

rhBMP-2 release was approximately 90, 64, and 64% for 1, 2.5, and 5 μg of rhBMP-2.mL^{-1}, respectively, at day 21. PLGA scaffolds were almost completely degradated at 6 weeks postoperative. ASCs modulated inflammatory response induced by low dose of rhBMP-2 (2.5μg) in muscle sites, decreasing the number of inflammatory foci, giant cells, and neovascularization. The results of part 4 showed that PRP+ASCs promoted the fewest formation of granulation tissue, the highest amount of bone neoformation (primary and secondary bone), and the highest level of bone maturation in bone deffects, compared to autogenous bone and PRP, alone. In summary, supplementation of osteogenic medium with rhBMP-2 did not improve the osteogenic differentiation of ASCs; the assotiation rhBMP-2+RA was more efficient on osteodifferentiation process compared to rhBMP-2 or RA alone; ASCs modulated the inflammatory process and increased neovascularization in muscle sites with low dose of rhBMP-2; the assotiation ASCs+PRP promoted the lowest number of granulation tissue and increased the amount of bone neoformation (primary and secondary bone) and maturation in bone deffects, compared to autogenous bone and PRP, alone.

Keywords: Bone morphogenetic protein 2. Mesenchymal stem cells. Osteogenesis. Retinoic acid. Cell differentiation. Scaffolds.

SUMÁRIO

1. INTRODUÇÃO

1.1 PRINCÍPIOS GERAIS

Procedimentos de enxerto ósseo são frequentemente necessários em cirurgias bucais, maxilofaciais e ortopédicas. Em decorrência deste fato, a busca por um substituto ósseo ideal vem sendo objeto de pesquisa por anos. Os substitutos ósseos, quanto à origem, são classificados em: Autógeno - obtido do próprio indivíduo, de sítios intrabucais ou extrabucais; Alógeno ou homógeno - obtido de indivíduos da mesma espécie, contudo geneticamente diferentes; Xenógeno ou heterógeno - advém de doadores de espécie diferente do receptor, como osso bovino; e Aloplástico - material sintético, de natureza polimérica, metálica ou cerâmica (ABBAS; LICHTMAN, 2004).

Quanto ao mecanismo de ação, os substitutos ósseos podem ser agrupados em: Osteoindutores - capazes de estimular células ectomesenquimais indiferenciadas a se diferenciarem em osteoblastos e/ ou condroblastos; Osteocondutores - servem como um arcabouço para a proliferação de vasos sanguíneos, tecido perivascular e células osteoprogenitoras do leito, que trarão os componentes necessários à formação e deposição óssea; e Osteogenitores - capazes de formar tecido ósseo por si mesmo (apresentam osteoblastos viáveis) (AL RUHAIMI, 2001).

Enquanto o osso autógeno apresenta-se como uma excelente alternativa biológica pelas suas propriedades osteocondutora, osteoindutora e osteogenitora, ele também possui alguns fatores desfavoráveis bem significativos, como a necessidade de uma área doadora, maior período de convalescença, morbidade e susceptibilidade à infecção no sítio doador, limitada quantidade de tecido ósseo, custo elevado (especialmente de sítios extrabucais), e um procedimento cirúrgico adicional (YOUNGER; CHAPMAN, 1989).

Materiais homógenos e heterógenos, apesar de serem utilizados há muito tempo, apresentam limitações, como a necessidade de um banco de ossos, reabsorção prematura (especialmente o medular), alta variabilidade das propriedades de osteoindução (de acordo com a idade do doador, área obtida, tipo de tecido ósseo, processamento do tecido e técnica de descontaminação empregada), e potencial de transmissão de

doenças infectocontagiosas (SOGAL; TOFE, 1999; PINHEIRO, 2001) e príons (proteínas infecciosas), tratando-se de osso heterógeno (SOGAL; TOFE, 1999).

Tendo em vista as desvantagens apresentadas pelos substitutos autógenos, homógenos e heterógenos, três abordagens têm sido propostas para a regeneração óssea: implantação de arcabouços sintéticos ou autógenos, sozinhos ou em combinação com fatores de crescimento; transdução de genes responsáveis pelos fatores de crescimento relacionados à capacidade osteogênica; e transplante de cultura de células osteogênicas, associadas ou não a arcabouços (FRANCESCHI, 2005; YUAN et al., 2009; LEE et al., 2010).

Em função do presente trabalho avaliar algumas dessas abordagens para a regeneração óssea, serão apresentados a seguir algumas características das proteínas ósseas morfogenéticas, com ênfase no tipo 2 (rhBMP-2), das células-tronco mesenquimais (MSCs), do uso de polímeros à base de poli(ácido láctico-co-glicólico) (PLGA) e de plasma rico em plaquetas (PRP) como carreadores para células e fatores de crescimento, bem como do papel do ácido retinóico, isolado ou em associação com a rhBMP-2, na diferenciação osteogênica.

1.2 PROTEÍNAS ÓSSEAS MORFOGENÉTICAS

Dentre os vários fatores de crescimento utilizados para tratamento de defeitos ósseos em cirurgias bucais, maxilofaciais e ortopédicas, as proteínas ósseas morfogenéticas recombinantes humanas (rhBMPs) têm atraído bastante interesse, em decorrência de sua capacidade osteoindutora (BISHOP; EINHORN, 2007; ALONSO et al., 2010). As proteínas ósseas morfogenéticas (BMPs) pertencem à superfamília do Fator de crescimento Tumoral β (TGF-β), e foram descobertas por Urist (1965), que verificou que matrizes ósseas implantadas em sítios ectópicos promoviam a formação óssea. Dependendo do gradiente de concentração, essas proteínas podem atuar na quimiotaxia, proliferação, inibição, diferenciação e maturação de uma variedade de células (SYKARAS; OPPERMAN, 2003).

As BMPs apresentam uma região com sete resíduos de cisteína na porção carbóxi-terminal, sendo que esses resíduos são preservados em todos os membros da família TGF-β. As

BMPs são sintetizadas na forma de um precursor com uma porção hidrofóbica e uma sequência polipeptídica ligada à região madura. As clivagens proteolíticas liberam a região madura, que pode então se dimerizar com outras BMPs. As diferenças químicas e estruturais nos homodímeros e heterodímeros podem justificar as variações biológicas e as características de ligação entre as diferentes BMPs. Atualmente, estão descritas aproximadamente 20 BMPs, sendo que as de maior destaque para a regeneração óssea são as BMP-2 e BMP-4 (semelhantes entre si, variando apenas alguns aminoácidos na região N-terminal), as BMP-5, BMP-6 e BMP-7 (igualmente semelhantes, sendo também denominadas como OP-1, proteínas osteogênicas) e a BMP-8 (OP-2) (LAURENT et al., 2004).

O processo de mineralização óssea é induzido pelas proteínas Smads em conjunto com as BMPs e seus receptores específicos. Os receptores para BMPs podem ser divididos em tipo IA (BMPR-IA), tipo IB (BMPR-IB) e tipo II (BMPR-II), sendo que todos são necessários para a transdução do sinal. Quando os receptores do tipo I estão presentes, a atividade das BMPs é aumentada, sendo que a atividade ótima exige a presença dos receptores dos tipos I e II (WAN et al., 2006; SONG et al., 2009). Estes receptores apresentam um domínio extra-celular (rico em cisteína), um domínio transmembranário e outro domínio citoplasmático (rico em serina/treonina) (SONG et al., 2009).

Na via clássica de sinalização, a BMP se liga ao receptor tipo II e a ativação deste receptor promove a fosforilação dos resíduos de serina nos receptores tipo I, ativando-os. Dando continuidade ao processo, as Smads 1, 5 e 8 se tornam fosforiladas no resíduo carbóxi-terminal serina e se translocam para o núcleo, onde interagem com proteínas ligantes ao DNA, ou exibem atividade direta de transcrição dos genes alvos, tanto como monômero quanto em associação com a Smad 4 (LAGNA et al., 1996; YANG et al., 1998; QING et al., 2000).

Durante a sinalização, utilizando as BMP-2, BMP-4 e BMP-7, as Smads 1, 5 e 8 são reguladas por receptores, sendo designadas de RSmads. Na sinalização com a BMP-3, as Smads 2 e 3 são as RSmads. Como já salientado acima, as Smads 4 são o mediador comum, ou seja, quando as RSmads são ativadas, elas se associam com as Smads 4 (co-Smads) no

citoplasma e são translocadas para o núcleo, onde ativam os genes específicos. As Smads 6 e 7 são inibidoras (ISmads) das RSmads, sendo as Smads 6 inibidoras da sinalização e as Smads 7 inibidoras da via de ativação. A atividade das BMPs é autorregulada e pode sofrer a interferência de fatores inibitórios, que podem se acoplar às BMPs, impedindo-as de exercerem atividade estimulatória junto aos receptores específicos de membrana, o que parece compor um sistema de *feedback* na formação óssea. Os fatores inibidores mais importantes, até o momento, são o *Noggin* e o *Chordin,* sendo que o primeiro tem grande afinidade por BMP-2 e BMP-4, impedindo que elas se acoplem aos receptores de superfície (BACHILLER et al., 2000).

A rhBMP-2 é um dos fatores de crescimento mais estudados para regeneração óssea. Apesar da sua atividade osseoindutora promissora (BISHOP; EINHORN, 2007; ALONSO et al., 2010), este fator de crescimento apresenta custo elevado, tem um tempo de meia-vida curto *in vivo*, requer altas doses terapêuticas e tem sido relacionado com efeitos colaterais relevantes nas áreas médica e odontológica, tais como dificuldade respiratória, disfalgia, edema, eritema, dor e rinite (BOYNE et al., 1997; VAIDYA et al., 2007; SHAH et al., 2008). Estes efeitos têm sido associados à inflamação tecidual e edema provocados por elevadas doses de rhBMP-2 (LEE et al., 2011a). Outra limitação da rhBMP-2 é a necessidade do uso de um carreador, uma vez que a formação de tecido ósseo não pode ocorrer simplesmente pela injeção de uma solução aquosa de rhBMP-2 no sítio alvo, pois essa proteína seria rapidamente eliminada do local de aplicação (DATE et al., 2004; LIANG et al., 2005; KUO et al., 2006; HABRAKEN et al., 2007; SOKOLSKY-PAPKOV et al., 2007; BASMANAV et al., 2008; LIM et al., 2009).

Dessa maneira, existe um interesse crescente em buscar agentes capazes de diminuir a resposta inflamatória causada pela rhBMP-2 *in vivo* e com isso otimizar os procedimentos de regeneração óssea. Outra abordagem para reduzir os efeitos indesejáveis da rhBMP-2 poderia ser a utilização de um agente que potencializasse a atividade osteogênica desta proteína, permitindo a utilização de uma menor quantidade desta proteína *in vivo*.

1.3 ÁCIDO RETINÓICO

O ácido retinóico (AR) deriva do retinaldeído, o qual é derivado da vitamina A. O metabolismo da vitamina A e os diversos efeitos de seus metabólitos são rigorosamente controlados por diferentes enzimas, proteínas ligantes do ácido retinóico e receptores nucleares do ácido retinóico (DUESTER, 2008). O AR controla a diferenciação e o metabolismo celular, atuando como um ligante para duas famílias de receptores nucleares. Os receptores do AR (RARα, RARβ e RARγ) que se ligam à forma abundante do AR conhecida como *all-trans*-AR (ATRA) e os receptores X (RXRα, RXRβ e RXRγ) que se ligam ao isômero 9-cis-AR (9CRA). A ligação do AR aos heterodímeros RAR/RXR promove uma cascata de eventos, que resulta no recrutamento de co-ativadores transcripcionais e no início da transcrição (MARK et al., 2006). Vários trabalhos têm associado o AR à regulação de genes osteogênicos e ao aumento da diferenciação osteogênica de diferentes tipos celulares, incluindo osteoblastos e células-tronco mesenquimais (GAZIT et al., 1993; ZHANG et al., 2010). Além disso, o AR tem sido relacionado com o aumento dos efeitos da rhBMP-2, através do aumento da atividade da fosfatase alcalina (ALP), fator de transcrição RunX2 e da expressão do mRNA da osteopontina (LI et al., 2003; WAN et al., 2006).

Entretanto, as informações relacionadas ao papel do AR na diferenciação osteogênica e à sua capacidade de potencializar os efeitos das BMPs são controversas. Li et al. (2003) observaram que o AR estimulou a diferenciação condrogênica e aumentou os efeitos da BMP-9. Cowan et al. (2005) demonstraram que a associação entre BMP-2 e AR acelerou a formação óssea *in vivo*. Por outro lado, Hoffman et al. (2006) verificaram que a ativação da BMP-4 envolveu a atenuação da sinalização do AR, enquanto Wang et al. (2008) demonstraram que o AR inibiu a diferenciação osteogênica de células estromais da medula óssea de ratos.

1.4 CÉLULAS-TRONCO MESENQUIMAIS

Os mamíferos são compostos por cerca de 220 tipos celulares especializados e todos esses são formados a partir do zigoto. Durante o desenvolvimento embrionário, ocorre a geração de células filhas, através de divisões sucessivas, e a organização

dessas células em três folhetos germinativos. De acordo com a localização no embrião, estes folhetos são classificados em ectoderma, mesoderma e endoderma. O ectoderma constitui a camada mais externa, enquanto o mesoderma representa a camada intermediária, e o endoderma constitui o folheto mais interno. As células destes três folhetos germinativos continuam a se dividir e se diferenciam, ou seja, se especializam gradativamente em função e estrutura (GENESER, 2003).

Em geral, concomitantemente à diferenciação celular, ocorre perda de outras possibilidades de diferenciação (perda da potência), ou seja, durante o desenvolvimento embrionário as células se tornam mais comprometidas com uma linhagem celular e, consequentemente, menos potentes. O comprometimento precede a diferenciação funcional e morfológica, sendo que no momento em que a célula se compromete com uma linhagem celular, ela estabelece seu destino. Tendo em vista que todas as células possuem material genético idêntico, modificações na atividade desse material conduzem ao fenômeno de diferenciação celular. Assim, a função e a morfologia de cada tipo celular ocorrem em função da expressão de certos genes específicos (USA, 2001).

Em relação à potência, as células indiferenciadas podem ser totipotentes (capazes de se diferenciar em todos os tecidos humanos, incluindo placenta e anexos embrionários); pluripotentes (capazes de se diferenciar em quase todos os tecidos, mesoderma, ectoderma e endoderma, com exceção da placenta e anexos embrionários); multipotentes (capazes de se diferenciar em múltiplos tipos celulares, restritos a uma camada germinativa ou sublinhagens específicas); e oligopotentes (aptas a se diferenciar em poucos tipos celulares). Por sua vez, em relação à origem, as células-tronco podem ser divididas em células-tronco embrionárias (derivadas do tecido embrionário isolado das células germinativas primordiais, massa interna do blastocisto, de embriões com 5-10 semanas); células germinais embrionárias (derivadas da região da prega gonodal, onde se originam as células germinais do adulto); células de carcinoma (encontradas em teratomas e teratocarcinomas); e células-tronco do adulto (células indiferenciadas encontradas em um tecido diferenciado) (USA, 2001).

As células-tronco do adulto têm capacidade de se renovar mantendo-se indiferenciadas e/ou podem se diferenciar em qualquer célula do tecido que as originou. As células-tronco hematopoiéticas, que originam todos os tipos de células sanguíneas, são as células-tronco conhecidas há mais tempo. Entretanto, vários outros tipos de células-tronco do adulto são conhecidos, incluindo epitelial, muscular, neuronal, mesenquimal, dentre outras. Estas células já foram isoladas de vários tecidos, tais como rim, pele, córnea, retina, cérebro, pâncreas, medula óssea, tecido adiposo, polpa dentária e músculo esquelético, dentre outros (WAGNER et al., 2005; ARVIDSON et al., 2011). As células-tronco do adulto podem atuar na renovação de células danificadas, células de tecidos doentes e células senis (HALVORSEN et al., 2000), sendo que a diferenciação das mesmas ocorre em virtude de estímulos hormonais, alterações fisiológicas, desenvolvimento neonatal ou em decorrência da necessidade de reparo tecidual (ZUK et al., 2002; DA SILVA MEIRELLES et al., 2008).

Inicialmente, as células-tronco mesenquimais (MSCs) atraíram atenção na regeneração óssea pelo fato de serem capazes de se diferenciar *in vivo* e *in vitro* em vários tipos celulares, incluindo osteoblastos e condrócitos. Os primeiros relatos da utilização de MSCs envolviam células isoladas da medula óssea. Friedenstein et al. (1971) confirmaram a presença de células-tronco não hematopoiéticas na medula óssea, ao observarem que essas células aderentes, de forma fibroblastóide, e com multiplicação rápida, eram dotadas da habilidade de se transformarem em colônias, que se assemelhavam ao osso e à cartilagem. Todavia, a denominação de MSCs e a observação de que estas células eram multipotentes ocorreu posteriormente.

Até recentemente, a medula óssea era considerada a principal fonte das MSCs. Posteriormente, essas células passaram a ser isoladas de outras fontes. Dentre elas, as células-tronco derivadas do tecido adiposo (ASCs) são consideradas uma opção interessante para aplicações clínicas por serem disponíveis em grandes quantidades, serem facilmente acessadas e isoladas, e apresentarem rápida adesão e proliferação em cultura (ZUK et al., 2001). Somam-se a todas

estas características o fato de que milhares de procedimentos de lipoaspiração são realizados constantemente em todo o mundo, com diferentes volumes de tecido removidos de cada paciente, e grande parte deste material ainda é descartado.

Várias nomenclaturas vinham sendo utilizadas para descrever a população de células isoladas do tecido adiposo e com capacidade de aderirem em superfícies plásticas. Com o intuito de padronizar estas terminologias, a *International Fat Applied Technology Society* adotou o termo "células-tronco derivadas de tecido adiposo" para identificar a população de células multipotentes, isoladas do tecido adiposo, e com capacidade de aderirem em superfícies plásticas (BUNNELL et al., 2008).

Outro ponto de divergência no meio científico vinha ocorrendo em relação à caracterização das células-tronco mesenquimais. Algumas características estavam bem estabelecidas, como a morfologia similar à dos fibroblastos, adesão em plásticos, heterogeneidade morfológica e capacidade de diferenciação em vários tipos celulares, de acordo com as condições de cultivo. Todavia, outras características eram controversas. Neste contexto, a *International Society for Cellular Therapy* se posicionou e estabeleceu como critérios mínimos para definir célula-tronco mesenquimais: a capacidade de aderência em superfícies plásticas, quando mantidas em cultura; a expressão das moléculas de superfície CD105, CD73 e CD90; a não expressão de CD45, CD34, CD14 ou CD11b, CD79a ou CD19 e HLA-DR; e a diferenciação em osteoblastos, condroblastos e adipócitos (DOMINICI et al., 2006).

Recentemente, um aspecto que vem chamando bastante atenção nas MSCs é o efeito parácrino das mesmas. Tal efeito está relacionado à uma modulação nas respostas imune e inflamatória, à uma melhora na cicatrização dos tecidos, e também à prevenção da rejeição alogênica, tanto em modelos animais quanto em humanos. Inclusive, trabalhos vêm mostrando a utilização de MSCs humanas em vários modelos animais, tais como camundongos, ovelhas e cães (LIECHTY et al., 2000; PLOTNIKOV et al., 2007; BONFIELD et al., 2010). Com base nestas informações, as MSCs têm sido clinicamente estudadas como uma nova abordagem para a regeneração tecidual e o

tratamento de doenças inflamatórias e mediadas pelo sistema imune (RYAN et al., 2005; VAN POLL et al., 2008; PAREKKADAN; MILWID, 2010).

Até o momento, o efeito parácrino das MSCs tem sido atribuído a basicamente três mecanismos; a ausência de expressão do complexo principal de histocompatibilidade (MHC) de nível II; a capacidade de prevenir a resposta de células T; e a produção de prostaglandinas e interleucina 10 (RYAN et al., 2005; PAREKKADAN et al., 2007; PAREKKADAN; MILWID, 2010). Desta maneira, tendo em vista os efeitos parácrinos das MSCs, poderia-se propor a utilização das ASCs para a regeneração de defeitos ósseos e para a modulação da resposta inflamatória causada pela rhBMP-2 *in vivo*.

1.5 CARREADORES PARA CÉLULAS E FATORES DE CRESCIMENTO

Com o progresso da engenharia tecidual, o conceito de carreadores vem sofrendo modificações, evoluindo da idéia inicial de que deveriam atuar apenas como uma estrutura biocompatível e inerte, que permitisse a proliferação celular e a migração dos vasos sanguíneos (SOKOLSKY-PAPKOV et al., 2007; GULDBERG et al., 2008). Atualmente, o carreador ideal deve ser capaz de preservar o volume tecidual; fornecer resistência mecânica; apresentar poros, que permitam a entrada e a proliferação de células e dos vasos sanguíneos; ser biocompatível; ser degradado através de processos controlados de dissolução e reabsorção, permitindo a substituição pelo tecido neoformado; possuir características físico-químicas adequadas para adesão, proliferação e diferenciação celular; carrear células e/ou fatores de crescimento; permitir a liberação de substâncias bioativas agregadas; ter um custo acessível; estar prontamente disponível; e ser biodegradável (YOKOTA et al., 2006; CRUZ et al., 2008).

Os arcabouços sintéticos representam componentes importantes nas abordagens da engenharia tecidual, seja como carreadores para fatores de crescimento ou para culturas de células. Além da ausência do potencial imunogênico e da possibilidade de transmissão de doenças infectocontagiosas,

estes materiais apresentam vantagens, tais como o controle da composição química, das características físico-químicas e das propriedades mecânicas. As características físico-químicas são de extrema importância para a regeneração óssea. Dentre elas, as mais relevantes são a composição química, cristalinidade, superfície, porosidade, interconectividade de poros e tamanho de partículas. Tais características estão diretamente relacionadas com biocompatibilidade, resistência mecânica, degradação, reabsorção e migração celular (CRUZ et al., 2006; YUAN et al., 2007; CRUZ et al., 2008).

Em relação à composição química, os carreadores podem ser divididos em metálicos, cerâmicos e poliméricos, sendo os dois últimos mais comumente empregados em engenharia tecidual óssea. Dentre os poliméricos, pode-se citar os ácidos polilático e poliglicólico, o copolímero dos ácidos polilático e poliglicólico, o ácido hialurônico, a quitosana, o colágeno, dentre outros. No grupo das cerâmicas estão hidroxiapatita, fosfato de cálcio bifásico, β-fosfato tricálcico, e vidros bioativos, dentre outros (HABRAKEN et al., 2007). Os arcabouços à base de poli(ácido láctico-co-glicólico) (PLGA) têm sido amplamente utilizados em regeneração óssea e vêm se mostrando interessantes em decorrência da sua biocompatibilidade, porosidade, propriedades mecânicas, habilidade em resistir às pressões causadas pelos tecidos moles, e capacidade de manutenção da estrutura tridimensional, após a implantação nos organismos vivos (HU et al., 2008; BUDYANTO et al., 2009; GE et al., 2009).

Outra opção de arcabouço é o plasma rico em plaquetas (PRP), uma suspensão concentrada de sangue centrifugado. O resultado é uma concentração acentuada de plaquetas em um reduzido volume plasmático (KAUX et al., 2010). Até o momento, tem-se conhecimento de que o PRP contém vários fatores de crescimento, tais como fator de crescimento derivado de plaquetas (PDGF), fator de crescimento transformador-β (TGF-β), fator de crescimento similar à insulina (IGF), fator de crescimento vascular endotelial (VEGF), e fator de crescimento de fibroblasto (FGF). O PRP também contém proteínas, dentre

elas fibrina, fibronectina e vitronectina. Estes fatores de crescimento e proteínas são armazenados nos α-grânulos e são liberados quando as plaquetas são ativadas, desempenhando um papel importante na proliferação, migração e diferenciação celular (LEE et al., 2011b).

Apesar dos resultados controversos, as terapias baseadas em PRP vêm recebendo crescente atenção em cirurgias maxilofaciais, bucais e ortopédicas, com intuito de melhorar a incorporação do enxerto ósseo. Em dermatologia, o PRP vem sendo usado para tratamento de úlceras, e na medicina desportiva, para tratamento de tendinopatias crônicas. Basicamente, essas terapias envolvem a injeção do PRP nos locais de injúria para promover a regeneração tecidual. Uma das propriedades clínicas mais importantes do PRP é o fato deste material ser autógeno e, por isso, não apresentar risco de transmissão de doença infectocontagiosa ou priônica. Além disso, o PRP apresenta uma consistência gelatinosa após sua ativação, a qual facilita sua manipulação e acomodação nos defeitos ósseos. Da mesma maneira, trata-se de um material biocompatível, com fatores de crescimento inerentes à sua composição, de fácil obtenção e baixo custo. Por outro lado, o PRP não apresenta resistência mecânica. Dessa maneira, todas estas características apontam o PRP para ser usado como arcabouço em áreas sem pressão excessiva, tais como lesões císticas, defeitos periodontais e levantamento do seio maxilar.

Desse modo, tanto o PLGA quanto o PRP, poderiam ser empregados para engenharia tecidual óssea como carreadores de células e/ou fatores de crescimento, respeitando o fato do PRP não apresentar resistência mecânica.

Assim sendo, a realização desta tese pode ser justificada pelo elevado número de procedimentos clínicos para aumento de tecido ósseo realizados anualmente nas áreas médica e odontológica, aliado ao fato de que os substitutos ósseos autógenos, homógenos e heterógenos apresentam limitações. Assim, esta pesquisa visou avaliar *in vitro* e *in vivo* os efeitos da rhBMP-2 e das ASCs humanas na osteogênese.

De acordo com os resultados obtidos, esta tese foi elaborada em capítulos, na forma de quatro artigos para publicação.

O **capítulo 1** descreve os efeitos da rhBMP-2 na diferenciação osteogênica *in vitro* de ASCs suplementadas com ascorbato e β-glicerofosfato, e os efeitos da BMP-4 e BMP-7 endógenas nesse processo. Para a realização desta parte do trabalho, foi necessário o estabelecimento de uma cultura primária de osteoblastos humanos para serem utilizados como controle positivo da diferenciação osteogênica. Também se fez necessário o isolamento das ASCs humanas.

Considerando a potencial capacidade do AR na indução osteogênica e a possibilidade de usar essa substância para substituir e/ou potencializar os efeitos da rhBMP-2, o **capítulo 2** descreve os efeitos do AR, rhBMP-2 e associação de ambos na diferenciação osteogênica *in vitro* de ASCs humanas. Estes diferentes tratamentos foram comparados entre si, através da caracterização bioquímica, histológica e molecular dos diferentes grupos celulares, e também comparando-os com osteoblastos humanos de cultura primária e pré-osteoblastos murinos comercialmente disponíveis (MC3T3-E1 subclone 4).

No **capítulo 3** são apresentados os efeitos da administração de ASCs humanas, incorporadas em arcabouços de PLGA, na resposta inflamatória causada por uma baixa dose de rhBMP-2. Para esta parte do trabalho, foi fundamental comprovar se as ASCs eram MSCs, através da citometria de fluxo, avaliando-se determinados marcadores de superfície, e por meio da diferenciação em multilinhagens celulares. De maneira similar, foi fundamental realizar a caracterização físico-química dos arcabouços de PLGA e avaliar a quantidade de rhBMP-2 agregada e liberada, bem como os modelos cinéticos envolvidos na liberação da rhBMP-2.

Por sua vez, o **capítulo 4** avaliou os efeitos *in vivo* das ASCs humanas incorporadas em arcabouços autógenos de PRP, na formação e maturação óssea, em defeitos criados na tíbia de cães, em 6 semanas de pós-operatório.

Estes capítulos são seguidos de uma discussão geral e das considerações finais sobre os trabalhos desenvolvidos.

O ineditismo deste trabalho se comprova em cada um dos capítulos, tendo em vista que não há relatos na literatura de estudos que avaliem os efeitos da BMP-2 exógena associados à expressão endógena da BMP-4 e BMP-7 na diferenciação osteogênica *in vitro* de ASCs, suplementadas com ascorbato e β-glicerofosfato. Da mesma maneira, não se tem conhecimento de trabalhos que comparem os efeitos do AR, rhBMP-2 e associação de ambos na diferenciação osteogênica *in vitro* de ASCs humanas. Também trata-se do primeiro estudo que avalia os efeitos da administração de ASCs humanas, incorporadas em arcabouços de PLGA, na modulação da resposta inflamatória causada por uma baixa dose de rhBMP-2. Este é, ainda um estudo inédito que avalia os efeitos das ASCs humanas, incorporadas em arcabouços de PRP autógeno, na neoformação e maturação óssea, em defeitos criados na tíbia de cães.

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2. OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar *in vitro* e *in vivo* os efeitos da proteína óssea morfogenética recombinante humana tipo 2 (rhBMP-2) e das células-tronco derivadas do tecido adiposo (ASCs) humano na osteogênese.

2.2 OBJETIVOS ESPECÍFICOS

Etapa 1 – Estabelecimento das culturas primárias:

- Estabelecer uma cultura primária de osteoblastos a partir de tecido ósseo humano remanescente da instalação de implantes dentários, para serem utilizados como controle positivo da diferenciação osteogênica;
- Avaliar a viabilidade da cultura de osteoblastos estabelecida;
- Isolar células-tronco derivadas de tecido adiposo (ASCs) humano obtidos de procedimentos de lipoaspiração;
- Avaliar a viabilidade e proliferação das ASCs;

Etapa 2 – Fenotipagem e diferenciação das ASCs:

- Comprovar se as ASCs são células-tronco mesenquimais (MSCs), através da citometria de fluxo avaliando-se determinados marcadores de superfície;
- Induzir a diferenciação das ASCs em adipócitos, por meio da suplementação do meio de cultivo;
- Comprovar a diferenciação das ASCs em adipócitos, através de coloração com Óleo Vermelho O;

Etapa 3 – Caracterização bioquímica, histológica e molecular:

- Induzir a osteodiferenciação das ASCs, utilizando rhBMP-2, ácido retinóico e a combinação de ambos;
- Caracterizar bioquímica-, histologica- e molecularmente as ASCs e os osteoblastos induzidos, comparando-os com osteoblastos humanos, obtidos de cultura primária e com pré-osteoblastos murinos comercialmente disponíveis (MC3T3-E1 subclone 4);

Etapa 4 – Confecção e caracterização dos arcabouços:

- Desenvolver arcabouços à base de poli(ácido láctico-co-glicólico) (PLGA), através da técnica de evaporação do solvente para agregar rhBMP-2 e ASCs;
- Caracterizar a estrutura físico-química dos arcabouços de PLGA, por meio de difração de raios-X (DRX) e microscopia eletrônica de varredura (MEV), determinando a presença da fase cristalina, estrutura de superfície, formato e tamanho de poros;
- Determinar a citotoxicidade e a degradação *in vitro* dos arcabouços de PLGA;

Etapa 5 – Agregação de rhBMP-2 e ASCs aos arcabouços:

- Agregar rhBMP-2 aos arcabouços de PLGA e avaliar a efetividade de agregação e liberação, em diferentes intervalos de tempo;
- Determinar os modelos cinéticos para explicar os mecanismos de liberação da rhBMP-2 dos arcabouços;
- Semear ASCs sobre os arabouços de PLGA e observar a adesão celular, através de MEV;

Etapa 6 – Administração *in vivo* das ASCs para modulação da inflamação:

- Implantar arcabouços de PLGA, arcabouços de PLGA com rhBMP-2 e arcabouços de PLGA com rhBMP-2 e ASCs no tecido muscular de cães;
- Avaliar a quantidade de focos inflamatórios, neovascularização e células gigantes no tecido muscular em 6 semanas de pós-operatório;

Etapa 7 – Implantação de ASCs em arcabouços de PRP no tecido ósseo:

- Confeccionar arcabouços de plasma rico em plaquetas (PRP);
- Agregar ASCs nos arcabouços de PRP;
- Implantar arcabouços de PRP, arcabouços de PRP com ASCs, e tecido ósseo autógeno em defeitos ósseos criados em tíbias de cães;
- Avaliar a quantidade de osso primário, osso secundário e tecido de granulação, em 6 semanas de pós-operatório.

Capítulo 1

Journal of Applied Oral Science

Addition of bone morphogenetic protein type 2 to ascorbate and β-glycerophosphate supplementation did not enhance osteogenic differentiation of human adipose-derived stem cells *in vitro*

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Running title: BMP-2 did not improve osteodifferentiation

ABSTRACT

Bone morphogenetic protein type 2 (BMP-2) is a potent local factor, which promotes bone formation and has been used as osteogenic supplement for mesenchymal stem cells. Objectives: This study evaluated the effect of recombinant BMP-2 as well as

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endogenous BMP-4 and BMP-7 in the osteogenic differentiation of adipose-derived stem cells (ASCs) in medium supplemented with ascorbate and β-glycerophosphate. Material and Methods: Human ASCs were treated with osteogenic medium in the presence (ASCs+OM+BMP-2) or absence (ASCs+OM) of BMP-2. Alkaline phosphatase (ALP) activity was determined and extracellular matrix mineralization was evaluated by Von Kossa staining and calcium quantification. The expressions of BMP-4, BMP-7, Smad1, Smad4, and phosphorylated Smad1/5/8 were analyzed by western blotting. Relative mRNA expressions of Smad1, BMP receptor type II (BMPR-II), osteonectin, and osteocalcin were evaluated by qPCR. Results: ASCs+OM demonstrated the highest expression of BMP-4 and BMP-7 at days 21 and 7, respectively, the highest levels of BMPR-II mRNA expression at day 28, and the highest levels of Smad1 mRNA at days 14 and 28. ASCs+OM+BMP-2 demonstrated the highest levels of Smad1 mRNA expression at days 1, 7, and 21, the highest expression of Smad1 at day 7, the highest expression of Smad4 at day 14, the highest ALP activity at days 14 and 21, and expression of phosphorylated Smad1/5/8 at day 7. ASCs+OM and ASCs+OM+BMP2 showed similar ALP activity at days 7 and 28, similar osteonectin and osteocalcin mRNA expression at all time points, and similar calcium depositions at all time points. Conclusions: Human ASCs expressed endogenous BMP-4 and BMP-7. Moreover, the supplementation of ASCs with BMP-2 did not increase the level of osteogenic markers in initial (ALP activity), intermediate (osteonectin and osteocalcin), or final (calcium deposition) phases suggesting that the exogenous addition of BMP-2 did not improve the *in vitro* osteogenesis process of human ASCs.
Keywords: Stem cells. Cell differentiation. Osteogenesis. Bone morphogenetic protein 2. Bone morphogenetic protein 4. Bone morphogenetic protein 7.

INTRODUCTION

Mesenchymal stem cells (MSCs) have been receiving considerable attention in bone regeneration, both for tissue

engineering or cellular therapy. These cells are capable of self-renewal or differentiation into multilineage cells, including osteoblasts and condroblasts, under appropriate conditions. MSCs have been isolated from different tissues, such as bone marrow, dental pulp, fat, and gingiva[7,18,26,34,36]. Adipose-derived stem cells (ASCs) represent one interesting cellular option for clinical applications since these cells are available in large amount, are easily accessible and isolated, and they demonstrate a fast attachment and proliferation in culture[36].

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor β (TGF-β) super-family and were identified by their ability to induce bone formation at ectopic sites[28]. In particular, BMP-2, BMP-4, and BMP-7 have been associated with bone development[20]. BMP-2 has been used for treatment of bone defects in orthopedic, spine, and maxillofacial surgeries[1,5]. Interestingly, it was observed during fracture repair that an increase of BMP-4 and BMP-7 occurs in response to the presence of BMP-2[27], and it was suggested that BMP-4 and BMP-7 might be able to substitute each other during bone healing, as has been shown in other tissues where these BMPs are co-expressed[10]. Therefore, the co-expression of a panel of BMPs exert strong synergy, meaning that combination of certain BMPs may be more efficacious than single BMP to stimulate osteogenic differentiation, and different BMPs may act at different nodal points of osteogenesis[5,20]. Besides the exogenous BMPs, it is important to consider the possibility and the effects of biologically active endogenous BMPs expressed by human MSCs. Seib, et al.[22] (2009) observed that bone marrow-derived stem cells promoted osteogenesis by endogenous BMP-2, BMP-4, and BMP-6.

Classical signaling pathway for BMPs begins with the binding of BMP to a dimeric complex of transmembrane serine–threonine kinase receptors, the type I and type II receptors. Type I receptors include BMP receptors (BMPRs) -IA, -IB, and activin receptor type 1 (ACTR1), while type II receptors include BMPR-II, ACTR2, and -2b, which are constitutively active kinases. These BMPRs subtypes have different BMP affinities and form receptor complexes prior to or after BMP binding, which is thought to regulate BMP signaling (Smad-dependent versus Smad-

independent). Heteromeric receptor complexes comprising type I and II receptors lead to ligand-induced phosphorylation of type I receptors. Following the activation of this receptor, receptor kinases phosphorylate the transcription factors Smad1, 5, or 8 that subsequently form heteromeric complexes with Smad4 and activate the expression of target genes in the nucleus[21,23,9]. Reports have shown that mitogen-activated protein kinase (MAPK)[12] and phosphatidylinositol 3-kinase (PI-3K)[16,22] activity are critical switches for osteogenic differentiation, indicating that other pathways, than the Smad pathway, regulate the mesenchymal stem cells differentiation after BMP exposure.

The most usual supplementation for *in vitro* osteogenic differentiation includes ascorbate, β-glycerophosphate, and dexamethasone[17,31,36]. Several studies have used BMP-2 instead of dexamethasone for osteogenic induction[14,22,32]. Differently, some reports have demonstrated that *in vitro* and *in vivo* treatments of ASCs with BMP-2 had no consistent effect on osteogenic differentiation[6,35]. Therefore, this study evaluated the effect of a recombinant BMP-2 as well as the endogenous BMP-4 and BMP-7 in the osteogenic differentiation of adipose-derived stem cells (ASCs) in medium supplemented with ascorbate and β-glycerophosphate.

MATERIALS AND METHODS

Reagents

Antibiotics/antifungic was purchased from Cultilab (São Paulo, SP, Brazil). Ascorbate, β-glycerophosphate, BMP-2 (recombinant human BMP-2), eosin, paraformaldehyde, ρ-nitrophenol phosphate (ρNPP), and silver nitrate were obtained from Sigma-Adrich (St. Louis, MO, USA). α-modified Eagle's minimal essential medium (αMEM modified) and Dulbecco's modified Eagle's medium (DMEM) were from Nutricell (São Paulo, SP, Brazil). Fetal bovine serum, SuperScript™ III, and Trizol® were purchased from Invitrogen (Carlsbad, CA, USA). Kit Calcium Assay was from BioAssay Systems (Hayward, CA, USA). Antibodies anti -BMP4 (MAB1049), -BMP7 (MAB4350), -β actin (04-1116), -Smad1 (05-1459), -phosphorylated Smad1/5/8 (AB3848), -Smad4 (04-1033), anti-rabbit, anti-goat and anti-

mouse peroxidase-conjugated antibodies, and Immobilon-P membranes were obtained from Millipore (Danvers, MA, USA). Enhanced chemiluminescence Pierce ECL was from Thermo scientific (Rockford, IL, USA). Step One® detection system and SYBR® Green PCR Master Mix were purchased from Applied Biosystems (Carlsbad, CA, USA). All chemicals were of analytical grade.

Isolation and culture of human adipose-derived stem cells

After approval by the Institutional Human Ethics Committee (No. 194/06 for adipose-derived stem cells and No. 568/10 for human osteoblasts) and with the understanding and written consent of the voluntaries, human lipoaspirate tissues from three health patients (average age 21), with normal body mass indexes, non-smokers, and not taking any medication, were processed to isolate ASCs, as described by Zuk, et al.[36] (2001). ASCs were sub-cultured and used at passage 3, according to groups: *ASC+OM:* Osteogenic medium (OM) (DMEM supplemented with 250μM ascorbate and 10mM β-glycerophosphate); *ASCs+OM+BMP2:* OM with 50ng/mL BMP-2. MC3T3-E1 pre-osteoblasts subclone 4 *(American Type Culture Collection,* Manassas, VA, USA, 2593™) were used as positive control for the determination of alkaline phosphatase activity, the evaluation of matrix mineralization, and for the western blotting assays. MC3T3-E1 cells were cultured according to the recommendations in αMEM modified with 50μg/mL ascorbate and 10mM β-glycerophosphate[33]. Human osteoblasts were used as positive control in qPCR experiments. Human osteoblast explant cultures[13] were obtained from three patients through biopsies of cortical-porous bone tissue from the mandible, using dental implant drills. Human osteoblasts (passage 6) were cultured with OM. Culture media were supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) antibiotic/antifungic.

Determination of alkaline phosphatase activity

MC3T3-E1 and ASCs ($9.4x10^4$ cells/well in 24-well plates) were cultivated for 7, 14, 21, and 28 days. Trizol® was used for total protein extraction, and the concentrations were determined according to Lowry's method[19]. ALP activity was determined by

releasing ρ-nitrophenol (ρNP) from ρ-nitrophenol phosphate (ρNPP). Concisely, 0.5mL of diethanolamine buffer (1.02M, pH 9.8, 0.6mM magnesium chloride) and 0.5mL of ρNPP were incubated for 2min at 37°C. Afterward, 0.1mL of the extracted protein was added and incubated at 37°C. Following 30min, 1mL of 3N NaOH was used to stop the reaction. Absorbances were measured (Infinite M200, TECAN, Salzburg, Grödig, Austria) at 405nm, and ALP activity was calculated from a ρNP standard curve and all values were normalized against total protein concentration.

Evaluation of matrix mineralization

Von Kossa staining determined the presence of phosphate at extracellular matrix. MC3T3-E1 and ASCs ($2x10^4$ cells/well in 96-well plate) were cultivated for 21 days. Cells were fixed for 1h with 3% (v/v) aqueous paraformaldehyde and stained with 1% (w/v) silver nitrate under light exposure for 1h, and counterstained with eosin.

MC3T3-E1 and ASCs ($2x10^4$ cells/well in 96-well plates) were cultivated for 5, 12, 23, and 32 days, for calcium quantification at extracellular matrix. Cells were harvested in 200μL of 0.1N HCl at 4°C for 4h and centrifuged at 10,000 x *g* for 5min. Kit Calcium Assay was used for measuring supernatant total calcium.

Western Blotting

MC3T3-E1 and ASCs ($9.4x10^4$ cells/well in 24 well-plates) were cultivated for 7, 14, 21, and 28 days. Following cell lysis, protein quantification was performed. For western blotting experiments, 5μg of denatured protein were loaded onto an SDS/polyacrylamide gel. Proteins were transferred to Immobilon-P membranes, which were incubated overnight with

anti -Smad1 (1:500), -phosphorylated Smad1/5/8 (1:500), -Smad4 (1:1000), -BMP4 (1:500), -BMP7 (2μg/mL), or -β actin (1:2000) antibodies. After incubation with each corresponding secondary antibody, the enhanced chemiluminescence Pierce ECL substrate was used for detection, according to the manufacturer's protocol.

qPCR

Human osteoblasts and ASCs ($9.4x10^4$ cells/well) were cultured for 1, 7, 14, 21, and 28 days in 24-well plates. T*otal RNA* was *isolated* with *Trizol*® reagent, and cDNA transcriptions were performed using oligo (dT) primers and SuperScript™ III. Step One® detection system and SYBR® Green PCR Master Mix were used for qPCR. The comparative threshold cycle (Ct) method was used in order to quantify changes in Smad1, BMPR-2, Osteonectin, and Osteocalcin gene expression between control (human osteoblasts) and treated ASCs. The fold difference between control and treatments was calculated according to $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (\Delta Ct \text{ control}) - (\Delta Ct \text{ treatment})$ and $\Delta Ct = (Ct \text{ target gene}) - (Ct \text{ internal control, ribosomal 18s})$. BMPR-II mRNA expression. Primer forward 5'-3' TGAAAAGATCAAGAAACGTGTGAAA. Primer reverse 5'-3' GCCCTGTTACTGCCATTATTGTT; Smad1 mRNA expression. Primer forward GGGACTGCCTCATGTCATTTACT. Primer reverse CAGACCTCCTTCTGCTTGGAA; Osteocalcin mRNA relative expression. Primer forward AGGGCAGCGAGGTAGTGAAG. Primer reverse AACTCGTCACAGTCCGGATTG; Osteonectin mRNA expression. Primer forward CGGGTGAAGAAGATCCATGAG. Primer reverse CTGCCAGTGTACAGGGAAGATG.

Statistical analysis

All experiments were performed in triplicate to confirm the reproducibility of the results. Results of ALP activity and calcium quantification in all groups were compared through non-parametric one-way analysis of variance (ANOVA) followed by Student Newman–Keuls (SNK) post hoc test. Results of mRNA relative expression in all groups were compared through Student's *t* test. Statistical analyses were performed comparing all treatments at each time point separately using GraphPad Prism 4 software (*Graph Pad Software Inc*, San Diego, CA, USA) at 95% confidence interval.

RESULTS

BMP-2 improved alkaline phosphatase activity

ASCs+OM and ASC+OM+BMP2 demonstrated similar ALP activities, and MC3T3-E1 cells (positive control) showed the highest activity (ρ=0.0004) at day 7. ASCs+OM+BMP2 up-regulated ALP activity compared to ACS+OM and MC3T3-E1 at day 14 (ρ=0.00379). At day 21, ASCs+OM+BMP2 and MC3T3-E1 demonstrated the highest activities (ρ=0.0087). There was no statistically significant difference among groups at day 28 (ρ>0.05) (Figure 1).

BMP-2 did not improve calcium deposition at extracellular matrix

Von Kossa staining demonstrated that all groups presented phosphate at extracellular matrix at day 21 (Figure 2A-C).

At day 5, calcium deposition was not observed at extracellular matrix. At day 12, MC3T3-E1 demonstrated the highest calcium amount (ρ<0.0001), ASCs+OM and ASCs+OM+BMP2 showed similar results. There was no statistically significant difference among groups (ρ>0.05) at days 23 and 32 (Figure 2D).

Expression of osteoblast-related proteins

ASCs+OM+BMP2 expressed more Smad1 when compared to MC3T3-E1 and ASCs+OM at day 7. At days 14 and 21, all groups demonstrated similar Smad1 expressions. ASCs+OM demonstrated the lowest expression at day 28 (Figure 3).

Only MC3T3-E1 expressed phosphorylated Smad1/5/8 at day 7. All groups demonstrated similar expression of these proteins at day 14. At day 21, ASCs+OM+BMP2 expressed more than ASCs+OM, while MC3T3-E1 stopped these expressions. All groups had no expression of these phosphorylated proteins at day 28 (Figure 3).

ASCs+OM showed the highest Smad4 expression at day 7. At day 14, MC3T3-E1 demonstrated the highest expression followed by ASCs+OM+BMP2 and then by ASCs+OM. MC3T3-E1 demonstrated the highest level of Smad4 at day 21, followed by ASCs+OM and then by ASCs+OM+BMP2. At day 28, MC3T3-E1 showed the highest expression, while ASCs+OM and ASCs+OM+BMP2 demonstrated similar levels of Smad4 (Figure 3).

All groups had no expression of BMP-4 at day 7. At day 14, ASCs+OM and ASCs+OM+BMP2 demonstrated similar expressions, while MC3T3-E1 had no expression. ASCs+OM demonstrated the highest BMP-4 expression followed by ASCs+OM+BMP2 and then MC3T3-E1 at day 21. Figure 3 did not show differences between ASCs+OM and ASCs+OM+BMP2 groups at day 28, while MC3T3-E1 demonstrated a weak expression.
ASCs+OM+BMP2 expressed the lowest BMP-7 levels at day 7. The expressions were similar for all groups at day 14. At days 21, ASCs+OM+BMP2 showed the highest expression of this protein. ASCs+OM BMP-7 expression was smaller than other groups at day 28 (Figure 3).

Relative expression of osteoblast-related mRNA
Figure 4A shows that BMPR-II relative mRNA expressions were similar for ASCs+OM and ASCs+OM+BMP2 ($\rho>0.05$) at days 1, 7, 14, and 21. At day 28, ASCs+OM demonstrated the highest expression ($\rho=0.0118$).
ASCs+OM+BMP2 showed the highest levels of Smad1 relative mRNA expression at days 1 ($\rho<0.0001$), 7 ($\rho=0.0002$), and 21 ($\rho=0.0008$). ASCs+OM demonstrated the highest expressions at days 14 ($\rho=0.0014$) and 28 ($\rho=0.0003$) (Figure 4B).
Osteocalcin and Osteonectin relative mRNA expressions were similar for all groups at all time points ($\rho>0.05$) (Figure 4C and 4D).

DISCUSSION

BMP-2 is considered a potent local factor that promotes bone formation[1] and has been used as osteogenic supplementation[14,22,32]. This report evaluated the effects of BMP-2 associated with ascorbate and β-glycerophosphate to induce osteogenic differentiation of human ASCs during the entire *in vitro* osteogenic process (day 1 to 32), and the effects of endogenous BMP-4 and BMP-7 in this process. Herein, efforts were made to study osteogenic markers, such as ALP activity, Smads, BMPR-II, osteonectin, osteocalcin, and calcium deposition, as discussed below.

An enzymatic hydrolysis activity of ALP is necessary to initiate biological mineralization by hydrolyzing β-glycerophosphate, which serves as an additional source of phosphate ions[25]. Some proteins are involved in extracellular matrix deposition and mineralization during bone formation, such as osteonectin and osteocalcin. Osteonectin is an intermediate phase glycoprotein that binds calcium and type I collagen with high affinity. Osteocalcin is produced exclusively in bone, dentin, and cementum and it represents approximately 20% of non-collagenous proteins[11]. Finally, a functional assay of terminally differentiated osteoblasts is the extracellular matrix mineralization, which can be evaluated by the calcium quantification.

In general, we observed that the supplementation with BMP-2 did not increase the levels of osteogenic markers in initial, intermediate, or final phases of human ASCs differentiation. Our results demonstrated that these cells expressed endogenous BMP-4 and BMP-7, and we were encouraged to suggest that these endogenous BMPs were biologically active due to the observed induction of ALP activity, up-regulation of osteogenic genes (osteocalcin and osteonectin), and increase of calcium deposition in cells treated without BMP-2. Conversely, Seib, et al.[22] (2009) observed that human bone marrow-derived mesenchymal cells were able to express endogenous BMP-4, but not BMP-7. These observed differences may be attributed to the different cell lines used.

Herein, the peak of ALP activity, which indicates the initial phase of extracellular matrix mineralization, occurred at day 7. At that stage, ASCs treated in the presence or absence of BMP-2 demonstrated similar results, suggesting that BMP-2 supplementation did not improve the initial phase of osteogenic differentiation, in accordance with Song, et al.[24] (2007) (human bone marrow-derived stem cells) and differing from Wang, et al.[30] (2010) (rat bone marrow stromal cells). It is important to appreciate that explanations made with BMPs in human cells can be extrapolated to the human system with more precision[22]. The presence of BMP-2 in medium supplementation did not up-regulate the BMPR-II mRNA expression, similarly to that found by Wang, et al.[30] (2010). We also observed similar expression of osteocalcin and osteonectin mRNA relative expression for ASCs

treated with or without BMP-2. Zuk, et al.[35] (2011) demonstrated that induction of ASCs with OM containing BMP-2 for 7 days significantly augmented expression of osteopontin and osteocalcin, and decreased ALP and osteonectin gene expressions. However, when the exposure time to BMP-2 was increased to either 21 or 28 days (the terminal differentiation stages of ASCs) any changes to gene expression attributable to BMP-2 was lost. Wan, et al.[29] (2006) observed similar osteonectin expression in mouse ASCs cultures using control medium and osteogenic medium with BMP-2. Complementary, our results demonstrated that ASCs treated with or without exogenous BMP-2 showed similar calcium deposition at extracellular matrix, as found by Song, et al.[24] (2007) and Zuk, et al.[35] (2011). Conversely, Beloti and Rosa[3] (2005) demonstrated that osteoblast differentiation of human bone marrow cells did not occur only in the presence of ascorbate and β-glycerophosphate. Khanna-Jain, et al.[15] (2010) observed that addition of BMP-2 or BMP-6 to dexamethasone, ascorbic acid, and β-glycerophosphate did not enhance osteogenic differentiation of human periodontal ligament cells, since they observed that neither of the BMPs induced *in vitro* mineralization, despite the increase of ALP activity.

BMP-2 supplementation led to the highest levels of Smad1 relative mRNA expression at days 1, 7, and 21, the highest expression of Smad1 at day 7, and the highest expression of Smad4 at day 14. Chang, et al.[4] (2009) demonstrated that the treatment of osteoblast-like cells with BMP-4 increases Smad1/5 phosphorylation. Aoki, et al.[2] (2001) also observed that the BMP-4 activation in C2C12 mouse muscle myoblast cell line occurs via Smad pathway. We did not observe association between BMP-4 expression and Smads1/5/8 phosphorylation. Supporting our results, Seib, et al.[22] (2009) demonstrated that osteogenesis by endogenous BMP-4 and BMP-6 was independent of Smad activation and dependent on phosphatidylinositol 3-kinase (PI-3K) activation in bone marrow-derived stem cells. Therefore, BMP-4 activation has been related to pathways dependent or independent of Smads. These different findings may be due to the different lineage cell type, species, and culture conditions. Zuk, et al.[35] (2011) did not observe any significant changes in

Smad1/5/8 phosphorylation levels upon BMP-2 induction. Additionally, these authors observed that the removal of dexamethasone from the BMP-2 did not significantly change the mineralization capacity of ASCs from that measured when samples were induced with BMP-2 and OM containing dexamethasone. Such a finding would suggest that the lack of effect of BMP-2 was not due to any interference by the presence of dexamethasone.

Despite the presence of BMP-2 in the osteogenic supplementation led to the highest expression of Smads, this protein did not increase the ALP activity at the peak of this enzyme, the expression of osteonectin and osteocalcin mRNA, or the calcium deposition at extracellular matrix, an indicator of osteoblast final maturation. Conversely, Dragoo, et al.[8] (2003) observed that ASCs cultured with BMP-2 demonstrated more ALP and matrix calcification than the positive osteoblast control. Considering that both treatments (ASCs+OM and ASCs+OM+BMP2) led to similar effects in osteogenic differentiation, the effects of exogenous BMP-2 could be counterbalanced by the highest expression of endogenous BMP-4 and BMP-7. Herein, probably endogenous BMP-4 and BMP-7 pathways were independent of Smad activation, corroborating Seib, et al.[22] (2009). There are a number of possible explanations for the reasons that ASCs had demonstrated similar osteogenic differentiation in the presence or absence of BMP-2, including the maturation of osteogenic progenitors during culture, emergence of a dominant cell type or spontaneous differentiation of MSCs into osteoblasts[22,24]. Although a combination of these factors could be responsible for the results herein, endogenous BMPs production could be a critical factor in any of these scenarios.

CONCLUSIONS

We conclude that human ASCs expressed endogenous BMP-4 and BMP-7. Moreover, the supplementation of ASCs with BMP-2 did not increase the level of osteogenic markers in initial (ALP activity), intermediate (osteonectin and osteocalcin), or final (calcium deposition) phases suggesting that the exogenous

addition of BMP-2 did not improve the *in vitro* osteogenesis process of human ASCs.

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FIGURES

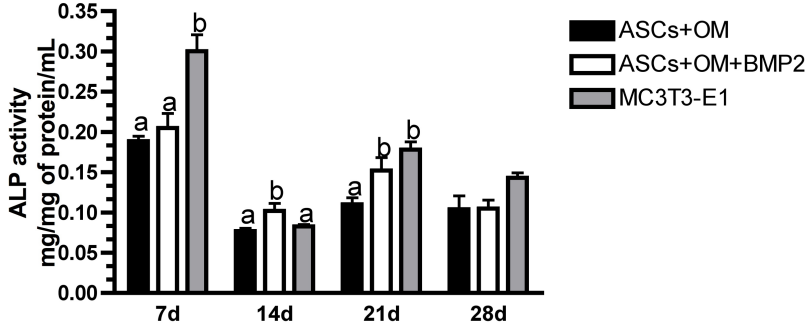

Figure 1

Figure 1: Alkaline phosphatase (ALP) activity. Enzymatic activity at days 7, 14, 21, and 28. Different letters refer to statistical significant differences (ANOVA/SNK, $\rho<0.05$).

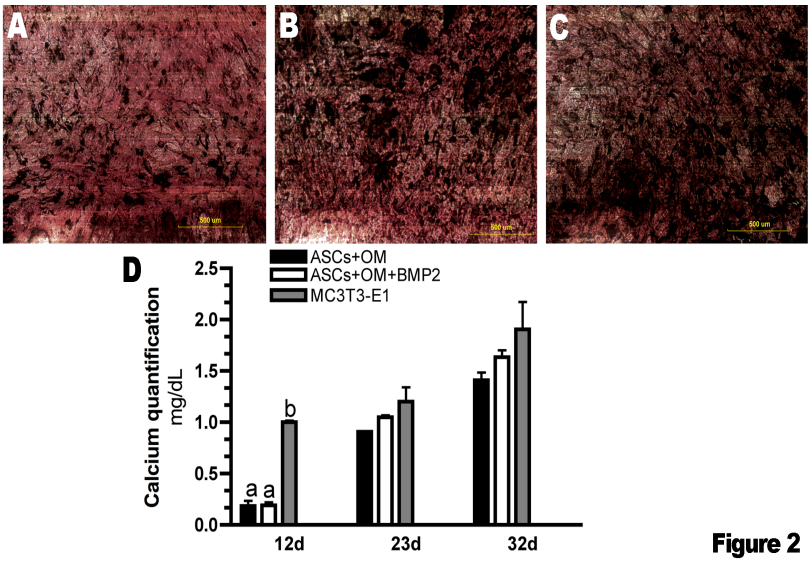

Figure 2

Figure 2: Extracellular matrix mineralization. A) Von Kossa staining. ASCs with osteogenic medium (OM). Bar=500µm; B) ASCs with OM supplemented with BMP-2; C) MC3T3-E1 cells (positive control); D) Calcium quantification at days 12, 23, and

32. Different letters refer to statistical significant differences (ANOVA/SNK, $\rho<0.05$).

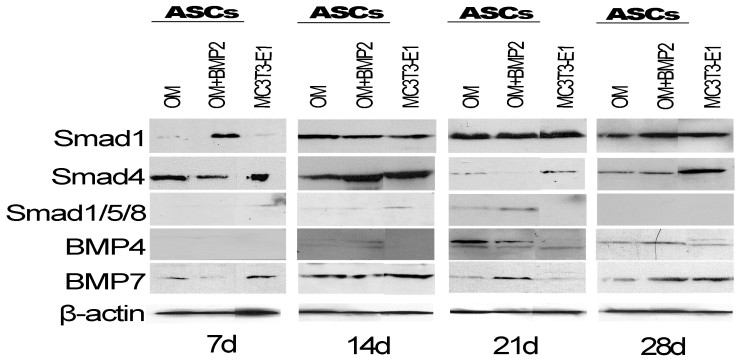

Figure 3

Figure 3: Expression of osteoblast related proteins. Protein expression evaluated by western blotting, at days 7, 14, 21, and 28.

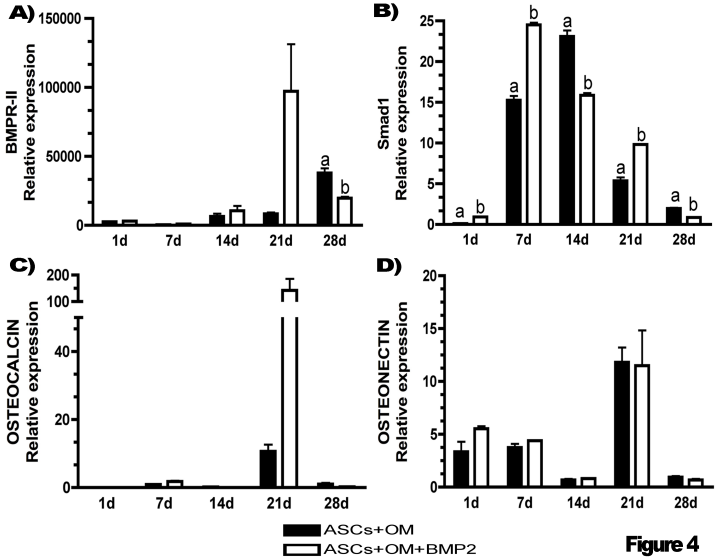

Figure 4: mRNA relative expression. A) BMPR-II mRNA expression; B) Smad1 mRNA expression; C) Osteocalcin mRNA relative expression; D) Osteonectin mRNA expression. Different letters refer to statistical significant differences (T test, $\rho<0.05$).

Capítulo 2

Tissue and Cell

Retinoic acid and bone morphogenetic protein-2 association improves osteogenic differentiation of human adipose-derived stem cells in vitro

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Running title: Retinoic acid+rhBMP-2 improves osteodifferentiation.

ABSTRACT

Considering the potential capacity of retinoic acid (RA) on osteogenic induction and the possibility of using this compound to

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substitute/ potentiate recombinant human bone morphogenetic protein type 2 (rhBMP-2) effects, this study compared the effect of RA, rhBMP-2, and rhBMP-2+RA on osteodifferentiation of human adipose-derived stem cells (ASCs) during entire *in vitro* osteogenic process. ASCs treated with osteogenic medium (OM), rhBMP-2, RA, or rhBMP-2+RA were assessed for inducing osteogenic differentiation through the evaluation of osteogenic markers, such as alkaline phosphatase (ALP), calcium, Smads, BMPs, BMP-2 receptor type II, osteocalcin, and osteonectin. rhBMP-2 led to a high Smad1 and BMP-7 expression (day 7), promoted the highest BMPR-II and osteocalcin mRNA relative expressions (day 21). RA exhibited the highest ALP activity (all time points), the highest BMPR-II mRNA expression (day 14), the highest Smad1 mRNA expression (day 21), and the highest osteonectin relative mRNA expressions (days 14, 21, and 28). rhBMP-2+RA promoted the highest levels of calcium deposition at extracellular matrix (days 12 and 32), a considerable Smad4 (days 21 and 28), a similar expression of phosphorylated Smad1/5/8 to MC3T3-E1 (all time points), the highest Smad1, osteocalcin, and osteonectin mRNA relative expressions (days 14, 7, and 7, respectively). Since rhBMP-2+RA positively acts in most osteogenic markers, especially calcium deposition, a matrix mineralization indicator characteristic of the final stage of progenitor cell development into osteogenic lineage, we were led to conclude that this association was more effective than rhBMP-2 or RA alone on osteodifferentiation of human ASCs.

Keywords: stem cells; cell differentiation; osteogenesis, retinoic acid, bone morphogenetic protein 2.

1. INTRODUCTION

Autogenic and allogenic bone grafting has been clinically employed in dental and maxillofacial surgery, especially in large bone defects, which have a limited regeneration potential. Concerns associated with autografts include limited anatomic bone sources and patient morbidity from additional surgery (Younger and Chapman, 1989). Major drawbacks regarding

allogenic grafting are risk of diseases transmission, host immunological reactions, and poor osteogenic capacity of the transplanted bone (Sogal and Tofe, 1999).

Implantation of synthetic biomaterial alone or in association with growth factors, transplantation of osteogenic cells, and gene therapy have been the main strategies applied in bone regeneration studies (Franceschi, 2005; Lee et al., 2010; Yuan et al., 2009). Recombinant human bone morphogenetic protein type 2 (rhBMP-2) is a potent osteogenic growth factor (Urist, 1965), which has been used for osteogenic differentiation of mesenchymal stem cells and treatment of animal and human bone defects in orthopedic, spine, and maxillofacial surgeries (Alonso et al., 2010; Bishop and Einhorn, 2007; Cancedda et al., 2007; Seib et al., 2009; Zheng et al., 2006). Additionally, United States Food and Drug Administration has approved the clinical use of rhBMP-2 since 2002, including sinus elevation and localized alveolar ride augmentation in 2006. Despite its promising activity on bone regeneration, rhBMP-2 is expensive, has a short half-life in vivo, requires a high therapeutic dose, and enhances patient's initial inflammatory response (Lee et al., 2011; Shah et al., 2008; Vaidya et al., 2007). Therefore, studies on novel osteogenic inductors with similar osteogenic capacity to rhBMP-2 are necessary to collaborate on development of bone regeneration therapy.

Retinoic acid (RA) has been reported to be involved in up-regulation of osteogenic genes, increasing bone formation of different cell types including osteoblasts and mesenchymal stem cells (Gazit et al., 1993; Zhang et al., 2010). Furthermore, RA has been related to enhance rhBMP-2 effects by increasing alkaline phosphatase (ALP) activity, RunX2 transcription factor and osteopontin mRNA expression (Li et al., 2003; Wan et al., 2006). BMP-2 classical signaling pathway begins with the binding of BMP-2 to the type II receptor (BMPR-II). Following transphosphorylation by BMPR-II, the type I receptors (BMPR-I) phosphorylate receptor-regulated Smads (R-Smads). Afterward, two phosphorylated R-Smads (Smad1, Smad5, or Smad8) form a heterodimer with the common Smad4 that translocates into the nucleus and activates the expression of genes involved in

osteodifferentiation (Qing et al., 2000; Song et al., 2009; Wang et al., 2008).
Since adipose-derived stem cells (ASCs) are easily isolated, cultured, and exhibit great plasticity, these cells represent an interesting alternative for osteogenic studies, besides their potential for therapeutic applications (Zuk et al., 2001). Considering the potential capacity of RA on osteogenic induction and the possibility of using this compound to substitute/ potentiate the rhBMP-2 action, this study compared the effect of RA, rhBMP-2, and rhBMP-2+RA on osteodifferentiation of human adipose-derived stem cells (ASCs) during entire (32 days) *in vitro* osteogenic process.

2. MATERIALS AND METHODS

2.1 ASCs isolation and cell culture conditions

Approval by the Human Ethics Committee of Federal University of Santa Catarina and written consent of the voluntaries were obtained before commencement of this study (No. 194/06 for adipose-derived stem cells and No. 568/10 for human osteoblasts). Human lipoaspirate tissues were processed to isolate ASCs, as described by Zuk et al. (2001). Briefly, lipoaspirate tissues were washed with PBS and digested with 0.075% collagenase type I (*Sigma Aldrich*, St Louis, MO, USA) at 37 ^{o}C for 30 min. Enzyme activity was neutralized with Dulbecco's modified Eagle medium (DMEM, Cultilab, Campinas, SP, Brazil), containing fetal bovine serum (FBS, Gibco, São Paulo, SP, Brazil) and centrifuged at 1200 x g for 10 min. The pellet was resuspended in 160 mM NH_4Cl (Sigma Aldrich) for 10 min to lyse the red blood cells. Another centrifugation was performed, the tissue was filtered to remove cellular debris and incubated with DMEM (Cultilab), 10 % FBS (Gibco), and 1 % penicillin-streptomycin-amphotericin (PSA, Gibco) at 37 ^{o}C and 5% CO_2. Afterward, the plates were washed with PBS to remove residual non-adherent red blood cells. ASCs were maintained at sub confluent levels and used at passage 3 for all experiments, according to the groups: OM- ASCs cultured with osteogenic medium (*OM*, DMEM, 10% FBS, 1% PSA, 250 µM ascorbate, 10 mM β-glycerophosphate); rhBMP-2: ASCs with OM and 50 ng/ml rhBMP-2; RA: ASCs with OM and 2.5 µM all-trans retinoic acid

(RA); rhBMP-2+RA: ASCs with OM, 50 ng/ml rhBMP-2, and 2.5μM RA (Wan et al., 2006). MC3T3-E1 pre-osteoblasts subclone 4 (American Type Culture Collection, Manassas, VA, USA) or human osteoblasts were used as positive control. MC3T3-E1 cells were cultured according to the recommendations in αMEM modified (Nutricell, Campinas, SP, Brazil) with 50 μM ascorbate and 10 mM β-glycerophosphate. Human osteoblast explant cultures were obtained through biopsies of cortical-porous bone tissue from the mandible, using dental implant drills. The bone fragments were rinsed in PBS, cut into small pieces (1-2 mm in diameter), incubated with DMEM, 10% FBS, 1% PSA, and left undisturbed for 4 days before the first medium change. The medium was thereafter changed once every week. Cells were detached with trypsin when primary cultures were confluent. For qPCR experiment, human osteoblasts (passage 3) were cultured with OM. All osteogenic supplementation were purchased from Sigma Aldrich.

2.2 Alkaline phosphatase activity

ASCs and MC3T3-E1 (9.4×10^4 cells/well) were cultivated in 24-well plates for 7, 14, 21, and 28 days. Total protein was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) and quantified, according to Lowry's method (Lowry et al., 1951). ALP activity was determined by releasing ρ-nitrophenol (ρNP) from ρ-nitrophenol phosphate (ρNPP) as described by Ogston et al. (2002). Briefly, 0.1ml of each protein extract was added to a mixture of 0.5 ml of ρNPP (Sigma-Aldrich) and 0.5ml of diethanolamine buffer, and maintained at 37 ^{o}C for 30 min. Subsequently, the reactions were stopped by addition of 3 N NaOH and absorbances were measured at 405 nm (TECAN, Salzburg, Grödig, Austria). ALP activity was calculated from a ρNP standard curve and all values were normalized against total protein concentration.

2.3 Evaluation of matrix mineralization

Von Kossa staining was performed to evaluate the presence of phosphate at extracellular matrix, which is used as an indicator of mineralization. ASCs and MC3T3-E1 (2×10^4 cells/well) were cultivated in 96-well plate for 21 days. Cells were fixed with 4%

(v/v) paraformaldehyde, stained with 1% (w/v) nitrate silver (Sigma Aldrich) under light exposure, and counterstained with eosin (Sigma Aldrich).
In order to confirm and quantify the mineralization at extracellular matrix, calcium quantification was performed. ASCs and MC3T3-E1 (2 x 10^4 cells/well) were cultivated in 96-well plates for 5, 12, 23, and 32 days. Cells were harvested in 0.1 N HCl (4 h at 4 ^oC) and centrifuged (10 000 x *g*, 5 min). Total calcium was measured using a Kit Calcium Assay (*BioAssay Systems*, Hayward, CA, USA).

2.4 Western Blotting
Samples (5 µg of protein) were separated electrophoretically on an SDS/polyacrylamide gel and transferred to Immobilon-P membranes (Millipore, Danvers, MA, USA). After blocking, membranes were incubated overnight with either anti- BMP7 (MAB4350, 2 µg/ml), or -BMP4 (MAB1049, 1:500), or -Smad1 (05-1459, 1:500), or -Smad4 (04-1033, 1:1000), or -Smad1/5/8 (AB3848, 1:500), or -β actin (04-1116, 1:2000) antibodies. After incubation with each corresponding secondary antibody, the Enhanced chemiluminescence *Pierce ECL* (Thermo scientific, Rockford, IL, USA) substrate was used for detection, according to the Manufacturer's protocol. Antibodies were purchased from Millipore.

2.5 qPCR
ASCs and human osteoblasts (9.4 x 10^4 cells/well) were cultured in 24-well plates for 1, 7, 14, 21, and 28 days. Total RNA was isolated using Trizol (Invitrogen), according to the manufacture's instructions. Samples were treated with DNAse (Invitrogen) according to the manufacture's instructions. RNA concentration was estimated by NanoVue (GE Healthcare, Little Chalfont, Buckingmshire, UK). Only samples with an optical density at 260 nm/280 nm greater than 1.8 were employed. Afterward, total RNA (1 µg) was transcribed into cDNA using oligo (dT) primers and SuperScript (Invitrogen) according to the manufacturer's instructions. PCR amplifications were performed in a 20 µl reaction mix containing 10 ng cDNA, 50 nM primers, and 10 µl Power SYBR1 Green PCR Master Mix (Applied Biosystems). The comparative threshold cycle (Ct) method was used to quantify

changes in gene expression between control (human osteoblasts) and treated ASCs. The fold difference between control and treatments was calculated according to $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct$ = (ΔCt control) - (ΔCt treatment) and ΔCt = (Ct target gene) - (Ct internal control, ribosomal 18s). Primer sequences are listed in Table 1.

2.6 Evaluation of adipogenic differentiation

ASCs (9.4 x 10^4 cells/well) were submitted to adipogenic induction (DMEM, 10% FBS, 1% PSA, 10^{-7} M dexamethasone, 2.5 µg/ml insulin, 5 µM indometacin, and 5 µM rosiglitazone) for 14 and 21 days in order to evaluate their multipotentiality.

The intracellular lipid deposits, characteristic of adipocytes, were identified by Oil Red O staining (Zuk et al., 2001). Briefly, cells were fixed with 4% (v/v) paraformaldehyde and stained with three volumes of 3.75% Oil Red O (Sigma Aldrich) in isopropanol plus two volumes of distilled water for 5 min. This analysis was also performed with OM, rhBMP-2, RA, rhBMP-2+RA, and MC3T3-E1 groups to observe their capacity to differentiate into adipocytes under osteogenic stimulus.

2.7 Statistical analysis

All experiments were performed in triplicate to confirm the reproducibility of the results. Statistical evaluation was performed with Graph Pad Software Inc. (San Diego, CA, USA). Results of ALP activity, protein quantification, calcium quantification, and mRNA relative expression in all groups were compared through non-parametric one-way analysis of variance (ANOVA) followed by Student Newman–Keuls (SNK) post hoc test. Differences between datasets with $\rho < 0.05$ were regarded as statistically level. Statistical analyses were performed comparing all treatments at each time point separately.

3. RESULTS

3.1 RA increased ALP activity

The highest ALP activity was observed at day 7 for all groups including the mock-treated (ASCs treated with OM) and the positive control (MC3T3-E1). RA promoted the highest ALP

activity at all time points (7, 14, 21, and 28d) ($\rho < 0.001$) in comparison to rhBMP-2 and rhBMP-2+RA (Fig. 1A).

3.2 rhBMP-2+RA increased the calcium deposition

Von Kossa staining demonstrated extracellular matrix mineralization at day 21 (Fig. 1B1-B5) for all groups. Calcium quantification data are presented in Figure 1C. Calcium deposition was not observed in extracellular matrix mineralization at day 5. At day 12, MC3T3-E1 showed the highest calcium amount ($\rho < 0.001$). At day 23, OM, rhBMP-2, and MC3T3-E1 demonstrated the highest calcium levels ($\rho < 0.001$), while rhBMP-2+RA exhibited the highest mineralization at day 32 ($\rho < 0.01$).

3.3 Expression of osteoblast-related proteins

Figure 2 shows that rhBMP-2 expressed more Smad1 compared to other groups at day 7. At other time points, the Smad1 expressions were similar for all groups, except for MC3T3-E1, which demonstrated the lowest level at day 21.

All groups demonstrated comparable Smad4 expression at days 7 and 14. At later time points (21 and 28d), rhBMP-2+RA showed higher Smad4 expression levels than rhBMP-2 and RA, and similar levels to the positive control (MC3T3-E1).

RA, rhBMP-2+RA, and MC3T3-E1 were the only groups that expressed phosphorylated Smad1/5/8 at days 7 and 14. At day 21, rhBMP-2+RA and MC3T3-E1 stopped this expression. All groups had no expression of these phosphorylated Smads at day 28.

Analyzing BMP-4 expression, all groups expressed this protein from day 14 on, except for MC3T3-E1, which had BMP-4 expression only at day 28. Furthermore, rhBMP-2 and RA, alone or associated, demonstrated similar BMP-4 expressions. rhBMP-2 expressed more BMP-7 compared to RA and rhBMP-2+RA at day 7, while OM and MC3T3-E1 showed the lowest levels. At days 14, 21, and 28, rhBMP-2, RA, and rhBMP-2+RA exhibited equivalent BMP-7 expressions.

3.4 Relative expression of osteoblast-related mRNA

At day 1, rhBMP-2 and rhBMP-2+RA exhibited the highest levels of BMPR-II mRNA relative expression ($\rho = 0.007$), while at day 7,

rhBMP-2+RA showed the highest expression ($\rho = 0.015$). Maximal levels of BMPR-II mRNA relative expression were observed from days 14 to 28. RA led to the highest BMPR-II mRNA relative expression at day 14 ($\rho = 0.004$), rhBMP-2 at day 21 ($\rho = 0.005$), and OM at day 28 ($\rho = 0.0001$) (Fig. 3A).
Smad1 mRNA expression was highest for rhBMP-2 at day 1 ($\rho = 0.018$). At day 7, the groups exhibited similar expression, excepting OM, for which the lowest Smad 1 mRNA expression level was quantified ($\rho = 0.002$). rhBMP-2+RA showed the highest expression at day 14 ($\rho < 0.0001$) and RA exhibited the highest level at day 21 ($\rho < 0.0001$). All groups had a similar behavior at day 28 ($\rho = 0.153$) (Fig. 3B).
At day 1, all groups exhibited irrelevant osteocalcin mRNA relative expression. At day 7, rhBMP-2+RA showed the highest osteocalcin mRNA expression ($\rho < 0.0001$), while RA demonstrated the highest expression at day 14 ($\rho = 0.002$). At day 21, rhBMP-2 achieved the highest osteocalcin mRNA level ($\rho = 0.001$). At day 28, there was no difference among all groups ($\rho = 0.345$) (Fig. 3C).
The osteonectin mRNA relative expression was higher for RA followed by rhBMP-2 ($\rho = 0.041$) at day 1. At day 7, this expression was highest in rhBMP-2+RA ($\rho = 0.0008$). At days 14 ($\rho = 0.001$) and 28 ($\rho = 0.044$), RA demonstrated the highest expression (Fig. 3D). Although no statistically significant differences were observed among OM, rhBMP-2, and RA, RA resulted in a higher osteonectin expression.

3.5 ASCs differentiated into adipocytes

ASCs showed negative staining for lipid-filled intracellular vacuoles with Oil red O after 21-day culture, confirming that the obtained cells were not adipocytes, and did not differentiate into adipocytes under osteogenic stimulus (Fig. 4A-E).
After 14 days of adipogenic induction, the presence of lipid-filled vacuoles (Fig. 4F) confirmed the ASCs capability to differentiate into adipocytes. At day 21, the number and size of the vacuoles increased (Fig. 4G).

4. DISCUSSION

In this study, attempts were made to compare the RA, rhBMP-2, and rhBMP-2+RA activities on *in vitro* osteogenic differentiation of human adipose-derived stem cells (ASCs).

There is previous conflicting information concerning the role of RA on osteogenic differentiation and the RA capacity to potentiate BMP osteogenic effects. For instance, Li et al. (2003) observed that RA stimulates chondrocyte differentiation and enhances BMP effects. Cowan et al. (2005) demonstrated that the association BMP-2+RA accelerates *in vivo* bone formation. Conversely, Hoffman et al. (2006) verified that BMP action involves attenuation of retinoid signaling and Wang et al. (2008) demonstrated that RA inhibits osteogenic differentiation of rat bone marrow stromal cells. However, to the best of our knowledge, this is the first study that compares the effects of RA, rhBMP-2, and the association of RA and rhBMP-2 in osteogenesis process from day 1 to 32. Herein, the osteodifferentiation process was characterized by osteogenic markers, such as ALP activity, calcium at extracellular matrix, Smads, BMPs, BMP-2 receptor, osteocalcin, and osteonectin, as discussed bellow.

ALP is an ectoenzyme tissue non-specific mostly known for its crucial role in active bone mineralization (Togari et al., 1993). In this study, the initial phase of extracellular matrix mineralization, characterized by the peak of ALP activity, occurred at day 7, which is an early activity, according to other studies (Beck et al., 2000; Luu et al., 2007; Ogston et al., 2002). When comparing the ALP activity among all groups, RA exhibited higher activity than rhBMP-2 and rhBMP-2+RA at all time points, and higher than MC3T3-E1 cells (positive control) at most of time points. Skillington et al. (2002) also demonstrated that RA stimulates the ALP activity. Conversely, Ogston et al. (2002) and Takahashi et al. (2008) showed a reduction on ALP activity by RA. As suggested by these authors, RA might have differential effects on ALP expression, which are species and cell-specific, depending on the level of constitutive expression. There are some reports describing the potentiation between RA and rhBMP-2 on ALP activity (Skillington et al., 2002; Wan et al., 2006; Zhang et al., 2009). Herein this potentiation was clearly observed for other osteogenic markers, such as calcium (12 and 32d), Smad4 (21

and 28d), Smad1 mRNA relative expression (14d), osteocalcin mRNA (7d), and osteonectin mRNA (7d), but not for ALP activity. Osteocalcin and osteonectin are important noncollagen bone matrix components considered markers of osteoblast mineralization (Ivanovski et al., 2001). Analyzing expressions of osteocalcin mRNA, rhBMP-2 and RA presented similar results at beginning and end of the osteogenic process, days 7 and 28, respectively. At the osteocalcin peak expression (21d), rhBMP-2 demonstrated the highest osteocalcin mRNA level. Since osteonectin binds calcium and type I collagen with high affinity, it is believed to play a positive role in hydroxyapatite crystals deposition. Here, osteonectin mRNA was detected from day 1 to 28, corroborating that osteonectin expression is not limited to the initiation of mineralization (Wang et al., 2008). RA demonstrated similar (1, 7, and 21d) or higher (14 and 28d) osteonectin mRNA relative expressions compared to rhBMP-2. Despite rhBMP-2+RA did not demonstrate the highest osteocalcin and osteonectin mRNA levels at their corresponding expression peak (21d), it promoted the highest calcium deposition at maturated extracellular matrix (32d). This fact could be a response to the early stimulation of osteocalcin and osteonectin mRNA expressions (7d) by rhBMP-2+RA. Skillington et al. (2002) studying preadipocytes observed that BMP-2, alone or associated with RA, was not able to induce neither osteocalcin mRNA expression nor extracellular matrix mineralization. Wang et al. (2008) verified that bone marrow stromal cells treated with RA, alone or in combination with OM, did not enlarge osteonectin mRNA expression or ALP activity. It is important to mention that cell lineage and origin, including donor characteristics, and experimental parameters, such as culturing conditions and experimental time, must be considered when comparing results. Furthermore, we do not have knowledge about any previous report in which human cells were applied to assess the effect of RA on osteogenic differentiation, as well as any study evaluating the entire osteogenic differentiation process (from day 1 to 32).

Concerning mineralization evaluation, all groups were able to mineralize extracellular matrix from day 12 on, including ASCs mock-treated (OM). In agreement with other studies (Wan et al., 2006; Zhang et al., 2010), rhBMP-2 and RA seemed to potentiate

each other's activities on calcium secretion, as observed at the mineral deposition peak (32d), when the calcium concentration was higher even than the positive control (MC3T3-E1). This cooperation might be due to the inhibition of adipocyte differentiation, as stated by Skillington et al. (2002) In Von Kossa technique, the stain reacts with phosphate in the presence of acidic material and does not react with calcium. Despite this staining method has been used in several reports, this is not a sensitive assay to evaluate mineralization. For this reason we used the calcium quantification, which is a quantitative analysis (Bonewald et al., 2003).

The results obtained from the present study show that tested groups exhibited similar Smad1 expressions, except at the beginning of the osteogenic process (7d), when rhBMP-2 up-regulated this protein. Analyzing Smad1 mRNA expression, rhBMP-2+RA and RA promoted an up-regulation at days 14 and 21, respectively. In contrast, Wang et al. (2008) reported that RA inhibited osteogenic differentiation of rat bone marrow stromal cells and did not influence Smad1 mRNA expression.

Regarding Smad4 evaluation, rhBMP-2+RA displayed promising results since it promoted Smad4 expression levels more similar to the positive control (MC3T3-E1) and higher than the other treated groups at later time points (21 and 28d).

Therefore, the analysis of phosphorylated Smads is more important than the non-phosphorylated ones, since the phosphorylation means activation of these proteins and signaling pathways. Results show that RA and rhBMP-2+RA up-regulated the phosphorylated Smad1/5/8 expressions at the beginning of differentiation process (7 and 14d). Cells treated with rhBMP-2+RA displayed interesting results since it demonstrated a similar expression pattern to MC3T3-E1 (positive control), discontinuing the expression of phosphorylated Smads from day 21 on, which might be related to a osteoblast lineage commitment. The observed results regarding Smads expressions corroborate the hypothesis that the molecular mechanism behind the cross talk of RA and rhBMP-2 signaling pathway is the activation of BMP receptors Smad-mediated transcription (Li et al., 2003; Wan et al., 2006). Nevertheless, further investigations are necessary to completely understand this process.

Given that ASCs are able to express endogenous BMPs and the

co-expression of BMPs has been reported to be more efficacious than single BMPs (Cho et al., 2002; Katagiri et al., 1998; Tsuji et al., 2010), we evaluated BMP-4 and BMP-7 expressions. rhBMP-2 and RA, alone or in association, similarly acted on BMP-7 expression at days 14, 21, and 28. rhBMP-2, RA, and rhBMP-2+RA equally stimulated BMP-4 expression. Moreover, BMP-4 expression was not detected at the beginning of differentiation process (7d) for both MC3T3-E1 cells and ASCs. Differing from our findings, Seib et al. (2009) observed that human bone marrow-derived mesenchymal cells were able to express endogenous BMP-4, but not BMP-7. The capacity of ASCs to express endogenous BMP-4 and BMP-7 (Seib et al., 2009) potentiated by ascorbate and β-glycerophosphate present in OM could explain the fact that OM treatment demonstrated similar results compared to rhBMP-2, rhBMP-2+RA, and MCT3T-E1 cells, at some time points. Chang et al. (2009) showed that MC3T3-E1 and MG63 cells treated with BMP-4 induced a rapid increase in Smad1/5 phosphorylation. We also observed this correlation between the expression of endogenous BMP-4 and Smad phosphorylation, especially for OM, rhBMP-2, and RA.

All treatments led ASCs to BMPR-II relative mRNA over-express (at least 218 fold-increase, rhBMP2+RA, day 28), especially at late time points (14, 21, and 28d). Since the BMP-4 signaling pathways in osteogenic differentiation process involves BMPR-II (Leong and Brickell, 1996), the endogenous BMP-4, might had stimulated BMPR-II, explaining the high BMPR-II mRNA expression levels observed after the BMP-4 expression had started. Although Gamer et al. (2011) had found that BMPR-II is not required for endochondral ossification, other works also demonstrated that osteogenic supplementation, BMP-2, or RA increased BMPR-II mRNA expression (Wang et al., 2008, 2010). The results from this study showed that the association of rhBMP-2+RA promoted a decrease on BMPR-II mRNA expression at late time points (from 14 to 28d) in comparison to the other treatments. Since the current understanding concerning the cross talk of RA signaling and rhBMP-2 pathway is relatively limited, we do not have a concrete justification for this BMPR-II mRNA decrease. One possible explanation might be that RA and BMP-2 could share each other's receptors related to signaling pathways

during osteogenic process, fact that remains to be determined.
ASCs showed negative staining for lipid-filled intracellular vacuoles under osteogenic stimulus or regular medium (data not shown), adding further evidence that the obtained cells were not originally adipocytes and did not differentiate into adipocytes under these conditions. In addition to the osteogenic differentiation, ASCs were able to differentiate into adipocytes, under adipogenic stimulus, confirming their multipotent ability.
In general, RA and rhBMP-2 had similar effects for several osteoblast-related markers, such as calcium quantification (12 and 32d), Smad1 (14, 21, and 28d), Smad 4 (all time points), BMP-4 (all time points), BMP-7 (14, 21, and 28d), Smad1 mRNA (7, 14 and 28d), osteocalcin mRNA (1, 7, and 28d), osteonectin mRNA (1, 7, and 21d), and BMPR-II mRNA (7d). RA displayed better results than rhBMP-2 for some other markers, for instance ALP activity (all time points), BMPR-II mRNA (14d), Smad1 mRNA (21d), osteocalcin mRNA (14d), osteonectin mRNA (14 and 28d). On the other hand, rhBMP-2+RA seemed to be the most promising osteoinductor, showing the most favorable results for the biomarkers calcium (12 and 32d), Smad4 (21 and 28d), phosphorylated Smad1/5/8 (all time points), Smad1, osteocalcin, and osteonectin (14, 7, and 7d, respectively). Still, additional studies are needed to investigate points that remain unknown, such as the exact mechanisms of action of these compounds and the cross talk between RA and rhBMP-2.

5. CONCLUSION

RA, rhBMP-2, and rhBMP-2+RA had multiple effects in processes involved in osteogenesis depending on the stage of differentiation. Since rhBMP-2+RA positively acts in most osteogenic markers, especially calcium deposition, a matrix mineralization indicator characteristic of the final stage of progenitor cell development into osteogenic lineage, we were led to conclude that this association seems to be an excellent option for osteogenic differentiation procedures.

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mRNA	Primer Forward (5'-3')	Primer Reverse (5'-3')
BMPR-II	TGAAAAGATCAAGAAACGTGTGAAA	GCCCTGTTACTGCCATTATTGTT
Osteocalcin	AGGGCAGCGAGGTAGTGAAG	AACTCGTCACAGTCCGGATTG
Osteonectin	CGGGTGAAGAAGATCCATGAG	CTGCCAGTGTACAGGGAAGATG
Smad1	GGGACTGCCTCATGTCATTTACT	CAGACCTCCTTCTGCTTGGAA
Ribosomal 18s	CACGGCCGGTACAGTGAAAC	CCCGTCGGCATGTATTAGCT

Table 1: Primer sequences of evaluated mRNA.

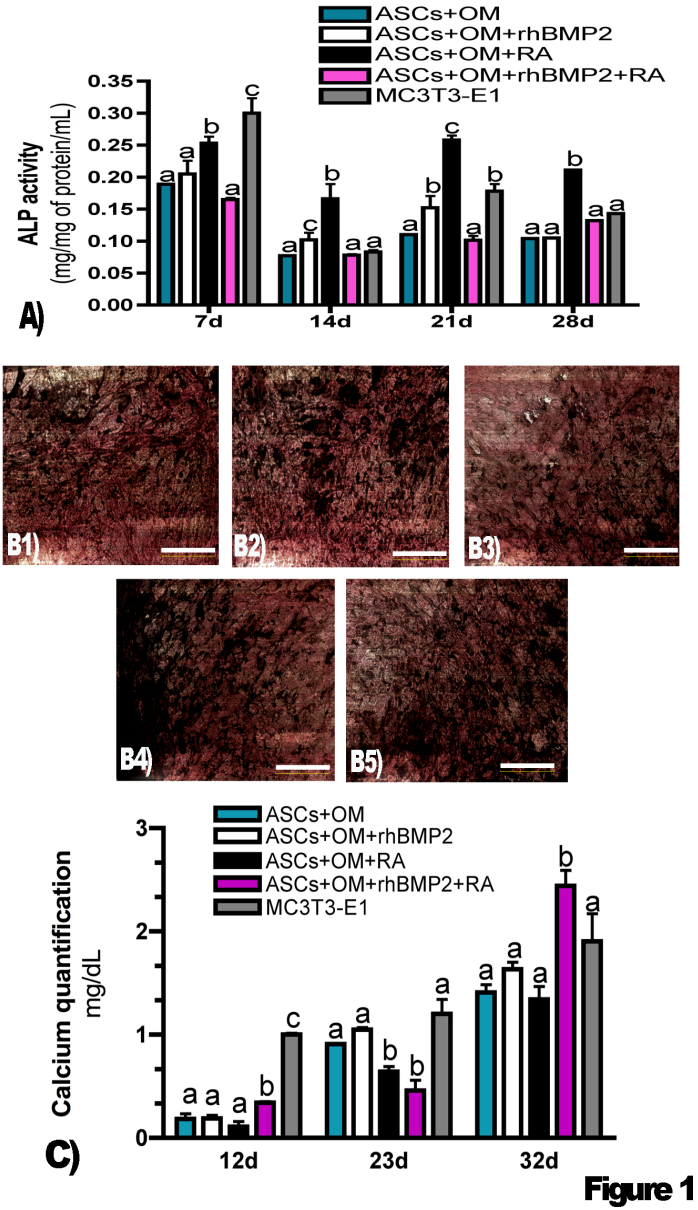

Figure 1

Fig. 1 Extracellular matrix evaluation. A) Alkaline phosphatase activity expressed as mg of ρNitrophenol (ρNP) per mg of protein per ml, at days 7, 14, 21, and 28; B) Von Kossa staining of ASCs

cells after 21-days culture. Cells were treated with osteogenic medium (OM) alone (B1) or supplemented with rhBMP-2 (B2), retinoic acid (RA) (B3), or rhBMP-2 associated to RA (B4). B5) MC3T3-E1 cells (positive control). Bar = 500 μm. Slides are representative of three independent experiments; C) Calcium quantification, expressed as mg per dl, at days 12, 23, and 32. Different letters represent statistically significant differences among treatments at each time point (ANOVA/SNK, $\rho < 0.05$). Bars represent the mean ± SD of three samples. Experiments were performed in triplicate.

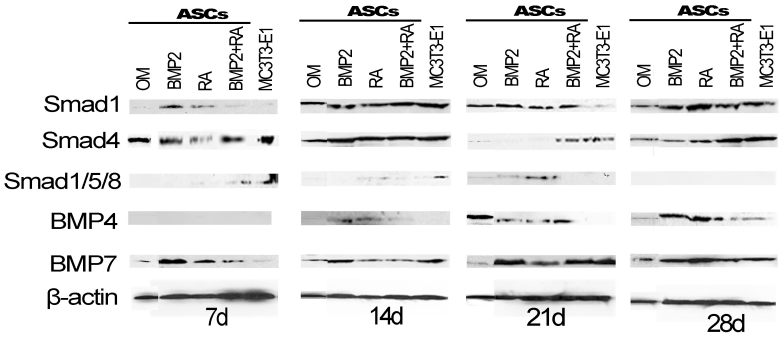

Figure 2

Fig. 2 Expression of osteoblast related proteins. Proteins expressions evaluated by western blotting at days 7, 14, 21, and 28. Bands are representative of three independent experiments.

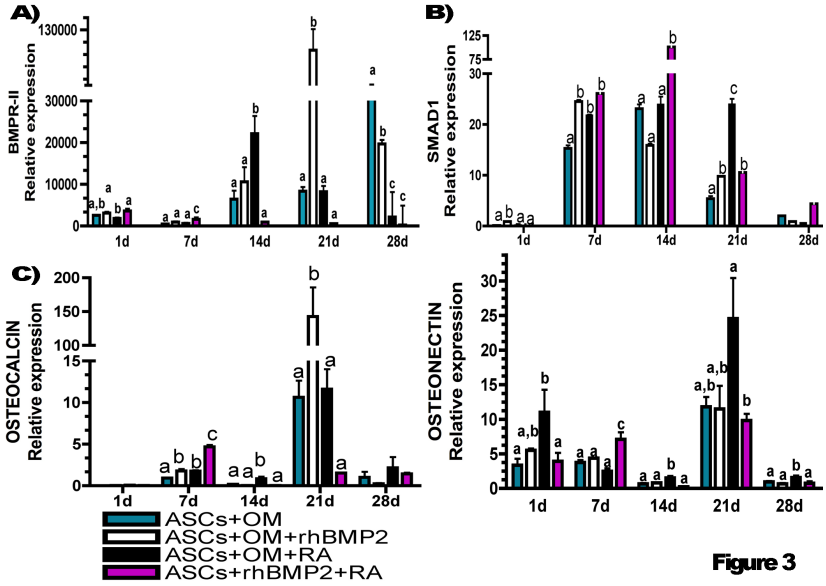

Fig. 3 mRNA relative expression. Different letters represent statistically significant differences among treatments at each time point (ANOVA/SNK, $\rho < 0.05$). Bars represent the mean ± SD of three samples. Experiments were performed in triplicate.

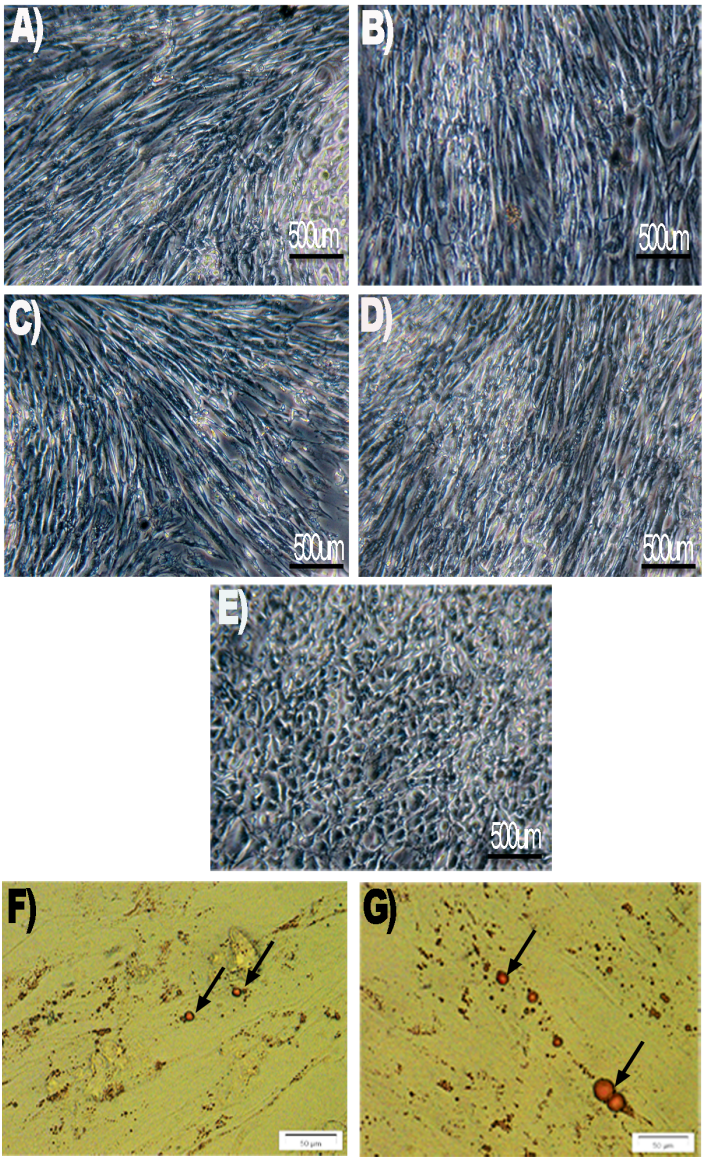

Fig. 4 Oil red O staining. A) ASCs cells treated with osteogenic medium (OM) after 21-days culture; ASCs cells treated with OM

supplemented with B) rhBMP-2; C) RA; D) rhBMP-2 and RA; E) MC3T3-E1 cells (negative control). Bar = 500 μm; ASCs cells cultured with adipogenic medium for F) 14 days and G) 21 days. Bar = 50 μm. Arrows indicate lipid-filled intracellular vacuoles, confirming the ASC differentiation into adipocytes. Slides are representative of three independent experiments.

Capítulo 3

Oral Surgery Oral Medicine Oral Pathology Oral Radiology

Adipose-derived stem cells reduced the inflammation promoted by a low dose of recombinant bone morphogenetic protein-2 (rhBMP-2)

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Abstract

Objective. This study evaluated the effect of *in vivo* administration of human adipose-derived stem cells (ASCs) incorporated into poly-lactic-co-glycolic acid (PLGA) scaffolds on the reduction of inflammatory response induced by a low dose of recombinant bone morphogenetic protein type 2 (rhBMP-2).

Study design. PLGA scaffolds were characterized and loaded with 1, 2.5 or 5μg of rhBMP-2/scaffold (n = 3), and the released protein amounts were quantified at 7h, 1, 7, and 21 days after loading (n = 3). The muscle tissue of six beagle dogs received the following treatments: PLGA, PLGA+rhBMP-2 (2.5 μg), and PLGA+rhBMP-2+ASCs (1 x 10^6 ASCs). Results were compared through ANOVA followed by Student Newman–Keuls post hoc test.

Results.

PLGA+rhBMP-2+ASCs reduced the number of inflammatory foci ($\rho<0.001$) and giant cells ($\rho<0.001$), and increased the neovascularization sites ($\rho<0.001$).

Conclusions.

ASCs reduced the inflammatory process and increased the neovascularization in muscles sites with a low dose of rhBMP-2.

Keywords Bone morphogenetic protein 2; Inflammation; Mesenchymal stem cells; Scaffolds.

INTRODUCTION

Recombinant human bone morphogenetic protein type 2 (rhBMP-2) has been used for treatment of bone defects in maxillofacial, oral, orthopedic, and spine surgeries.[1-3] Despite its promising activity in bone regeneration,[4] rhBMP-2 has been clinically related to some relevant adverse effects in dentistry and medical procedures, including breathing difficulty, dysphagia, edema, erythema, pain, and rhinitis.[5-7] These effects have been attributed to the soft tissue inflammation and swelling at the surgical site.[8]

The inflammatory response induced by rhBMP-2 is not unexpected, since this protein is chemoattractant for lymphocytes, monocytes, and macrophages.[9] Therefore, reports have suggested that rhBMP-2 does not provide advantages over autograft bone because of the undesirable postoperative symptoms.[6,10] In this sense, there has been increasing interest in evaluating agents capable of reducing the rhBMP-2-induced inflammatory response and improve its use in bone regeneration procedures.

Studies have shown that the systemic administration of mesenchymal stem cells (MSCs) can modulate immune-inflammatory response and attenuate tissue damage caused by excessive inflammation,[11,12] probably due to a paracrine effect of these cells.[13] Additionally, the administration of MSCs can suppress the production of pro-inflammatory cytokines and increase the levels of anti-inflammatory interleukins.[14]

MSCs have been isolated from several tissues, including bone marrow, dental pulp, periodontal ligament, and fat.[13,15] The adipose-derived stem cells (ASCs) seem to be an interesting cellular option for clinical applications because they are abundant, easily accessible and isolated, and present a fast attachment and proliferation in culture.[16] Therefore, this study evaluated the effect of *in vivo* administration of human ASCs incorporated into poly-lactic-co-glycolic acid (PLGA) scaffolds on the reduction of inflammatory response induced by a low dose of rhBMP-2, assuming that MSCs may modulate the inflammatory response.

MATERIALS AND METHODS

Scaffolds manufacturing and characterization

Scaffolds were obtained by solvent evaporation technique.[17] Briefly, polymers of poly-lactic-co-glycolic acid (PLGA) (Böehringer, Ingelheim, Germany) (82/18 w/w) were dissolved in methyl chloride (Merck, Whitehouse Station, NJ, USA) (10% w/v) followed by sucrose (Synth, São Paulo, SP, Brazil) addition (70% w/v). This solution was put into 0.5 x 1.0 x 1.0 cm molds. After the solvent evaporation, the scaffolds were immersed in polyvinyl alcohol (Synth, São Paulo, SP, Brazil) to remove the sugar. Finally, the scaffolds were vacuum dried (60°C, 24 hours) and sterilized (25 kGy gamma rays, Embrarad, Cotia, SP, Brazil).

Scaffold's surface, pore size, and internal pore morphology were evaluated by scanning electronic microscopy (SEM) (JSM T330TM JEOL, Tokyo, Japan). The pore size was measured by Digimizer® analysis image software (*MedCalc software*, Broekstraat, Mariakerke, Belgium). X-ray diffraction spectrum (XRD, Bruker-AXS D5000, Madison, WI, USA) was obtained by the powder diffraction method (CuK_α radiation, wavelength of 1.54056 Å, scanning range 2θ = 1- 40°, step time of 2 s, and 0.02° of step size). Gel permeation chromatography (*Waters Corporation*, Milford, MA, USA) with a refraction index detector (model 241) was used to determine the average molecular weight (Mw), average molecular number (Mn), and the polydispersity index (PI). The scaffolds were solubilized in 3 mg ml^{-1} tetrahydrofuran (*Tedia Company Inc.*, Fairfield, OH, USA), which was the mobile phase (1 mL min^{-1} flow rate), and a column model HR 4E and 5E of Waters Corporation was the stationary phase. The Mw was calculated using monodisperse polystyrene standards (Mw 1,000 and 150,000 Da).

rhBMP-2 loading and release

The scaffolds (0.5 x 1.0 x 1.0 cm) (n = 6) were immersed in 1 ml of ultra-pure water containing 1, 2.5 or 5 µg of rhBMP-2 (Sigma Aldrich), pH 7.0 at 37°C for 18 hours. Scaffolds in ultra-pure water without rhBMP-2 were used as negative control. Different rhBMP-2 solutions (1, 2.5 or 5 µg rhBMP-2.ml^{-1}) were also added into plates without scaffolds to estimate the degraded rhBMP-2 amount and the rhBMP-2 adhesion on plates.

After the loading period, supernatants were collected and stored at -80 °C. The scaffolds were transferred to other wells, squeezed, 1ml of phosphate buffered saline (PBS) was added, and the supernatants were collected and stored (Initial squeezed sample). BMP-2 Quantikine Elisa Kit (*R&D Systems*, Minneapolis, MN, USA) was used for protein quantification. Additionally, pH values were monitored over time in order to evaluate scaffolds' degradation.

After the loading step, the scaffolds were transferred to other wells and 1ml of PBS was added. Following each incubation period (7 hours, 1, 7, and 21 days), the supernatants were collected and stored (-80 °C), the scaffolds were transferred to other wells, and PBS was added. At the end of the release time

(21 days), the same procedure described above for initial squeezed sample was used.
Kinetic models as zero order kinetic model, Higuchi model, and erosion or diffusion-dependent release were considered in order to explain the mechanism involved in rhBMP-2 release from scaffolds. The most appropriate model was selected by linear regression analysis (concentration over time).

ASCs isolation, culture, and immunophenotyping
After approval by the Human Ethics Committee (no. 194/06, Universidade Federal de Santa Catarina) and the understanding and written consent of the voluntaries, in compliance with the Helsinki Declaration, the human lipoaspirate tissues were processed to isolate ASC, according to Zuk et al.[16] Briefly, lipoaspirate tissues were washed extensively with PBS and extracellular matrix was digested with 0.075% collagenase type A (Sigma Aldrich) at 37°C for 30 minutes. Enzyme activity was neutralized with Dulbecco's modified Eagle medium (DMEM, Cultilab, Campinas, SP, Brazil), containing fetal bovine serum (FBS, Gibco, São Paulo, SP, Brasil) and centrifuged at 1200 x g for 10 minutes. The pellet was resuspended in 160 mM NH_4Cl (Sigma Aldrich) at room temperature for 10 minutes to lyse the red blood cells. The cells were collected by centrifugation (1200 x g for 10min), filtered through a 100μm nylon mesh to remove cellular debris, and incubated overnight with DMEM (Cultilab), 10% FBS (Gibco), and 1% penicillin-streptomycin-amphotericin (PSA, Gibco) at 37°C and 5% CO_2. Afterward, the plates were washed with PBS to remove residual non-adherent red blood cells. ASCs were maintaining at subconfluent levels and used at passage 3 for the experiments.
Flow cytometry was performed for detection of surface antigens, as previously described by da Silva Meirelles et al.[18] Concisely, ASCs were trypsinized, centrifuged, and incubated for 30 minutes at 4°C with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, peridinin chlorophyll (PerCP)-, PerCP with a cyanine dye (PerCP-Cy5.5)-conjugated antibodies against human CD14, CD19, CD34, CD45, CD73, CD90, CD105, and HLA-DR (Pharmingen BD, San Diego, CA, USA). Excess antibody was removed by washing. The cells were analyzed using a

FACScalibur cytometer equipped with 488 nm argon laser (Becton Dickinson, San Diego, CA, USA) with the Cell Quest software. At least 10,000 events were collected. The FlowJo 8.6.3 (Three star Inc., Ashland, OR, USA) software was used for building histograms.

ASCs multilineage differentiation

ASCs (2 x 10^4 cell/well in 96 well-plates) were analyzed for their capacity to differentiate toward the adipogenic and osteogenic lineages after 21 days of induction. Adipogenic differentiation was induced by culturing ASCs with DMEM (Cultilab) supplemented with 10% FBS (Gibco), 1% PSA (Gibco), 10^{-7} M dexamethasone, 2.5 µg ml^{-1} insulin, 5 µM indometacin, and 5 µM rosiglitazone. Afterward, the presence of intracellular lipid deposits was evaluated by Oil Red O staining.[18] This analysis was also performed with ASCs cultured with regular medium (DMEM, 10 % FBS, and 1 % PSA) for 21 days to confirm that the isolated cells were not adipocytes.

ASCs were cultured with DMEM (Cultilab) supplemented with 10 % FBS (Gibco), 1 % PSA (Gibco), 250 µM ascorbate, 10 mM β-glycerophosphate, and 50 ng ml^{-1} rhBMP-2 to induce osteogenic differentiation. Von kossa staining was performed to evaluate extracellular matrix mineralization.[16] Items for adipogenic and osteogenic induction were purchased from Sigma-Aldrich.

Scaffold cytotoxicity

The scaffolds cytotoxicity was evaluated by MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide] colorimetric assay. 2.5 x 10^4 ASCs/well were cultivated in 96-well plates for 24 hours. Subsequently, the scaffolds (0.5 x 0.5 x 1.0 cm) or polyethylene fragments (negative control) were placed into the wells, and the percentages of viable cells were verified after 1, 3, and 7 days. The scaffolds' dry weight values were determined and SEM was performed (see *Scaffolds characterization* item) to evaluate the scaffolds *in vitro* degradation.

***In vivo* assay**

After approval by the Animal Ethics Committee (no. 196/11, Universidade Federal de Santa Catarina), the potential of ASCs in reducing the rhBMP-2-induced inflammatory response was

evaluated in six beagle dogs (approximately 1.5-year-old) by histopathological analysis with respect to the humane care and treatment of animals used in the study.

General anesthesia was obtained by intramuscular administration of 10 mg ketamine chlorate/kg and 4 mg xylazine chlorate/kg. The proximal tibia was shaved, treated with antiseptic iodine solution, and a 5 cm incision through the skin was used to access the muscle. Pouches were created and assigned to receive one of the following treatments: PLGA - scaffolds of PLGA (0.5 cm^3); PLGA+rhBMP-2 - scaffolds with 2.5 µg of rhBMP-2 (see *rhBMP-2 loading* section); PLGA+rhBMP-2+ASC - 20µL of a ASCs suspension (1 x 10^6 cells/20 µL) were seeded onto rhBMP-2-loaded scaffolds. In this last approach, the scaffolds were stored for 4 h and incubated overnight to ensure cell adhesion. The animals received all treatments in muscle pouches and the disto-proximal order of each treatment was randomly determined in each animal. The muscle and skin were sutured and a single dose of benzyl penicillin 20,000 UI benzatine /kg and 1 ml ketoprofen/5 kg was administered intramuscularly.

At 6 weeks, the animals were sacrificed by anesthesia overdose and sections of the muscle tissue were processed and stained with hematoxylin and eosin. Inflammatory foci, giant cells, and neovascularization sites were quantified in 12 microscope fields (1 x 100 magnification) per histopathological section randomly selected. Four sections were analyzed for each treatment and the individual data was determined as the mean result of these sections. An inflammatory infiltrate was considered present when ≥ 3 leukocytes were detected in each inflammatory focus.

Statistical analysis

Statistical analyses of the data were performed using Graph Pad Software (La Jolla, CA, USA). Intra-examiner reproducibility for pores size, inflammatory foci, giant cells, and neovascularization was tested with intra-class correlation coefficient (ICC). Results of rhBMP-2 loading and release, scaffolds cytotoxicity, inflammatory foci, giant cells, and sites of neovascularization in all groups were compared through non-parametric One-way Analysis of Variance (ANOVA) followed by Student Newman–Keuls (SNK) post hoc test (95% confidence interval). Statistical analyses were

performed at each time point separately. PLGA scaffolds degradation was analyzed by comparing the results of different time points through ANOVA/SNK (95 % confidence interval).

RESULTS

Scaffolds characterization

The ICCs were 0.99%. Scaffolds (0.5cm^3) presented 0.112 ± 0.003 g (Fig. 1a) and XRD analyses indicated a non-crystalline phase (Fig. 1b). SEM analyses showed an irregular surface with interconnected cubical irregular pores (size of 420 ± 121 μm) and high surface/volume ratio (Fig. 1c-f). After sterilization, the Mw was 135,883 g/mol, Mn was 61,860, and IP was 2.2.

rhBMP-2 loading and release

The scaffolds treatment with 1, 2.5, or 5μg of rhBMP-2 demonstrated incorporation rates of 78.76 ± 4.83%, 73.97 ± 8.60%, and 60.91 ± 9.33%, respectively, without significant differences among groups ($\rho = 0.07$). After the loading period (18 hours), the retained rhBMP-2 percentages in the void spaces were similar among groups ($\rho = 0.96$) (1.32 ± 0.81%, 1.50 ± 0.52% or 2.41 ± 0.20% for 1, 2.5, or 5μg of rhBMP-2, respectively) (Table I).

The percentages of released rhBMP-2 were highest for the group loaded with 1μg at all time points ($\rho < 0.05$). After the release periods, the rhBMP-2 that remained incorporated in void spaces was zero, 1.75 ± 3.03%, and 9.75 ± 10.18% for 1, 2.5 and 5μg of rhBMP-2, respectively ($\rho = 0.16$) (Table I).

Regarding the rhBMP-2 release from the scaffolds, the highest average correlation coefficient ($R^2 > 0.944$) was obtained with the anomalous kinetic transport, indicating that the rhBMP-2 release involved simultaneously diffusion and erosion mechanisms.

Scaffolds cytotoxicity

The scaffolds were not cytotoxic for ASC since the cell viability values were similar to those obtained from PLGA (79.71 ± 4.73, 100 ± 1.42, and 84.45 ± 0.03 % at days 1, 3 and 7, respectively) and from negative control (polyethylene fragments) (76.31 ± 4.96, 100 ± 4.96, and 92.28 ± 9.2 % at days 1, 3 and 7, respectively) ($\rho > 0.05$) at days 1, 3, and 7.

Characterization of ASC

The ASCs expressed the surface markers CD90, CD73, and CD105 and demonstrated less than 1% of expression for CD34, HLA-DR, CD19, CD14, and CD45 (Fig. 2a-h). The ASCs confirmed their mesenchymal stem characteristics by depositing a mineralized matrix as evidenced by Von Kossa staining, or by acquiring intracellular lipid deposits, confirmed by Oil Red O staining. Moreover, Oil Red O staining confirmed that the isolated cells were no adipocytes (Fig. 2i-k).

Scaffolds degradation

In vitro degradation of the scaffolds increased over time (7.48 ± 2.27, 14.91 ± 2.79, and 18.56 ± 0.22 % at days 1, 3, and 7, respectively, $\rho = 0.001$), in agreement with SEM results (Fig. 3a-b). Additionally, the scaffolds were almost completely degraded *in vivo* after 6 weeks (Fig. 4a-f).

Scaffolds, rhBMP-2 solutions, and PLGA+rhBMP-2 demonstrated a near-neutral pH after 1 day of the rhBMP-2 loading. After 7 days, the neutral pH was maintained in the rhBMP-2 solutions, while PLGA and PLGA+rhBMP-2 showed a pH reduction to 6.0. PLGA and PLGA+rhBMP-2 demonstrated a pH value of 4.0, while rhBMP-2 solutions showed a pH value of 5.0 at day 21.

***In vivo* effect of ASC on inflammatory response induced by rhBMP-2**

Qualitative analysis revealed that PLGA+rhBMP-2+ASCs demonstrated a hyaline necrosis of muscle fibers, while PLGA and PLGA+rhBMP-2 resulted in coagulation necrosis.

According to quantitative analyses (Fig. 4a-f and Fig. 5a-b), PLGA+rhBMP-2+ASCs promoted the lowest number of inflammatory foci followed by PLGA+rhBMP-2 and PLGA ($\rho < 0.001$). PLGA+rhBMP-2+ASCs demonstrated the lowest number of giant cells ($\rho < 0.001$). Additionally, PLGA+rhBMP-2+ASCs promoted the highest neovascularization ($\rho < 0.001$). Regarding the cicatricial muscle tissue, PLGA+rhBMP-2+ASCs demonstrated higher results than PLGA ($\rho < 0.001$).

DISCUSSION

In attempt to modulate the inflammatory response induced by a low dose of rhBMP-2, the present study evaluated the *in vivo* effect of the administration of ASCs incorporated into scaffolds by the quantification of inflammatory foci, giant cell, and neovascularization. Results from the flow cytometry and multilineage differentiation confirmed the MSCs characteristics of the ASCs.[19] Additionally, Oil Red O staining for ASCs cultured with regular medium added further evidence that the isolated cells were not originally adipocytes and differentiated into adipocytes only under appropriated stimulus.

Prior to the evaluation of the ASCs potential to modulate the rhBMP-2-induced inflammatory response, it was essential to confirm that this protein was delivered in amounts sufficient from scaffolds. The *in vitro* assays confirmed that most of this growth factor was effectively loaded and released. These results are in accordance with the observed physical characteristics for the scaffolds and previous report.[20,21]

The inflammatory response induced by rhBMP-2 is not unexpected, since this protein is chemoattractant for lymphocytes, monocytes, and macrophages. Moreover, rhBMP-2 can induce osteoclastogenesis by potentiating receptor activator of nuclear factor-kB ligand, a cytokine essential for inducing osteoclast differentiation.[9,22,23] The results herein demonstrated that ASCs promoted the least quantification of giant cells and inflammatory foci compared to the other groups. We have also observed that ASCs promoted the highest neovascularization compared to PLGA or PLGA+rhBMP-2. It has been suggested that all of these events are related to the paracrine signaling of MSCs.[13] In support of our results, Zhou et al.[14] demonstrated that the administration of human ASCs suppressed the production of the pro-inflammatory cytokines, including interferon-γ, tumor necrosis factor-α, interleukins (IL)-1α, IL-1β, IL -6, IL-12, IL-15, IL-17, and increased the levels of the anti-inflammatory IL-10. Furthermore, they observed that ASCs promoted a reduction of some chemokines, including monocyte chemotactic protein-1, Rantes, and keratinocyte cytokine.

The neovascularization results promoted by ASCs is a relevant finding since angiogenesis is related to essential aspects of tissue regeneration, including nutrients, waste, and oxygen transport.[24] Besides the paracrine effect promoted by ASCs, other

mechanism related to the neovascularization findings may be the expression of angiogenic and arteriogenic cytokines by ASCs, such as vascular endothelial growth factor, monocyte chemoattractant protein-1, and fibroblast growth factor. On the other hand, MSCs are also able to differentiate into cardiomyocytes and vascular lineage.[24] Consequently, at this stage, additional studies are necessary to better understand the mechanisms related to the decrease of the rhBMP-2-induced inflammation, and the increase of neovascularization. It is suggested that the rhBMP-2-induced inflammatory response is proportional to the applied amount of this growth factor.[9] Since we used a low dose of rhBMP-2, a confirmatory study of the ASC capacity to reduce the inflammatory response provided by high rhBMP-2 doses is warranted.

In conclusion, ASC reduced the inflammatory process and increased the neovascularization in muscles sites with a low dose of rhBMP-2.

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Group	Incorporated rhBMP-2 (%)	Released rhBMP-2 (%)				Retained rhBMP-2 in void spaces (%)	
		7h	1d	7d	21d	After loading	After release
1.0 µg	78.76±4.83	37.93*	63.46*	70.38*	90.21*	1.32±0.81	0
2.5 µg	73.97±8.60	31.28	34.60	37.86	63.60	1.50±0.52	1.75±3.03
5.0 µg	60.91±9.33	29.43	33.98	38.13	63.93	2.41±0.20	9.75±10.18

Table I Percentage of incorporated and released rhBMP-2. Values are expressed as Mean ± SD. Incorporation values are expressed as percentages of rhBMP-2 applied in the scaffolds. There was no statistically significant difference in incorporation among groups ($\rho = 0.07$; ANOVA). Release values are expressed as percentages of rhBMP-2 incorporated into scaffolds (1, 2.5, and 5µg of rhBMP-2 at 7h, 1, 7, and 21 days. (*) indicates statistically significant differences between 1µg of rhBMP-2 and other groups ($\rho < 0.05$; ANOVA with SNK post hoc test). There was no statistically significant difference in retained rhBMP-2 in void spaces after the loading period ($\rho = 0.96$; ANOVA) and after the release period among groups ($\rho = 0.16$; ANOVA). Experiments were performed in triplicate.

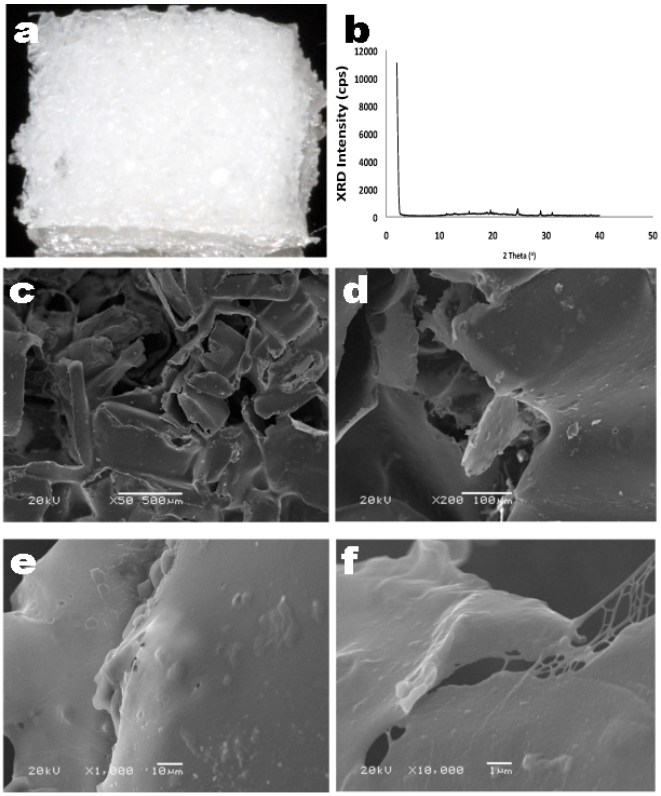

Figure 1

Fig. 1 a PLGA scaffold with 0.112 ± 0.003g. After sterilization, the Mw was 135,883 g/mol, Mn was 61,860, and IP was 2.2. **b** XRD spectrum of PLGA scaffold indicating a non crystalline phase. **c** SEM photomicrograph of this scaffold at 50x magnification (bar = 500 µm), indicating irregular pores with 420 ± 121µm. ICC was 0.99 %. **d** SEM photomicrograph at 200x magnification (bar = 100µm) with interconnected pores. **e** SEM photomicrograph at 1,000x magnification (bar = 10µm). **f** SEM photomicrograph at 10,000x magnification (bar = 1µm) indicating irregular surface scaffolds. Experiments were performed in triplicate.

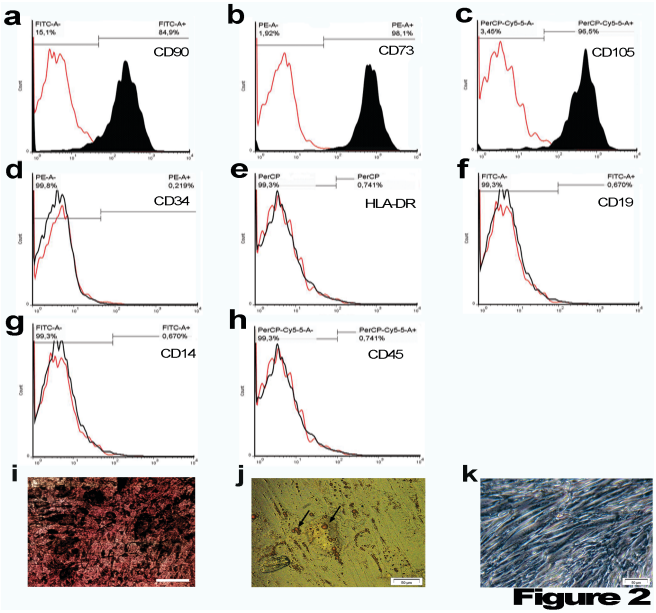

Fig. 2 Representative immunophenotypic profile of ASCs. ASCs were positive for **a** CD90, **b** CD73, and **c** CD105 and negative for **d** CD34, **e** HLA-DR, **f** CD19, **g** CD14, and **h** CD45. Flow cytometry histograms show the expression (black line) of cells' surface markers compared with controls staining (red line); **i** Von kossa staining of ASCs cultured with osteogenic medium for 21 days. Bar = 500μm. Black areas indicate extracellular matrix mineralization. **j** Oil Red O staining of ASCs cultured with adipogenic medium for 21 days. Bar = 50μm. Arrows indicate lipid-filled intracellular vacuoles in adipocytes. **k** Oil O staining of ASC after 21 days in culture with regular medium, confirming that the isolated cells were no adipocytes. Bar = 50μm. The images are representative of three independent experiments.

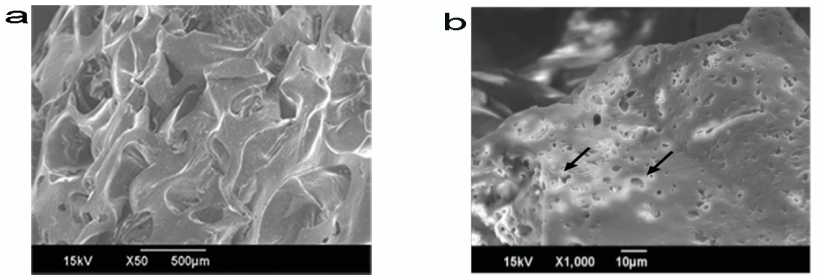

Fig. 3 **a** SEM photomicrograph of PLGA scaffold at 50x magnification (bar = 500µm). **b** SEM photomicrograph of this scaffold at 1,000x magnification (bar = 10µm). Arrows indicate areas of degradation at day 7. Experiments were performed in triplicate.

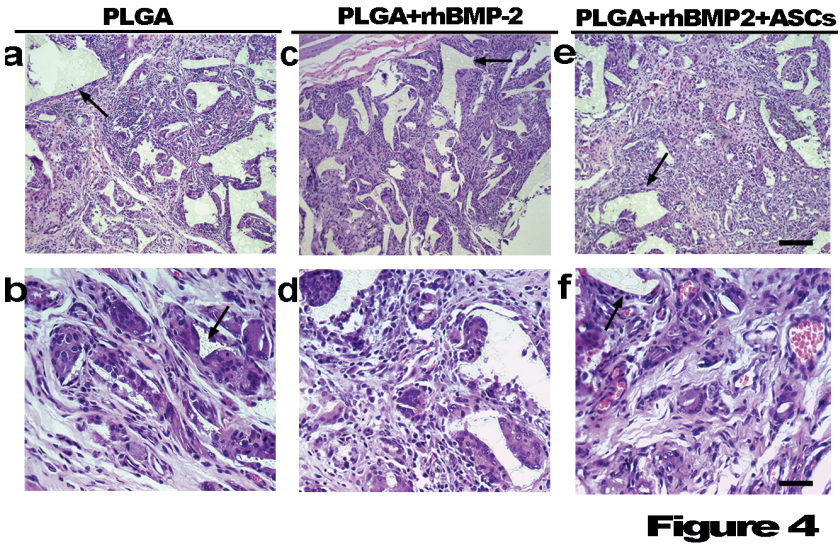

Fig. 4 Histopathological aspects of muscle tissue following treatment with **a-b** PLGA, **c-d** PLGA+rhBMP-2, and **e-f** PLGA+rhBMP-2+ASCs. Formalin fixed, paraffin-embedded

muscle tissue sections from day 45 after treatments were stained with hematoxylin and eosin (H&E). Slides shown are from six different dogs and represent typical findings obtained from the treatments. Slides from upper row are in magnification of 10x (scale bar = 50μm) and slides from lower row are magnification of 40x (scale bar = 200μm). Arrows indicate areas of PLGA scaffolds degradation.

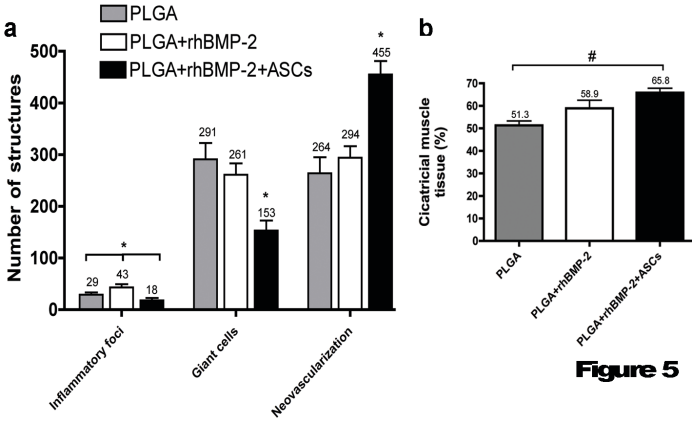

Figure 5

Fig. 5 a Quantification of inflammatory foci, giant cells, and neovascularization (n = 6). (*) indicates statistically significant differences on inflammatory foci among all groups ($\rho < 0.001$; ANOVA with SNK post hoc test). (*) indicates statistically significant differences on giant cells among PLGA+rhBMP-2+ASCs and other groups ($\rho < 0.001$; ANOVA with SNK post hoc test). (*) indicates statistically significant differences on neovascularization among PLGA+rhBMP-2+ASCs and other groups ($\rho < 0.001$; ANOVA with SNK post hoc test). **b** Quantification of cicatricial muscle tissue. (#) indicates statistically significant differences between PLGA+rhBMP-2+ASCs and PLGA ($\rho < 0.01$; ANOVA with SNK post hoc test).

In vivo effect of human adipose-derived stem cells incorporated into autogenous platelet-rich plasma in bone regeneration

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Short title: Bone regeneration by ASCs and PRP

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Abstract

Background and objective: The bone regeneration is often unsatisfactory and could be largely improved by mesenchymal stem cells therapy. However, the choice of the optimal scaffolds for these cells remains a problem. Therefore, the purpose of the present study was to evaluate the *in vivo* effect of human adipose-derived stem cells (ASCs) incorporated into autogenous platelet-rich plasma (PRP) in bone regeneration.

Material and Methods: Human ASCs were isolated from lipoaspirate tissues, subcultured, and used at passage 4. Immunophenotyping and multilineage differentiation of ASCs were initially performed and their mesenchymal stem cells characteristics confirmed. *In vivo* effect of ASCs incorporated into PRP scaffolds to regenerate bone defects was evaluated in the tibia of six beagle dogs at 6 weeks postoperative by the quantification of primary and secondary bone, and granulation tissue. For this purpose, animals were treated with PRP, PRP+ASCs, and autogenous bone grafts (ABG).

Results: PRP+ASCs promoted the highest bone formation (primary+secondary bone), highest bone maturation (secondary bone), and the lowest amount of granulation tissue.

Conclusion: In summary, PRP+ASCs increased the bone formation and maturation, and decreased the granulation tissue in bone defects created in canine tibia, even when compared to ABG.

Introduction

Several reports have focused their attention on the development of bone substitutes that match the excellent biological properties of autogenous bone grafts (ABG), lacking the main drawbacks of this biomaterial, which are the donor site morbidity and limited amount of grafted materials (1). The main approaches applied in bone regeneration include the tissue engineering by the use of

osteogenic cells, scaffolds, and/or growth factors either separately or in combination.

Mesenchymal stem cells (MSCs) initially received a lot of attention from bone tissue regeneration methods due to their capacity in differentiating into many cell types, including osteoblasts and chondrocytes (2). Recently, the paracrine effects of these cells have been highlighted both in tissue engineering and in the treatment of several immune-mediated diseases (3, 4). Several reports are associating paracrine signaling as the primary mechanism of action, and some studies have even demonstrated that direct injection of the molecules secreted by MSCs can provide an improved benefit compared to transplantation of whole cells (5, 6). Adipose-derived stem cells (ASCs) represent an excellent MSCs option for clinical applications because they are abundant, easily accessible and isolated, able to promote minimal donor site morbidity, and plus they demonstrate a fast attachment and proliferation in cell culture (2, 7).

One of the key factors in tissue engineering is the use of an appropriate scaffold. Scaffolds have been developed from the concept of inert and biocompatible materials, which allow adhesion and proliferation of cells and vessels. Currently, however, these biomaterials should also present characteristics such as being affordable, present capacity to load and release growth factors, preserving tissue volume, having capacity to be biodegradable and replaced by neo-bone tissue, high surface area to volume ratio for cell–matrix interaction, and sufficient space for extracellular matrix regeneration.

Platelet-rich plasma (PRP) is an autogenous blood product produced by the centrifugation of venous blood (8). This biomaterial is an interesting option as scaffolds for MSCs given that it does not present risk of disease transmission, besides being providing with the characteristics described above for scaffolds, and considering that its preparation technique is simple and presents a gel consistency after its activation, which facilitates the manipulation and accommodation in the bone defects. It is also important to mention that PRP does not present mechanical resistance, indicating PRP to be used as autogenous scaffolds in areas with no pressure such as sinus lifting, defects around dental implants, periodontal defects, and cystic lesions.

Moreover, PRP contains numerous growth factors, including platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and insulin-like growth factor (IGF), besides some proteins such as fibrin, fibronectin, and vitronectin. These growth factors and proteins, which are stored in the α-granules and released when the platelets are activated, play a pivotal role in mitogenesis, chemotaxis, and angiogenesis (9-12). Therefore, the purpose of the present study is to evaluate the effect of human ASCs incorporated into autogenous PRP in bone regeneration in a canine model.

Materials and Methods

Isolation and determination of adipose-derived stem cells viability and proliferation

The experiments were undertaken after approval by the Institutional Human Ethics Committee at Universidade Federal de Santa Catarina (No. 194/06) and with the understanding and written consent of the voluntaries. The human lipoaspirate tissues were processed to isolate ASCs, according to Zuk *et al.* (2). Concisely, lipoaspirate tissues were washed with PBS and digested with 0.075% collagenase type I (*Sigma Aldrich*, St Louis, MO, USA) at 37^{o}C for 30 min. Enzyme activity was neutralized with Dulbecco's modified Eagle medium (DMEM, *Cultilab*, Campinas, SP, Brazil), containing fetal bovine serum (FBS, *Gibco*, São Paulo, SP, Brazil) and centrifuged at 1200 x *g* for 10 min. The pellet was resuspended in 160 mM NH_4Cl (*Sigma Aldrich*) at room temperature for 10 min to lyse the red blood cells. Another centrifugation (1200 x *g* for 10 min) was performed, the tissue was filtered through a 100 µm nylon mesh to remove cellular debris and incubated overnight with DMEM (*Cultilab*), 10% FBS (*Gibco*), and 1% penicillin-streptomycin-amphotericin (PSA, *Gibco*) at 37^{o}C and 5% CO_2. Subsequently, the plates were washed with PBS to remove residual non-adherent red blood cells. ASCs were maintained at sub confluent levels and used at passage 4 for the experiments.

ASCs were seeded in 6-well plates to determine the cells viability and proliferation at three different sets up: $1x10^5$ cells/ well for days 1 and 2, $8x10^4$ cells/ well for days 3 and 4, and $6x10^4$ cells/ well for day 5. Afterwards cells were trypsinized and counted using an automated cell counter (Countess™, *Invitrogen*, Carlsbad, CA, USA).

Confirmation of MSCs characteristics

Immunophenotyping and multilineage differentiation of ASCs were performed in order to confirm the MSCs characteristics. Immunophenotyping was carried out for detection of surface antigens by flow cytometry, according to da Silva Meirelle (13). Briefly, ASCs were trypsinized, centrifuged, and incubated at 4°C for 30 min with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, peridinin chlorophyll (PerCP)-, PerCP with a cyanine dye (PerCP-Cy5.5)-conjugated antibodies against human CD14, CD19, CD34, CD45, CD73, CD90, CD105, and HLA-DR (*Pharmingen BD*, San Diego, CA, USA). Antibody excess was removed by washing. The cells were analyzed using a FACScalibur cytometer equipped with 488 nm argon laser (*Becton Dickinson*, San Diego, CA, USA) with the Cell Quest software. At least 10000 events were collected. The FlowJo 8.6.3 (*Three star Inc.*, Ashland, OR, USA) software was used to build histograms.

The capacity of ASCs to differentiate toward the adipogenic and osteogenic lineages were analyzed after 21 days of induction. For adipogenic induction, ASCs ($2x10^4$ cell/ well) were cultured in 96-well plates with DMEM, 10% FBS, 1% PSA, 100 µM dexamethasone, 2.5 $\mu g.mL^{-1}$ insulin, 5 µM indometacin, and 5 µM rosiglitazone. Subsequently, the presence of intracellular lipid deposits was evaluated by Oil Red O staining (13). This analysis was also performed in ASCs cultured with regular medium (DMEM, 10% FBS, and 1% PSA) for 21 days in order to confirm that the isolated cells were not adipocytes. ASCs were cultured with DMEM, 10% FBS, 1% PSA, 250 µM ascorbate, 10 mM β-glycerophosphate, and 50 $ng.mL^{-1}$ rhBMP-2 to induce osteogenic differentiation (14). The presence of phosphate at extracellular matrix was evaluated by Von kossa staining (2). Items for adipogenic and osteogenic induction were acquired from Sigma-Aldrich.

Preparation of autogenous platelet-rich plasma and human adipose-derived stem cells complex

After approval by the Institutional Animal Ethics Committee at Universidade Federal de Santa Catarina (no. 196/11), the capacity of the association PRP+ASCs to regenerate bone defects was evaluated in six beagle dogs (n = 6) (approximately 1.5-year-old) by histomorphometric analysis.

General anesthesia was obtained by intramuscular administration of ketamine chlorate (10mg.kg^{-1}) and xylazine chlorate (4mg.kg^{-1}). In the PRP preparation, 10 mL of intravenous blood of each animal were collected using two vacuum glass tubes containing 0.5 mL of 10% (w/v) trisodium citrate solution. The blood was centrifuged at 2000 rpm for 10 min at 22^{o}C for platelet separation (15). A triphasic solution was obtained in which the lower portion contains red cells and growth factors; the intermediate layer is the PRP; and the superior segment contains few platelets, red cells, and plasma. Since the largest number of platelets is in the inferior portion of the intermediate layer, 1-2 mm above the red portion, this part was transferred to another tube and a second centrifugation (2000 rpm for 10 min) was performed to concentrate the platelets. Finally, 500 µL of PRP was transferred to a tube with 50 µL of 10% (w/v) calcium chloride solution and 20 µL of a cellular suspension containing 1x10^{6} ASCs was added. The centrifugation of blood and surgical procedure were initiated at the same time. The colloid PRP was obtained after 15-20 min of the activation. The final concentration of platelets in the PRP was analyzed in an automatic counter (*Countess*™, Invitrogen, Carlsbad, CA, USA). A minimum count of 1x10^{6} platelets/ µL was required.

Surgery procedures

The proximal tibias were shaved, treated with antiseptic iodine solution, and 5 cm incision through the skin was used to access the mucoperiosteum, which was elevated for bone exposure. Standardized osteotomies were made with 10 mm trephine bur at 1200 rpm under abundant saline irrigation, resulting in a defect of both bone and periosteum (osteo-periosteal). The area was copiously washed to remove all bone debris. The first bone defect

was created 2 cm below the joint capsule line at the central medial-lateral portion of the proximal tibiae. The other three defects were created in a distal direction at distances of 2 cm along the central region of the bone. The bone defects were assigned to receive one of the following treatments: Clot; PRP- 500 µL of activated PRP; PRP+ASCs- 20 µL of a ASCs suspension ($1x10^6$ cells/ 20µL) in PRP scaffolds; ABG- autogenous bone graft removed during the bone defects creation. The animals received all treatments in bone defects and the disto-proximal order of each treatment was randomly determined in each animal. The periosteum was sutured with polyglactin 910 (*Ethicon*, Boston, MA, USA) and the skin was sutured with 4-0 nylon (Ethicon). Animals received a single dose of benzyl penicillin benzatine (20000 $UI.kg^{-1}$, intramuscularly) and 1% ketoprofen (0.2 $ml.kg^{-1}$). At 6 weeks, the animals were sacrificed by anesthesia overdose, and the tibiae were retrieved by sharp dissection. The bone tissue were decalcified in 10% Morse solution for 16 weeks, washed in running water for 24 h, and gradually dehydrated in a series of ethanol solutions ranging from 70% to 100% (v/v). Following dehydration, the samples were paraffin-embedded, and sections (5 µm) were routinely stained with hematoxylin and eosin (H&E). Areas of primary bone, secondary bone, and granulation tissue in the bone defects were measured by a single masked, previously calibrated operator using Olympus DP72 capture system and Super FL analysis (Richmond Hill, ON, Canada). Sixteen microscope fields were randomly measured (magnification 10 x and 40 x). Four sections were measured for each treatment and the individual data was determined as the mean result of the four sections.

Statistical analysis

Statistical analyses were performed using Graph Pad Software Inc. (La Jolla, CA, USA). Results of primary bone, secondary bone, and granulation tissue in all groups were compared through non-parametric One-way Analysis of Variance (ANOVA) followed by Tukey post hoc test (95% confidence interval).

Results

Viability and characterization of ASCs

The ASCs proliferation rates were 40, 50, 75, 104, and 150% at days 1, 2, 3, 4, and 5, respectively. The ASCs confirmed their MSCs characteristics by the expression of the surface markers CD90, CD73, and CD105 and less than 1% of expression for CD34, HLA-DR, CD19, CD14, and CD45 (Fig. 1A-H). In addition, ASCs acquired intracellular lipid deposits under appropriated stimuli, confirmed by Oil Red O staining and indicating the capacity to differentiation into adipocytes. Oil Red O staining also confirmed that the isolated cells were not adipocytes. Von Kossa staining indicated a mineralization of extracellular matrix, meaning the osteoblastic differentiation (Fig. 2A-C).

Histopathologic observations

PRP+ASCs promoted more fusion areas than PRP and ABG in bone defects. In addition, PRP induced a cicatricial area with layers of thin and incomplete lamellae bordering the limits of the defects. ABG promoted a pattern of osteogenesis and ossification from the extremities to center, with proportional amount of primary and secondary bone tissue. PRP+ASCs promoted a pattern of osteogenesis and ossification diffuse, which occurs from the extremities to the center and from the center to the peripheral region, with more proportions of secondary bone organization.

In the quantitative analysis, PRP+ASCs induced the highest bone neoformation (primary+secondary bone) compared to clot, PRP, and ABG ($\rho < 0.001$). Additionally, PRP+ASCs promoted the higher bone maturation than clot, PRP, and ABG, since PRP+ASCs showed the lowest levels of primary bone (immature bone) ($\rho < 0.001$) and the highest levels of secondary bone (mature) ($\rho < 0.001$). Furthermore, PRP+ASCs exhibited the lowest levels of granulation tissue ($\rho < 0.001$) (Fig. 3A-H and 4A-D).

Discussion

In this study, the capacity of human ASCs aggregated into PRP scaffolds to regenerate bone defects created in canine tibia was assessed by comparing the responses of ABG and PRP alone. Efforts were made to evaluate the primary and secondary bone,

and the granulation tissue. Furthermore, immunophenotyping and multilineage differentiation of ASCs were also performed and confirmed that the isolated cells were MSCs (16). We decided not to administer ASCs without scaffolds to prevent the possibility that these cells migrate from a bone defect to another and interfere with the findings, since MSCs administered alone demonstrated approximately half of the retention in the target site compared to cells administered with scaffolds (17). Moreover, tissue engineering is dependent on the ability to safely ensure sufficient delivery of cells within the target tissue, as well as adequate local engraftment and function at this site. The use of cells in combination with appropriate scaffold provides a physical support for cells orientation and proliferation, and molecular cues to direct cell survival, cell cycle progression and the expression of different phenotypes (17).

It is relevant to mention that one of the key implications of this study is related to a possible approach for tissue engineering based only in autogenous elements (cells, scaffolds, and growth factors presents in ASCs and PRP). In this context, we can emphasize some PRP advantages, such as the fact that this material does not promote immune reaction or contamination by prions, and that its preparation technique is affordable and minimally invasive. In addition, PRP is an interesting scaffold for periodontology, since it presents a gel consistency after activation that is ideal to be applied in areas as periodontal defects, sinus lifting, defects around dental implant, and cystic lesions.

Despite the numerous clinical and experimental data in the literature regarding the osteogenic potential of PRP, the function of this material in the wound healing process has not yet been clearly elucidated. Several studies showed that PRP significantly enhanced bone healing in surgical created critical-size defects (18-20). Additionally, other reports demonstrated that PRP associated with different scaffolds enhanced the *in vitro* osteogenic differentiation of MSCs (21, 22). Conversely, several studies found a significant lack of evidence to support the increasing clinical use of PRP as a treatment modality for bone regeneration (23, 24). According to the *in vivo* findings of the present study, PRP showed less capacity of bone formation and maturation compared to ABG. These conflicting PRP results may be due to different defect models, lack of control groups, different

evaluation periods and techniques, besides aspects related to the PRP, such as the choice of anticoagulant and activator, the speed of the centrifugation, and the time between activation of the PRP and its clinical use.

However, it is important to consider that the purpose of this study was not to use PRP conventionally, but as scaffolds for ASCs. In agreement with this premise, PRP+ASCs promoted the highest bone neoformation and maturation compared to ABG, which is the "gold standard" of bone grafts (25). Additionally, PRP+ASCs exhibited the lowest levels of granulation tissue. The most reasonable explanation for the outcomes promoted by PRP+ASCs in bone regeneration and maturation seems to be related to the several growth factors present in PRP and ASCs (3, 4, 9, 10, 26), such as VEGF, monocyte chemoattractant protein-1 (MCP-1), PDGF, TGF-β, IGF-1, and FGF (3, 10). PDGF seems to be the first growth factor present in a wound, and it initiates connective tissue healing, including bone regeneration and repair. The most important specific activities of PDGF include mitogenesis, angiogenesis, and macrophage activation (27). Additionally, some reports have demonstrated that PRP accelerates bone healing due to changes in bone morphogenetic protein type 2 (BMP-2) expression and activation (28, 29). Furthermore, TGF-β when released by platelet degranulation or actively secreted by macrophages acts as paracrine growth factors, which affect mainly fibroblasts, bone marrow stem cells, and pre-osteoblasts (30). TGF-β1 and TGF-β2 also have the ability to stimulate deposition of the collagen matrix of wound healing and bone, and they demonstrate important role in chemotaxis and mitogenesis of osteoblast precursors (31). Moreover, TGF-βs inhibit osteoclast formation and bone resorption (32). In addition, TGF-β contains several described BMPs and it has shown to promote osteogenic differentiation of human MSCs through nuclear factor-Kβ-mediated BMP-2 release (33).

Evidence suggests that the presence of certain pro-inflammatory mediators is required for bone healing and bone injury is followed by a choreographed cascade of events, some of which are dependent upon the presence of pro-inflammatory mediators.

Conversely, a persistent inflammation may result in suboptimal bone formation (34). In this sense, Mountziaris *et al.* (35, 36) reported that the modulation of the inflammatory response might represent a new direction in tissues engineering. They also affirm that novel strategies that exploit inflammatory signals have the potential to induce greater regeneration than current systems, which only deliver growth factors. It has been demonstrated that the administration of human ASCs is able to suppress the production of the pro-inflammatory cytokines, including interferon-γ, tumor necrosis factor-α, interleukins (IL)-1α, IL-1β, IL -6, IL-12, IL-15, IL-17; increase the levels of the anti-inflammatory IL-10; and promote a reduction of chemokines as MCP-1, Rantes, and keratinocyte cytokine (26).
Since PRP showed less capacity of bone formation and maturation compared to ABG and the association PRP+ASCs presented a higher capacity than ABG, these results indicate that the enhanced capacity of bone regeneration and maturation was promoted by ASCs or by the potentiation between ASCs and PRP. Corroborating this argument Marx *et al.* (27) pointed out that PRP help to initiate the actions of other growth factors and cells. Cenni *et al.* (22) showed that PRP in combination with freeze-dried bone allograft induced the production of FGF-2 by bone marrow stromal cells.

Another explanation for the optimistic outcomes promoted by PRP+ASCs in bone regeneration and maturation could be related to ASCs capacity to differentiate into multilineage cells, including osteoblastic (2, 13, 14) and/or endothelial phenotype, such as smooth muscle cells and endothelial cells (37, 38). Nevertheless, it is important to consider that we used human ASCs in dogs. MSCs can be transplanted between MHC-incompatible individuals due to the capacity of these cells to avoid allogeneic rejection, in humans and in animal models (7). However, allogeneic MSCs begin to induce immunogenicity after their differentiation (39). Consequently, cell differentiation probably was not one of the reasons for the findings of this study.

In summary, PRP+ASCs increased bone formation and maturation, and decreased the granulation tissue in bone defects created in canine tibia, even when compared to ABG. Multiple

biological mechanisms of ASCs and/or PRP, such as bone regeneration, paracrine effects and immuno-regulation, among others, could contribute to the findings observed in this study. The results obtained clearly suggest that PRP+ACSs implantation has the potential to improve bone regeneration. However, the results from this experimental study cannot be implemented in human patients so far. For this, safety experiments using the appropriate standard cell therapy protocols in humans must be carried out and further research is warranted to clarify the specific biological activity in the bone regeneration process of PRP+ASCs.

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FIGURES

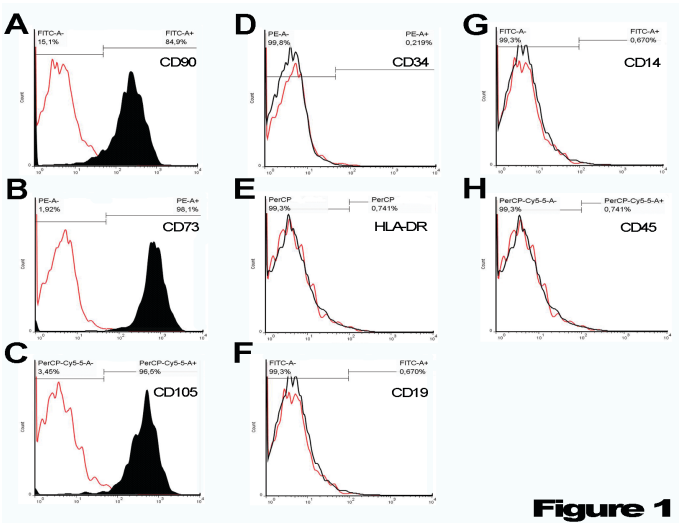

Figure 1: Representative immunophenotypic profile of ASCs. Flow cytometry histograms show the expression (black line) of

cells' surface markers (A) CD90, (B) CD73, (C) CD105, (D) CD34, (E) HLA-DR, (F) CD19, (G) CD14, and (H) CD45 compared with controls staining (red line). ASCs were positive for CD90, CD73, and CD105 and negative for CD34, HLA-DR, CD19, CD14, and CD45. Experiments were performed in triplicate.

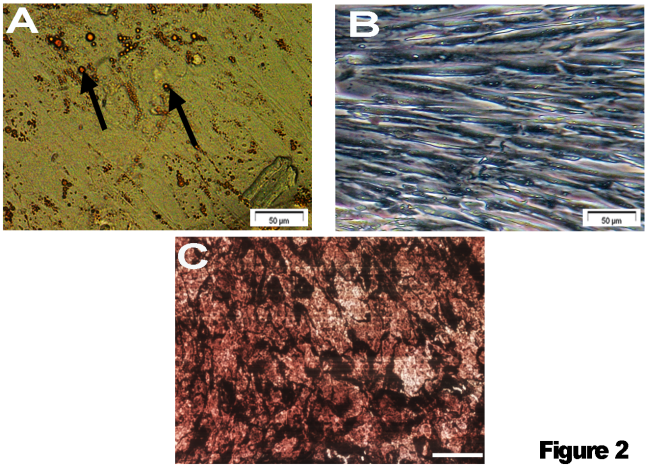

Figure 2

Figure 2: Representative images of multilineage differentiation of ASCs. (A) Oil Red O staining of ASCs cultured with adipogenic medium for 21 days. Bar = 50 µm. Arrows indicate lipid-filled intracellular vacuoles, confirming the differentiation into adipocytes; B) Oil O staining of ASCs cells after 21 days in culture with regular medium. Bar = 50 µm. The lack of lipid-filled intracellular vacuoles confirmed that the isolated ASCs were no adipocytes and did not differentiate into adipocyte spontaneously; C) Von kossa staining of ASCs cultured with osteogenic medium for 21 days. Bar = 500 µm. Black areas represent phosphate and indicate extracellular matrix mineralization, suggesting the differentiation into osteoblasts. Images are representative of three independent experiments.

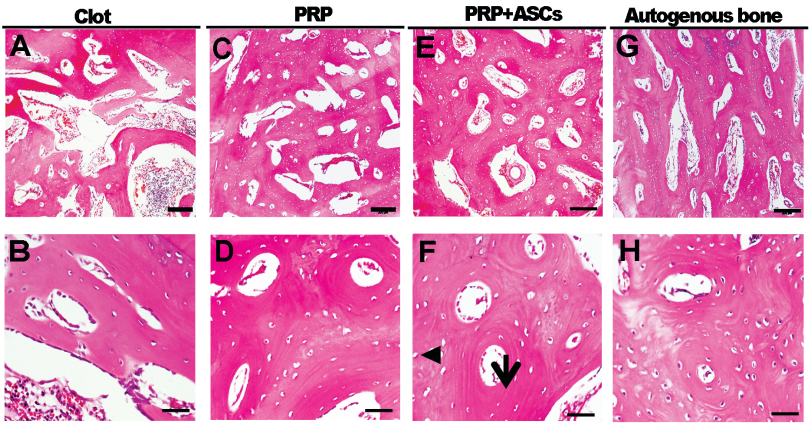

Figure 3

Figure 3: Histopathological aspects of bone tissue following treatment with Clot (A and B); PRP (C and D); PRP+ASCs (E and F); and Autogenous bone (G and H). Formalin fixed, paraffin-embedded tissue sections at 45 days after treatments were stained with hematoxylin and eosin (H&E). Slides shown are from six different dogs and represent typical findings obtained from the treatments. Slides from upper row are in magnification of 10x (scale bar = 200μm) and slides from lower row are magnification of 40x (scale bar = 50μm). Open arrow indicates areas of intense vascularization. Closed arrow indicates area of intense osteoblast activity, and primary and secondary ossification process. Images are representative of three independent experiments.

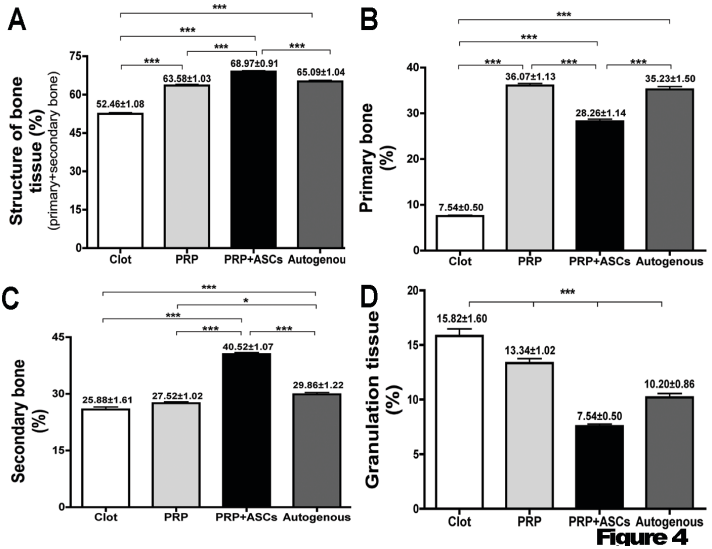

Figure 4: Quantification of structures of bone tissue. A) Primary and secondary bone: (***) indicates statistically significant differences among groups, since Clot < PRP = ABG < PRP+ASCs ($\rho < 0.001$; ANOVA with Tukey post hoc test); B) Primary bone: (***) indicates statistically significant differences among groups, since Clot < PRP = ABG < PRP+ASCs ($\rho < 0.001$; ANOVA with Tukey post hoc test); C) Secondary bone: (***) indicates statistically significant differences among groups, since Clot = PRP < ABG < PRP+ASCs ($\rho < 0.001$, except between ABG and PRP+ASCs, where $\rho < 0.05$; ANOVA with Tukey post hoc test); and D) Granulation tissue: (***) indicates statistically significant differences among all groups, since clot > PRP > ABG > PRP+ASCs ($\rho < 0.001$; ANOVA with Tukey post hoc test). Images are representative of three independent experiments.

3. DISCUSSÃO GERAL

O presente trabalho avaliou *in vitro* e *in vivo* os efeitos da rhBMP-2 e das ASCs humanas na osteogênese, através de diferentes abordagens.

Para inicar esse estudo foi necessário estabelecer uma cultura primária de osteoblastos humanos. Optou-se pela técnica do explante, utilizando fragmentos ósseos humanos remanescentes da perfuração para instalação de implantes dentários (MAILHOT; BORKE, 1998). Além do reaproveitamento deste tecido ósseo que seria descartado, outra vantagem dessa técnica é não promover morbidade adicional ao paciente.

Jonsson et al. (1999) compararam o potencial osteogênico de culturas de células estromais derivadas da medula óssea, osteoblastos derivados da técnica do explante e osteoblastos isolados por digestão enzimática. Após 2 semanas, os osteoblastos derivados da técnica do explante apresentaram o dobro de confluência comparados com os obtidos por digestão enzimática. Entretanto, a síntese de colágeno, osteocalcina e a atividade de fosfatase alcalina foi similar entre os dois grupos. Dessa maneira, a técnica do explante, além de ser metodologicamente mais simples, por não envolver as questões relacionadas à enzima, tais como a escolha da enzima, a concentração da mesma e o tempo de digestão, fornece resultados similares à técnica de digestão enzimática.

Além da técnica para isolamento das células, outro aspecto importante a ser destacado é a seleção dos voluntários. Nesse trabalho, os voluntários não poderiam ser fumantes, usar qualquer tipo de medicamento, apresentar alterações sistêmicas ou locais, além disso, deu-se preferência para pacientes jovens. Os osteoblastos humanos isolados apresentaram, em todas as passagens, viabilidades superiores a 90%. Além disso, as taxas de proliferação celular foram similares até a nona passagem, indicando que estas células ainda não haviam entrado em estado de senescência. Os osteoblastos humanos foram utilizados como controle positivo da diferenciação osteogênica somente nos experimentos de qPCR, uma vez que os iniciadores foram desenhados para células humanas. No restante dos

experimentos, utilizou-se como controle positivo os pré-osteoblastos murinos MC3T3-E1 subclone 4 (ATCC).

Em relação ao isolamento das ASCs humanas, utilizou-se a técnica de digestão enzimática (ZUK et al., 2001), que vem sendo descrita com bastante frequência e êxito na literatura (ZUK et al., 2002; WAN et al., 2006; NORA et al., 2011). Critérios rígidos de inclusão e exclusão de voluntários também foram seguidos para as ASCs, tendo em vista a importância desse estágio para a pesquisa. De maneira similar aos osteoblastos humanos, essas células mantiveram taxas de viabilidade e de proliferação celular elevadas, até a sétima passagem. Inclusive, Zuk et al. (2001) não observaram diminuição na duplicação cumulativa da população de ASCs avaliadas até a décima terceira passagem, que correspondeu a 165 dias em cultura. Entretanto, além do problema da senescência, elevados períodos em cultura podem permitir uma diferenciação espontânea das ASCs. Essa diferenciação pode estar associada ao emprego do soro fetal bovino nas culturas celulares, à expressão de BMPs endógenas, e/ou ao estado de confluência, em decorrência dos estímulos mecânicos (SONG et al., 2007; SEIB et al., 2009).

Como parte dos critérios mínimos propostos pela *Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy* para definir MSCs, essas células devem ser capazes de se diferenciar em multilinhagens celulares e precisam apresentar resultado positivo para os marcadores de superfície CD90, CD73 e CD105, e negativo para CD34, HLA-DR, CD79a ou CD19, CD14 ou CD11b, e CD45 (DOMINICI et al., 2006). No presente trabalho se comprovou as características de MSCs das ASCs isoladas, através da diferenciação em osteoblastos e adipócitos, e da expressão ou não dos marcadores específicos avaliados através de citometria de fluxo. Além disso, se comprovou que as ASCs isoladas não eram adipócitos e que não se diferenciaram espontaneamente em adipócitos, uma vez que as ASCs cultivadas com meio osteogênico ou meio regular não mostraram a presença de vesículas lipídicas na coloração com Óleo vermelho O.

Após o isolamento e a caracterização das células, avaliou-se os efeitos da rhBMP-2 na diferenciação osteogênica das ASCs suplementadas com ascorbato e β-glicerofosfato, e os

efeitos da BMP-4 e BMP-7 endógenas neste processo. Comprovou-se que as ASCs expressaram BMP-4 e BMP-7 endógenas. Os resultados dessa pesquisa sugerem que essas BMPs eram biologicamente ativas, uma vez que a expressão das BMPs estavam relacionadas com a indução na atividade de ALP, o aumento na expressão de genes osteogênicos (osteocalcina e osteonectina), e o aumento na deposição de cálcio na matriz extra-celular, nas células que não foram tratadas com rhBMP-2. Diferentemente dos resultados demonstrados nesse trabalho, Seib et al. (2009) observaram a expressão de BMP-4 endógena, mas não de BMP-7, em células mesenquimais derivadas da medula óssea humana. Uma das razões para tal discrepância pode ser a diferença na origem das MSCs.

Resumidamente, observou-se que a suplementação com rhBMP-2 associada ao ascorbato e ao β-glicerofosfato não aumentou os níveis de marcadores osteogênicos nas fases iniciais (atividade de ALP), intermediárias (osteocalcina e osteonectina) ou tardias (deposição de cálcio) da diferenciação das ASCs *in vitro,* comparado com as ASCs que receberam o mesmo tratamento sem a adição de rhBMP-2. Similar aos resultados desse trabalho, Wang et al. (2010) observaram que a presença de rhBMP-2 não promoveu aumento na expressão do mRNA do BMPR-II. Wan et al. (2006) também demonstraram similar expressão da osteonectina em ASCs murinas usando meio osteogênico com e sem rhBMP-2. Complementariamente, Song et al. (2007) também demonstraram que ASCs tratadas com e sem BMP-2 exógena promoveram similar deposição de cálcio na matriz extra-celular.

Apesar da suplementação do meio com rhBMP-2 não ter influenciado no aumento dos marcadores da diferenciação óssea, as ASCs tratadas com rhBMP-2 apresentaram os maiores níveis do mRNA da Smad 1 (dias 1, 7 e 21), a maior expressão da Smad 1 (dia 7), a maior expressão da Smad 4 (dia 14), e a expressão da Smad 1/ 5/ 8 fosforiladas precocemente (dia 7). Ou seja, o grupo que recebeu tratamento com rhBMP-2 apresentou maior expressão de elementos participantes da via Smad. Diferentemente desse trabalho, Chang et al. (2009) demonstraram que células tratadas com BMP-4 aumentaram a fosforilação de Smad 1/ 5. Complementariamente, Aoki et al.

(2001) observaram que o tratamento de células de linhagens mioblástica murinas, C2C12, com BMP-4 promoveu a diferenciação osteogênica das mesmas através da via de ativação das Smads. No presente trabalho não se observou associação entre a expressão de BMP-4 e a fosforilação de Smads 1/ 5/ 8. De acordo com estes resultados, Seib et al. (2009) demonstraram, em células-tronco derivadas da medula óssea, que a osteogênese promovida pela BMP-4 e BMP-6 foi independente da ativação das Smads e dependente da ativação da fosfatidilinositol 3-kinase (PI-3K). Ou seja, a ativação da BMP-4 tem sido relacionada com vias dependentes e independentes das Smads. Estas diferenças podem ser decorrentes dos distintos tipos celulares estudados, diferentes espécies e condições de cultivo.

Considerando que, tanto o tratamento com, quanto o sem rhBMP-2 promoveram efeitos similares na diferenciação osteogênica, pode-se sugerir que a rhBMP-2 exógena possa ter sido contrabalanceada pelas altas expressões de BMP-4 e BMP-7 pelas células que não receberam rhBMP-2. Entretanto, até o momento não temos explicações adequadas para justificar porque as células sem rhBMP-2 apresentaram maiores expressões de BMPs endógenas. Além da expressão e efeitos das BMPs endógenas, há várias explicações possíveis para justificar os resultados semelhantes da diferenciação osteogênica das ASCs tratadas com e sem rhBMP-2. Dentre eles, a maturação de progenitores osteogênicos na cultura, a emergência de um tipo celular dominante, e ainda a diferenciação espontânea das ASCs em osteoblastos (SONG et al., 2007; SEIB et al., 2009). Convém salientar que, embora uma combinação de todos os fatores acima citados possam ser responsáveis pelos resultados desta parte do trabalho, a produção de BMPs endógenas pode ser um fator crucial para cada um desses fatores.

Na sequência desse trabalho, comparou-se os efeitos da rhBMP-2, AR e associação de ambos na diferenciação osteogênica de ASCs. Tal proposta se justifica pelo fato de que a rhBMP-2, apesar de ser utilizada em inúmeras cirurgias para aumento de tecido ósseo, nas áreas médica e odontológica, apresenta algumas características desfavoráveis significativas, tais como tempo de meia-vida curto *in vivo*, custo elevado,

necessidade de elevadas doses para se alcançar efeito *in vivo*, e resposta inflamatória elevada (VAIDYA et al., 2007; SHAH et al., 2008; LEE et al., 2011a). Desse modo, estudos em busca de substâncias com capacidade osteoindutora similar à da rhBMP-2 poderiam colaborar para as abordagens de engenharia tecidual. Optou-se por estudar o papel do AR por essa substância ser relatada como capaz de aumentar a expressão de genes associados à osteogênese, de aumentar a formação óssea, e de provocar diferenciação osteogênica de vários tipos celulares, incluindo osteoblastos e MSCs (GAZIT et al., 1993; ZHANG et al., 2010).

No entanto, existem várias informações conflitantes no que diz respeito ao papel do AR na diferenciação osteogênica e na sua capacidade de potencializar os efeitos osteoindutores da rhBMP-2. Alguns trabalhos mostram que o AR estimula a diferenciação de condrócitos e aumenta os efeitos da BMP *in vitro* e *in vivo* (LI et al., 2003; COWAN et al., 2005). Por outro lado, outros estudos relataram que o AR inibe a diferenciação osteogênica e a ação da BMP envolve a atenuação do AR (HOFFMAN et al., 2006; WANG et al., 2008).

Nesse trabalho, observou-se que, a rhBMP-2 e o AR tiveram efeitos similares na atividade de certos marcadores ósseos, como a deposição de cálcio na matriz extra-celular (dias 12 e 32), a expressão da Smad 1 (dias 14, 21 e 28), da Smad 4 (dias 7, 14, 21 e 28), da BMP-4 (dias 7, 14, 21 e 28), da BMP-7 (dias 14, 21 e 28), do mRNA da Smad 1 (dias 7, 14 e 28), do mRNA da osteocalcina (dias 1, 7 e 28), do mRNA da osteonectina (dias 1, 7 e 21), e mRNA do BMPR-II (dia 7). Por sua vez, o AR apresentou melhores resultados quando comparado com a rhBMP-2 no que diz respeito aos outros marcadores, como a atividade da ALP (dias 7, 14, 21 e 28), a expressão do mRNA do BMPR-II (dia 14), do mRNA da Smad 1 (dia 21), do mRNA da osteocalcina (dia 14) e do mRNA da osteonectina (dias 14 e 28). Finalmente, a associação rhBMP-2+RA pareceu ser a melhor opção osteoindutora, tendo em vista que mostrou os melhores resultados na quantificação de cálcio na matrix extra-celular (dias 12 e 32), na expressão da Smad 4 (dias 21 e 28), e da Smad 1/ 5/ 8 fosforilada (dias 7, 14, 21 e 28),

na quantificação do mRNA da Smad 1, do mRNA da osteocalcina e do mRNA da osteonectina (dias 14, 7 e 7, respectivamente).

A rhBMP-2 demonstrou efeito superior ao do AR ou da associação rhBMP-2+AR somente na expressão da Smad 1 e da BMP-7 (dia 7), na expressão relativa do mRNA do BMPR-II e do mRNA da osteocalcina (dia 21). Assim, é importante ressaltar que, o AR isoladamente teve efeito similar ou melhor do que a rhBMP-2, na maioria dos marcadores ósseos avaliados. Quando o AR foi utilizado em associação com a rhBMP-2, estes efeitos nos marcadores ósseos foram otimizados, principalmente nos marcadores de diferenciação característicos de osteoblastos maduros, tais como a deposição de cálcio.

A partir dos resultados obtidos desta comparação entre rhBMP-2, AR e associação de ambos, pode-se sugerir uma nova abordagem que avalie a associação de quantidades menores de rhBMP-2 associada ao AR. Caso os resultados favoráveis observados forem mantidos com menor quantidade de rhBMP-2, poderia-se conseguir uma diminuição dos efeitos indesejáveis da utilização clínica da rhBMP-2. Outra abordagem interessante seria a agregação do AR e da rhBMP-2 em arcabouços para se avaliar a neoformação óssea.

A terceira parte do trabalho surgiu em decorrência do fato que a rhBMP-2, apesar de ser um agente osteoindutor potente, tem sido clinicamente relacionada com alguns efeitos colaterais importantes em cirurgias bucais, maxilofaciais e ortopédicas. Estes efeitos incluem dificuldade respiratória, disfalgia, edema, eritema, dor e rinite (BOYNE et al., 1997; VAIDYA et al., 2007; SHAH et al., 2008). A presença de inflamação tecidual e edema, provocados pela rhBMP-2, tem sido apontada como a principal causa destes efeitos colaterais (LEE et al., 2011a). Assim, como estudos pré-clínicos e clínicos têm mostrado que a administração sistêmica de MSCs pode modular as respostas imune e inflamatória e atenuar os danos teciduais causados por uma resposta inflamatória exacerbada (RYAN et al., 2005; PAREKKADAN et al., 2007; PAREKKADAN; MILWID, 2010), postulou-se a implantação de ASCs humanas agregadas em arcabouços de PLGA para modular a resposta inflamatória causada pela rhBMP-2. Esta resposta inflamatória não é inesperada, uma vez que a rhBMP-2 exerce quimiotaxia em linfócitos, monócitos e macrófagos. Além disto, a rhBMP-2 pode

induzir osteoclastogênese, através da potencialização do receptor do ativador do fator nuclear Kappa B (RANKL), uma citocina essencial para a indução de diferenciação de osteoclastos (CUNNINGHAM et al., 1992; CHAN et al., 2007).

Esperava-se observar uma diminuição da resposta inflamatória gerada pela rhBMP-2 em função do efeito parácrino das ASCs, uma vez que elas são MSCs (RYAN et al., 2005; PAREKKADAN; MILWID, 2010). Inclusive, Zhou et al. (2011) demonstraram que a administração de ASCs suprimiu a produção de citocinas pró-inflamatórias, dentre elas interferon-γ, fator de necrose tumoral-α, e interleucinas (IL)-1α, IL-1β, IL -6, IL-12, IL-15, IL-17, e aumentou os níveis da IL-10 anti-inflamatória. Estes autores também associaram as ASCs com a redução de algumas quimiocinas, incluindo MCP-1 e Rantes. Outro ponto interessante levantado na revisão de literatura de Parekkadan e Milwid (2010) é o fato de que a injeção direta de algumas moléculas secretadas pelas MSCs propicia mais benefícios, quando comparado com o transplante das células como um todo. Por esta razão, se discute o papel e a importância da proliferação e da diferenciação das MSCs para se alcançar os efeitos terapêuticos desejados na engenharia tecidual.

Para realmente cumprir os propósitos delineados se fez necessário avaliar as características físico-químicas dos arcabouços de PLGA relacionadas à adesão e proliferação celular, e determinar a quantidade de rhBMP-2 incorporada e liberada dos arcabouços. Resumidamente, observou-se que os arcabouços de PLGA, produzidos a partir de uma técnica simples, bem estabelecida na literatura e com baixo custo, apresentaram características físico-químicas compatíveis para adesão e proliferação celular e foram capazes de incorporar e liberar rhBMP-2. O ponto observado mais importante dessa parte do trabalho foi que as ASCs modularam a resposta inflamatória induzida por uma baixa dose de rhBMP-2 e aumentaram significativamente a neovascularização. Este achado é relevante para a engenharia tecidual, uma vez que a vascularização está relacionada com transporte de nutrientes, resíduos metabólicos e oxigênio (NADERI et al., 2011).

Tendo em vista as metodologias utilizadas, não foi possível afirmar se a neovascularização observada foi decorrente do

efeito parácrino, como já demonstrado na literatura, por meio da expressão de citocinas angiogênicas e arteriogênicas, tais como VEGF, MCP-1 e FGF; ou por meio da diferenciação das ASCs em cardiomiócitos e/ou outras linhagens celulares relacionadas ao sistema vascular (NADERI et al., 2011). Por esta razão, pesquisas adicionais são necessárias para explicar os mecanismos relacionados ao aumento da neovascularização e da diminuição da resposta inflamatória, associado à implantação de ASCs em arcabouços.

Um ponto a ser destacado é que optou-se pela administração das ASCs agregadas em arcabouços, tendo em vista que as MSCs administradas sistemicamente podem se diferenciar em sítios ectópicos e também provocar a formação de microembolos (AGUILAR et al., 2007; KUNTER et al., 2007; FIORINA et al., 2009). Entretanto, estudos futuros serão necessários para confirmar se a agregação das células em arcabouços realmente é capaz de impedir ou minimizar a formação de microembolos e a manutenção dessas células no sítio alvo.

A última parte do presente trabalho consistiu na avaliação dos efeitos das ASCs humanas, agregadas em arcabouços de PRP autógeno, na neoformação e maturação óssea em defeitos criados em tibias de cães, comparando-se com PRP isolado ou osso autógeno (ABG). A principal vantagem desta abordagem reside no fato de se utilizar arcabouços para as ASCs que são autógenos, biocompatíveis, com fatores de crescimento inerentes à sua composição, de fácil obtenção e baixo custo (OYAMA et al., 2004).

Resumidamente, os resultados obtidos mostraram que as ASCs agregadas ao PRP aumentaram a neoformação (osso primário e osso secundário) e a maturação óssea (osso secundário), além de diminuir a quantidade de tecido de granulação. O osso primário é o primeiro tecido ósseo a ser formado no desenvolvimento embrionário e na regeneração óssea. É um tecido imaturo, no qual percebe-se a deposição de fibras colágenas de maneira aleatória. Geralmente, o osso primário é substituído por osso secundário, um tecido mais maduro. O tecido de granulação, por sua vez, é um tecido formado por fibras conjuntivas que substitue o coágulo de fibrina na cicatrização de feridas.

Apesar da grande quantidade de trabalhos experimentais e clínicos relacionados ao PRP, a função deste material no processo de cicatrização ainda não foi completamente elucidado. Vários trabalhos indicam que não há evidências suficientes para justificar o uso do PRP como modalidade terapêutica de regeneração óssea. Dentre eles, o trabalho de Ozdemir et al. (2012) mostrou que o PRP associado a arcabouços de fosfato tricálcio não melhorou a regeneração óssea em modelos de defeito produzidos na calvária de coelhos. Garcia et al. (2010) mostraram que o PRP não melhorou a regeneração óssea peri-implantar. Em contrapartida, outros trabalhos mostraram achados favoráveis à utilização do PRP, tais como o de Mariano et al. (2010), que mostraram que o PRP aumentou significativamente a cicatrização óssea em defeitos críticos provocados na calvária de ratos diabéticos. Parizi et al. (2011) demonstraram que coral do golfo Pérsico associado ao PRP humano promoveu melhor cicatrização óssea em defeitos críticos provocados em coelhos, quando comparado aos animais que receberam somente o coral. Chen et al. (2012) observaram que o PRP associado ao cimento de fosfato de cálcio melhorou a atividade da ALP *in vitro* das MSCs e a regeneração óssea *in vivo*. Cenni et al. (2009), por sua vez, demonstraram que a diferenciação de células estromais da medula óssea foi acelerada com a adição de PRP ao osso alógeno desmineralizado e congelado. Estes resultados conflitantes em relação aos efeitos do PRP podem ser explicados pelos diferentes modelos experimentais empregados, pela falta de grupos controles adequados e, também, pelas diferentes técnicas e períodos de análise. De acordo com o presente trabalho, o PRP apresentou menor capacidade de formação e maturação óssea comparada com o ABG. Já o PRP+ASCs demonstrou maior capacidade de formação e maturação óssea, mesmo comparado com o ABG, o qual é considerado o "padrão ouro" dos substitutos ósseos. Estes achados indicam que a melhora na regeneração e maturação óssea foi promovida pelas ASCs ou pela potencialização dessas células com o PRP. Marx et al. (1998) apontou que o PRP ajuda a iniciar a ação dos outros fatores de crescimento e de células na regeneração óssea. Cenni et al. (2009) demonstraram que o PRP em combinação com osso alógeno desmineralizado e congelado induziu a produção de

FGF-2 pelas células estromais da medula óssea. Estes estudos indicam uma potencialidade entre os fatores de crescimento produzidos pelo PRP e os liberados pelas ASCs.

Em relação ao papel da ASCs nas abordagens propostas, poderia-se sugerir a capacidade dessas células se diferenciarem em múltiplas linhagens celulares, incluindo osteoblastos (ZUK et al., 2001; DA SILVA MEIRELLES et al., 2006; WAN et al., 2006) e/ou células com fenótipos endoteliais, dentre elas as células endoteliais e as células de músculo liso (OSWALD et al., 2004; SILVA et al., 2005). Entretanto, é importante ressaltar que foram utilizadas ASCs humanas em cães. As MSCs podem ser transplantadas mesmo entre indivíduos com complexo principal de histocompatibilidade (MHC) incompatíveis, pela capacidade destas células evitarem rejeição alogênica, tanto em modelos animais quanto humanos. Três mecanismos principais propiciam esse efeito: primeiramente, as MSCs apresentam baixa imunogenicidade, frequentemente pela falta do MHC-II e a co-expressão de moléculas estimulatórias; em segundo lugar, as MSCs evitam indiretamente a resposta de células T pela modulação das células dendríticas e diretamente pela interferência da atividade das células NK, CD8+ e CD4+; em terceiro lugar, as MSCs induzem uma supressão do microambiente através da produção de prostaglandinas, interleucina-10, e pela expressão de indolamina 2,3,-dioxigenase, a qual depleta o triptofano do meio (RYAN et al., 2005). Assim, em decorrência desta capacidade de evitar rejeição alogênica é possível transplantar células entre indivíduos com MHC incompatíveis. Porém, quando estas células se diferenciam elas passam a induzir imunogenicidade (HUANG et al., 2010). Por isso, como esse estudo empregou células alogênicas, pode-se sugerir que a diferenciação não foi o mecanismo responsável pelos resultados obtidos. Além disso, conforme já destacado, tem sido questionada a importância da “pega” do enxerto das MSCs, bem como da proliferação e/ou diferenciação das mesmas, para se alcançar os benefícios terapêuticos na regeneração óssea (PAREKKADAN; MILWID, 2010). Certamente, estudos adicionais são necessários para melhor compreender os mecanismos de ação das MSCs nestes processos de regeneração óssea.

Além disso, os resultados obtidos poderiam estar associados aos fatores de crescimento presentes no PRP e nas

ASCs (KAUX et al., 2010; PAREKKADAN; MILWID, 2010; LEE et al., 2011b; ZHOU et al., 2011). Tanto as MSCs quanto o PRP são capazes de expressar citocinas, tais como VEGF, MCP-1, PDGF, TGF-β, IGF-1, e FGF. O PDGF parece ser o primeiro fator de crescimento presente na ferida e inicia a cicatrização do tecido, incluindo o reparo e a regeneração óssea. As atividades mais importantes do PDGF incluem mitogênese, angiogênese e ativação de macrófagos (MARX et al., 1998).

Adicionalmente, alguns trabalhos têm demonstrado que o PRP acelera a cicatrização óssea pela expressão e ativação da BMP-2 (PARK et al., 2008; LEVI et al., 2011). Outro fator envolvido é o TGF-β, este fator de crescimento quando liberado pelas plaquetas ou ativamente secretado pelos macrófagos, atua como um fator de crescimento parácrino, principalmente sobre fibroblastos, células-tronco da medula óssea e pré-osteoblastos. Adicionalmente, cada uma destas células alvo tem a habilidade de sintetizar e secretar seu próprio TGF-β para atuar sobre células adjacentes ou atuar como um fator de crescimento autócrino (BECK et al., 1993). As funções mais importantes do TGF-β1 e TGF-β2 parecem ser a quimiotaxia e a mitogênese de precursores de osteoblastos. Além disso, eles também têm habilidade de estimular a deposição de matriz de colágeno do processo cicatricial e do tecido ósseo (PIERCE et al., 1992). Adicionalmente, os TGF-βs inibem a formação de osteoclastos e a reabsorção óssea (MOHAN; BAYLINK, 1991). Sem contar que a família TGF-β também inclui várias BMPs, as quais têm capacidade de promover diferenciação osteogênica de MSCs (HESS et al., 2009). Outro constituinte do PRP que pode ter efeito osteogênico nas ASCs é o TGF-α.

Ainda em relação à capacidade parácrina das ASCs, poderia-se sugerir outra explicação para os achados desse trabalho. Evidências indicam que a presença de certos mediadores pró-inflamatórios é necessária para a cicatrização óssea, ou seja, após a injúria óssea, a cicatrização compreende uma sequência de eventos, sendo que alguns são dependentes da presença de mediadores inflamatórios. Entretanto, se o processo inflamatório persistir ou estiver presente em uma intensidade acima do ideal, pode resultar em uma formação óssea deficiente (THOMAS; PULEO, 2011). Mountziaris et al.

(2008; 2011) propuseram que a modulação da resposta inflamatória poderia ser uma nova abordagem da engenharia tecidual. Eles ainda afirmaram que as novas estratégias exploratórias da modulação da resposta inflamatória têm potencial para induzir maior formação óssea, quando comparadas com as técnicas disponíveis atualmente. Neste contexto, deve-se destacar que a administração de ASCs pode suprimir a produção de citocinas pró-inflamatórias, conforme discutido anteriormente, sendo essa outra explicação plausível para justificar os resultados obtidos.

A vascularização é um dos maiores desafios das técnicas de engenharia tecidual. Quando um tecido ósseo resultante das técnicas de engenharia tecidual é implantado em um defeito ósseo, o fluído extra-celular fornece os nutrientes necessários. Tem sido relatado que as células podem se manter viáveis somente com um suprimento sanguíneo mínimo de 150 a 200μm (COLTON, 1995). A geração da rede de capilares e vasos de grande calibres é fundamental para a regeneração tecidual, tanto para o transporte de oxigênio, nutrientes, quanto de catabólitos (NADERI et al., 2011). Assim, a capacidade das ASCs de melhorar a neovascularização poderia também estar contibuindo para os resultados obtidos, conforme apresentado no capítulo 3. Em concordância, Li et al. (2009) demonstraram que células-tronco derivadas da medula óssea, associadas com matriz óssea desmineralizada e PRP, foram capazes de melhorar a vascularização na fáscia do tecido muscular. Essa capacidade das MSCs de melhorar a vascularização pode ser decorrente da expressão de citocinas angiogênicas e arteriogênicas, incluindo VEGF, MCP-1 e FGF. Além disso, o PRP também tem capacidade de expressar tais citocinas.

Resumidamente, esta abordagem para engenharia tecidual, envolvendo a associação do PRP e das ASCs, apresenta vantagens, como o fato de ser baseada somente em elementos autógenos (células, fatores de crescimento e arcabouços), bem como pelo fato da técnica usada para preparação do PRP apresentar um custo acessível e ser minimamente invasiva, além da consistência gelatinosa do PRP após sua ativação, a qual é ideal para ser aplicada em defeitos periodontais, levantamento de seio e preenchimento de lesões císticas. Além disso, o PRP não provoca reação imune, nem

contaminação microbiana ou priônica. Todas estas características comprovam que o PRP é um excelente material para ser utilizado como arcabouço das ASCs para a regeneração de defeitos ósseos, em áreas com pouca pressão mecânica.

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4. CONSIDERAÇÕES FINAIS

- Por meio da técnica de explante foi possível obter culturas primárias de osteoblastos humanos com viabiliade superior a 90% até a nona passagem;

- Através da técnica de digestão enzimática foi possível obter culturas primárias de ASCs com viabilidade superior a 90% até a sétima passagem;

- Foram comprovadas as características de células-tronco mesenquimais das ASCs isoladas, através da expressão dos marcadores de superfície e da capacidade de diferenciação em multilinhagens celulares;

- A associação de rhBMP-2+RA se mostrou mais eficiente na diferenciação osteogênica das ASCs *in vitro*, quando comparada com rhBMP-2 ou RA isoladamente;

- Os arcabouços produzidos à base de PLGA mostraram características físico-químicas compatíveis com seu uso em engenharia tecidual;

- Os arcabouços de PLGA não apresentaram citotoxicidade e demonstraram capacidade de degradação *in vitro*;

- Os arcabouços de PLGA apresentaram capacidade de incorporação e de liberação de rhBMP-2 maiores do que 60%;

- Os modelos cinéticos indicaram que a liberação de rhBMP-2 ocorreu por difusão e erosão;

- As ASCs agregadas em arcabouços de PLGA reduziram o processo inflamatório e aumentaram a neovascularização nos sítios musculares de cães, que receberam baixa dose (2,5ug) de rhBMP-2;

- As ASCs associadas com PRP apresentaram menor quantidade de tecido de granulação e promoveram maior quantidade e

maturação de tecido ósseo neoformado nos defeitos ósseos criados na tíbia de cães, comparado com osso autógeno e PRP isoladamente.

Parecer do Comitê de Ética em Pesquisa com Seres Humanos

Células-tronco derivadas de tecido adiposo

UNIVERSIDADE FEDERAL DE SANTA CATARINA
Pró-Reitoria de Pesquisa e Extensão
Departamento de Projetos e Extensão
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PARECER SUBSTANCIADO – 194/06

I – Identificação:
- **Título do Projeto:** Avaliação dos efeitos da associação de biovidros e proteína óssea morfogenética recombinante humana (rhBMP-2) na diferenciação de células mesenquimais indiferenciadas em osteoblastos.
- **Pesquisador Responsável:** Prof. Dr. Ricardo de Souza Magini
- **Pesquisador Principal:** Ariadne Cristiane Cabral da Cruz
- **Data prevista para a coleta dados:** de 01/10/2006 a 01/09/2008
- **Local onde a pesquisa será conduzida:** Fundições de Santa Catarina

II - Objetivos:
Geral: a presente pesquisa tem o propósito de avaliar os efeitos da agregação de proteína óssea morfogenética recombinante-2 (rhBMP-2) a diferentes tipos de biovidros na diferenciação de células mesenquinais indiferenciadas em osteoblastos.
Específicos:
- Desenvolver uma metodologia para agregação de rhBMP-2 aos biovidros;
- Avaliar efetividade da agregação de rhBMP-2 aos biovidros em diferentes intervalos de tempo;
- Avaliar a capacidade de osteoindução da rhBMP-2 agregada aos biovidros nas células mesenquinais indiferenciadas em diferentes intervalos de tempo;
- Obter células mesenquinais indiferenciadas a partir de tecido adiposo humano;
- Avaliar a viabilidade das culturas de células mesenquinais indiferenciadas obtidas;
- Submeter as células mesenquinais indiferenciadas à agregação de rhBMP-2 e biovidros;
- Avaliar a viabilidade das células mesenquinais indiferenciadas submetidas à agregação de rhBMP-2 e biovidros;
- Avaliar o comportamento dos diferentes biovidros em relação à agregação de rhBMP-2.

III - Sumário do Projeto: trata-se de um projeto que será desenvolvido no âmbito do depto de Estomatologia do CCS/UFSC, por uma equipe de quatro pesquisadores a pesquisadora principal, o pesquisador responsável e orientador, ambos do referido departamento e outros dois pesquisadores identificados como co-orientadores do projeto, sendo uma professora do Depto de Ciências Farmacêuticas da UFSC e outro pesquisador do Instituto de Biotecnologia Aplicada (Bauru- SP).
a) Descrição e caracterização da amostra: os participantes da pesquisa serão cinco voluntários submetidos a cirurgia de lipoaspiração que concordarem em doar o tecido adiposo retirado na cirurgia para que sejam realizados os procedimentos de pesquisa com este material. Serão incluídos na pesquisa apenas doadores com índice de massa corpórea normal que apresentarem prova sorológica negativa para hepatite B, C e síndrome de imunodeficiência adquirida (AIDS). b) Metodologia proposta: a pesquisa será realizada com 4 tipos de biovidros e uma proteína óssea morfogenética recombinante tipo 2 (rhBMP-2), que serão submetidos a dois métodos de agregação. Serão extraídas células mesenquinais indiferenciadas do tecido adiposo dos pacientes. Após o tratamento das células extraídas, esta amostra de material será dividida em seis grupos com diferentes combinações de materiais (cabe observar que o projeto anuncia

seis grupos, mas apresenta grupos que vão de GI a GIV). Por fim "a resposta celular aos diferentes biovidros e rhBMP-2 será avaliada pela medição de viabilidade celular, da proliferação celular e da diferenciação osteoblástica. O projeto prevê a realização de todos os experimentos em triplicata e a análise estatística dos dados obtidos.

IV – Comentários:

O protocolo da pesquisa contém todos documentos necessários para sua análise e exigidos pela legislação. Entretanto, apresenta duas versões de Termo de Consentimento Livre e Esclarecido (TCLE), ambas redigidas de forma considerada inadequada, uma versão utiliza linguagem técnica, inacessível a leigos, enquanto a outra não esclarece como será a coleta do material doado, nem esclarece de que se trata de material extraído por razões que independem dos objetivos da pesquisa. No orçamento apresentado há informação de que foi solicitado ao CNPq o custeio da pesquisa. Os pesquisadores apresentam declaração de compromisso com os preceitos éticos e com a resolução do CNS 196/96 e também uma declaração do chefe do departamento de Estomatologia reiterando estes compromissos e autorizando a pesquisa. A análise dos currículos dos pesquisadores, disponíveis no sistema Lattes do CNPq, revela sua sólida experiência no campo da pesquisa. Cabe ainda acrescentar que os pesquisadores estimam que a pesquisa não trará riscos aos participantes, mas não deixam claro quais benefícios surgirão em decorrência da investigação.

V – Parecer CEPSH:

(X) PENDENTE (detalhes pendência)*

Parecer:
Tendo em vista o exposto, sou de parecer que o presente projeto de pesquisa seja considerado pendente até a apresentação de nova versão do TCLE.

Informamos que o parecer dos relatores foi aprovado por unanimidade ou maioria, em reunião deste Comitê na data de 26 de junho de 2006.

Parecer final – Tendo em vista o atendimento das pendências, somos de parecer que o presente projeto e o TCLE sejam aprovados. Ressaltamos, no entanto, que tanto a "primeira via do entrevistador" como o "consentimento pós informado", sejam uma mesma peça e entregue uma via para o sujeito da pesquisa e outra via fique em poder do pesquisador.

(X) aprovado

Data da reunião:
Florianópolis, 14 de agosto de 2006.

Parecer prorrogação: O pesquisador responsável solicita a prorrogação do projeto, tendo em vista dificuldades de financiamento do projeto. A solicitação é que seja alterado o prazo de out/06 a set/08 para ago/08 a ago/10. Somos de parecer que a prorrogação seja aprovada.

Data da reunião:

Florianópolis, 28 de julho de 2008.

Washigton Portela de Souza
Coordenador CEPSH/UFSC

Fonte: CONEP/ANVS - Resoluções 196/96 e 251/97 do CNS.

Parecer do Comitê de Ética em Pesquisa com Seres Humanos

Osteoblastos

Parecer Consubstanciado Nº: 568/10
Data de Entrada no CEP: 03/02/2010
Titulo do Projeto: Estabelecimento de cultura primária de osteoblastos, a partir de tecido ósseo humano, obtido após perfuração para instalação de implantes dentários.
Pesquisador Responsavel: Cláudia Maria Oliveira Simões
Pesquisador Principal: Ariadne Cristiane Cabral da Cruz
Propósito: Projeto de pesquisa desenvolvido durante o doutorado, mas não sendo a pesquisa principal
Instituição onde se realizará: Outras

Último Parecer enviado
Enviado em: 23/02/2010

Objetivos (Preenchido pelo pesquisador)
Objetivo Geral -Estabelecer uma cultura primária de osteoblastos a partir de tecido ósseo remanescente de perfurações ósseas para a instalação de implantes dentários. Objetivo Específico - Localizar um paciente na clínica de Pós-graduação do Cepid, que necessite de implantes dentários para repor dente (s) perdido (s); - Explicar ao paciente os objetivos e etapas da presente pesquisa e solicitar que ele autorize a utilização do tecido ósseo removido durante a perfuração óssea para a instalação do implante dentário, que seria descartado; - Obter a amostra deste tecido ósseo; Etapa 2: -Processar o tecido ósseo em condições adequadas para estabelecer uma cultura primária de osteoblastos; - Cultivar a linhagem de cultura primária de osteoblastos humanos até a sexta passagem; Etapa 3: - Congelar os osteoblastos humanos da cultura primária estabelecida e utilizá-los posteriormente para avaliar a citotoxicidade e genotoxicidade de produtos e compostos em fase experimental.

Comentários
Apesar de utilizar material biológico humano para a pesquisa, o projeto descreve que este material utilizado é normalmente descartado durante o procedimento de perfuração óssea para instalação de implante dentário. Levando em consideração que o TCLE se encontra bem redigido e parece esclarecer devidamente

o sujeito de pesquisa sobre a sua participação neste projeto; e que o procedimento seria realizado por motivos alheios a esta pesquisa, damos por aprovado o presente projeto de pesquisa, estando os pesquisadores autorizados a realizar os procedimentos descritos.

Parecer
Aprovado "ad referendum"

Data da Reunião
22/02/2010

Parecer da Comissão de Ética no uso de Animais

23/02/12 14:35

Resultado de Solicitação de Protocolo

Protocolo
PP00679
Título
Avaliação da capacidade osteogênica e osteoindutora de células-tronco derivadas de tecido adiposo humano associadas com arcabouços de PLGA e PRP, no tecido muscular e ósseo de cães
Data de Entrada
03/11/2011

Resultado:
Aprovado
Data/Prazo
02/12/2011

Considerações
Oficio nº 196/CEUA/PRPE/2011
Do: Presidente da Comissão de Ética no Uso de Animais-CEUA
Ao(à): Prof(a) Dr(a) Cláudia Maria Oliveira Simões, Departamento de Ciências Farmacêuticas - CCS

Prezado(a) Professor(a),
Em relação ao protocolo de pesquisa sob sua responsabilidade a CEUA deliberou o seguinte:

- APROVADO, por dois anos para a utilização de seis animais da espécie canina (Canis familiares – Beagle).

- Procedência do animal: Biotério Central.

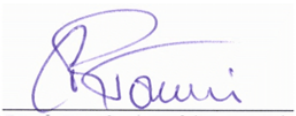
Por ocasião do término desse protocolo, DEVERÁ SER APRESENTADO RELATÓRIO detalhado relacionando o uso de animais no Projeto desenvolvido aos resultados obtidos, conforme formulário ON LINE CEUA.

Atenciosamente,

Relatório Final previsto para (90 dias após término da vigência do protocolo ou no momento da apresentação de um novo protocolo)
Data 07/03/2014

Data 07/12/2011

Parecer(es):



Prof. Assoc. Carlos Rogério Tonussi, D.Sc.
COMISSÃO DE ÉTICA NO USO DE ANIMAIS – PRPE – UFSC
PRESIDENTE

Abrir Solicitação | Criar Relatório

Parecer_1_PP00679.pdf